Comparison of pre-injury and improved passage methods for Schwann cells cultured *in vitro*★◆

Yang Jun[1], Qin Jian-qiang[2], Pei Guo-xian[3], Guo Xin-hui[1], Ye Han-shen[1], Chen Da-jun[1], Hong Jia-jin[1], Cai Wei-bin[1]

[1]Department of Orthopedics, the 421 Hospital of Chinese PLA, Guangzhou 510310, Guangdong Province, China; [2]Institute of Clinical Anatomy, Southern Medical University, Guangdong Provincial Key Laboratory of Tissue Construction and Inspection, Guangzhou 510515, Guangdong Province, China; [3]Xijing Hospital, the Fourth Military Medical University of Chinese PLA, Xi'an 710032, Shaanxi Province, China

Yang Jun★, Master, Attending physician, Department of Orthopedics, the 421 Hospital of Chinese PLA, Guangzhou 510310, Guangdong Province, China yangjun1267@sina.com

Correspondence to: Qin Jian-qiang, Ph.D., Professor, Institute of Clinical Anatomy, Southern Medical University, Guangdong Provincial Key Laboratory of Tissue Construction and Inspection, Guangzhou 510515, Guangdong Province, China jqqin@fimmu.com

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Abstract

BACKGROUND: Tissue engineering methods to construct nerve bridge graft for repairing nerve injury requires massive purified Schwann cells cultured *in vitro*.

OBJECTIVE: To compare the purity and quality of Schwann cells obtained with the pre-injury method and improved passage method.

METHODS: (1) Pre-injury method: Sciatic nerve of Sprague-Dawley neonatal rats was pre-injured for 3 days and removed, the epineurium was isolated and digested with trypsin and collagenase. Schwann cells were cultured in the culture medium after fibroblasts were eliminated with differential adhesion method. (2) Improved passage method: Sciatic nerve was directly removed from Sprague-Dawley neonatal rats and the epineurium was isolated. Double-enzyme digestion and single-enzyme digestion methods were adopted to primarily culture Schwann cells for 5−7 days. Schwann cells were purified with a single enzyme rapid digestion and centrifugation.

RESULTS AND CONCLUSION: Both pre-injury and improved passage methods obtained 95% purity of Schwann cells cultured *in vitro*, with no significant difference ($P > 0.05$). The resultant Schwann cells obtained by two methods showed normal morphology, high quantity, high purity and productive growth. These experimental findings imply that both pre-injury and improved passage methods are ideal approaches for *in vitro* culture of Schwann cells with high quality and high purity.

INTRODUCTION

After peripheral nerve injury, Schwann cells are essential for the survival and function of peripheral nerve fibers, these cells can secrete nerve growth factor and other active substances, also plays a synergic role in the macrophage's promotion of axonal maturation and reinnervation, and are effective to improve the repair of injured peripheral nerve. Currently, a tissue engineering approach for constructing nerve grafts to bridge nerve injury usually requires massive purified Schwann cells cultured *in vitro*, therefore, methods for Schwann cells culture and purification *in vitro* are the key for tissue-engineered nerve bridges. However, traditional methods of *in vitro* culture and expansion result in poor activity and limited number of seed cells due to long-term contact with digestive enzyme in nerve tissue. This study aims to compare the pre-injury and improved passage methods for *in vitro* culture of Schwann cells through the observations of cell purity and quality, in a broader attempt to lay a basis for nerve tissue engineering study.

MATERIALS AND METHODS

Design

A comparative observational experiment regarding cells.

Time and setting

Experiments were performed in Institute of Clinical Anatomy, Southern Medical University, Guangdong Provincial Key Laboratory of Tissue Construction and Inspection, China from May 2003 to June 2009.

Materials

Animals

Sixty Sprague-Dawley neonatal clean rats of 3 days old, irrespective of genders, were provided by the Animal Center at the First Military Medical University of Chinese PLA, China.

Reagents and instruments

DMEM/F12 medium, trypsin, type II collagenase and D-Hank's solution (Sigma Corporation, USA); fetal calf serum (HyClone, USA); rabbit anti-S-100 monoclonal antibody and SABC immunohistochemistry kit (Boster, Wuhan, China); 25-mL culture flasks (Orange, Belgium); Σ960 microplate reader (Metertech, Taiwan, China); superclean bench (Sanyo, Japan); autoclave (Hirayama, Germany); cell incubator (Heraeus, Germany); high-speed refrigerated centrifuge (Heraeus, Germany); DIC inverted fluorescence microscope and phase contrast inverted microscope (Olympus, Japan).

Methods

Pre-injury culture

Sprague-Dawley neonatal rats were housed for 3 days and then decapitated after sciatic nerve injury, then rats were immersed in ethanol solution at 75% volume fraction for 5 seconds and placed in sterile operating table. Distal sciatic nerve was harvested and epineurium was stripped under a microscope, then nerves were divided into single beams and cut into pieces, incubated at 37 ℃ in 5% CO_2 incubator, digested with 1.25 g/L trypsin 1 mL for 7 minutes, finally the reaction was terminated with serum. After samples were placed still for 7 minutes, the supernatant was transferred to No. 1 centrifugation tube, and digested with 1 g/L collagenase 2 mL for

20 minutes, diluted and triturated with D-Hank's solution 5 mL for 5 minutes. After some large undigested tissues were precipitated, the suspension was transferred to No. 1 tube and centrifuged at 1 000 r/min for 10 minutes, incubated with DMEM/F12 medium containing 10% fetal calf serum after supernatant was discarded. The tissue samples were differentially adhered to the culture flasks coated with rat tail collagen for 20 minutes to remove fibroblasts, the culture medium was transferred to No. 2 centrifugation tube at 1 000 r/min for 10 minutes. After supernatant was removed, cells were incubated with DMEM/F12 culture medium containing 10% fetal calf serum, cell concentration was adjusted to 2×10^8/L and then inoculated to culture dishes, 96-well plates and glass slides. Cells were cultured at 37 ℃ in 5% CO_2 incubator, and culture medium was replenished on the next day and changed every 3–5 days. At 5 days after primary culture, cells were subcultured. Centrifuge tube was added with 2 mL D-Hank's solution and 0.25% trypsin 500 μL, then the mixture was added into the culture flask. Cells were observed under an inverted microscope for 3–5 minutes. After Schwann cells shrunk back to a cluster, cells were triturated gently three times and cultured with DMEM/F12 medium containing 10% fetal calf serum, then cells were centrifuged in a tube at 1 000 r/min for 10 minutes. After supernatant was removed, the precipitates were cultured with DMEM/F12 medium 1.5 mL containing 10% fetal calf serum, then gently triturated and removed into a new culture flask. Cells were further cultured at 37 ℃ in 5% CO_2 incubator.

Improved subculture

Sprague-Dawley rats were directly sacrificed and immersed in 75% ethanol solution for 5 seconds. Bilateral sciatic nerve was dissected in sterile operating table at sterile conditions under a dissecting microscope. After epineurium was stripped, sciatic nerves were placed in two culture dishes containing DMEM/F12 medium. Nerve bundle was washed three times with DMEM/F12 medium and cut into pieces at a size of 0.1–0.2 mm, samples were cultured with 0.16% collagen enzyme 1 mL and 0.25% trypsin 1 mL, fully triturated and cultured at 37 ℃ in 5% CO_2 incubator for 5 minutes. The digestion procedure was terminated with DMEM/F12 medium containing 10% fetal calf serum, and standing for 5–10 minutes, the supernatant was transferred into the centrifuge tube. Tissues were again digest with 0.16% collagenase 1 mL for 15–20 minutes, and the digestion was terminated with 2.0–3.0 mL D-Hank's solution and fully triturated for 5 minutes. After some large undigested tissues were precipitated, the suspension was transferred to a centrifuge tube at 1 000 r/min for 10 minutes. After supernatant was removed, the sediments in the centrifuge tube were cultured with 1.5 mL DMEM/F12 medium containing 10% fetal calf serum at 37 ℃ in 5% CO_2 incubator. When both Schwann cells and fibroblasts synchronized to grow 5 days, cells were subcultured and digested with 0.3% trypsin 1.5–2.0 mL in the culture flasks, observed under an inverted microscope for 20–30 seconds. Schwann cells were shown to shrink back to a cluster, then gently triturated three times and cultured with 5 times volume of DMEM/F12 medium containing 20% fetal calf serum. Cells were centrifuged in a tube at 1 500 r/min for 5 minutes. The supernatant was discarded and the precipitates were cultured with DMEM/F12 medium 1.5 mL containing 20% fetal calf serum, gently mixed and triturated, then placed into a new culture flask at 37 ℃ in 5% CO_2 incubator[1].

Cell morphology observations

The inoculated cells were observed under phase contrast microscope at 40 × and 100 × magnification for cell morphology and growth.

Determination of cell growth curve

At 1, 3, 5, 7, 9, 11 days after the incubation, five culture dishes were collected and the medium was removed, samples were washed with D-Hank's solution twice and digested with 1.25 g/L trypsin, then observed under a microscope. After the cells contracted and became rounded, the digestive solution was removed and the original culture medium was pour back, cells were fully triturated and microscopically observed. All cells were seen to detach from the dish wall, the cell suspension was harvested to count the number of cells per milliliter.

MTT assay for cell activity

At 1, 3, 5, 7, 9, 11 days after the incubation, cells within five holes in 96-well plates were enrolled as a group, MTT solution 20 μL was added into each well. After cells were incubated for 4 hours, the culture medium was discarded and each well was added with 150 μL DMSO, oscillation for 10 minutes. The absorbance value at 570 nm was read with a microplate reader and the growth curve was plotted.

SABC immunohistochemistry

SABC kit (Wuhan Boster Company, China) was applied in this study for dyeing. In brief, Schwann cells cultured with these two methods were prepared into cell slices at 1×10^8/L, rinsed with 0.1 mol/L PBS three times; fixed with 40 g/L paraformaldehyde for 10 minutes and rinsed with PBS three times; blood-derived peroxidase was blocked with 1% H_2O_2, at 37 ℃ in an incubator for 20–30 minutes, rinsed with PBS; blocked with sheep serum (1: 20) at 37 ℃ in a incubator for 15–30 minutes, rinsed with PBS; samples were incubated with rabbit anti-S-100 monoclonal antibody (1: 100) 100 μL at 37 ℃ for 30–60 minutes and rinsed with PBS; samples were incubated with biotinylated goat anti-mouse IgG at 37 ℃ for 20 minutes and rinsed with PBS; cells were further cultured with SABC at 37 ℃ incubator for 20 minutes and rinsed with PBS; DAB chromogenic kit (AR1022) was used for DAB coloration and cementing.

Main outcome measures

The purity and quality of Schwann cells cultured with two methods were observed.

Statistical analysis

Data were analyzed with SPSS 10.0 software by chi-square test.

RESULTS

Phase-contrast microscope observation of the morphology of cultured Schwann cells

Schwann cells cultured with these two methods were spindle-shaped, with the marginal bright band, nucleus was oval or oblong, there were bipolar cell processes at varying length, cells often arranged as end to end or side by side. The fibroblasts showed large and flat cell body area, irregular shape, with no marginal bright band, and several processes interconnected into a flake (Figures 1−2).

Figure 1 Schwann cells cultured with pre-injury method (Inverted microscope, ×100)

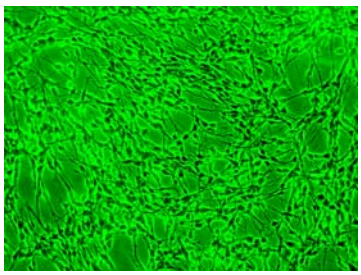


Figure 2 Schwann cells cultured with improved method (Inverted microscope, × 100)

The counting results of Schwann cells cultured with two methods are shown in Table 1.

Table 1 Number of Schwann cells cultured with pre-injury and improved passage methods

Item	Pre-injury method	Improved method
Number of Schwann cells (10^6/L)	563.5	575.0
Number of fibroblasts (10^6/L)	20.5	16.5
Total cell number (10^6/L)	584.0	591.5
Number of Schwann cells/ total cell number (%)	96.5	97.2

As shown in Table 1, there was no significant difference in the number of Schwann cells cultured with these two methods (x^2 = 9.245, P > 0.05).

Schwann cells' growth curve

The content of Schwann cells obtained from the harvested culture dishes at each time point was measured. In the pre-injury group, they were (1.85±0.21) ×10^8/L at day 1, (2.90±0.02) ×10^8/L at day 3, (5.95±0.15) ×10^8/L at day 5, (13.21±0.02) ×10^8/L at day 7, (45.30±2.00) ×10^8/L at day 9, (32.12±1.30) ×10^8/L at day 11; in the improved passage group, they were (2.00±0.01) ×10^8/L at day 1, (3.20±0.02) ×10^8/L at day 3, (6.90±0.35) ×10^8/L at day 5, (15.40±1.21) ×10^8/L at day 7, (48.60±2.81) ×10^8/L at day 9, (34.52±2.01) ×10^8/L at day 11 (Figure 3).

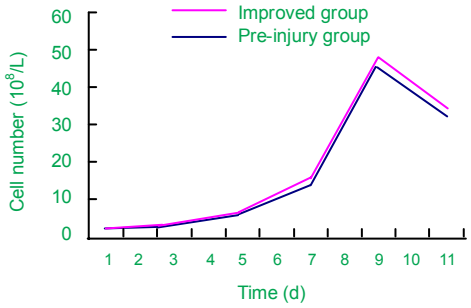


Figure 3 Schwann cells growth curve

MTT assay for the activity of Schwann cells

Schwann cells were cultured in 96-well plates and the absorbance value at different time points was read. In the pre-injury group, it was 0.430±0.005 on day 1, 0.455±0.008 on day 3, 0.502±0.005 on day 5, 0.630±0.021 on day 7, 1.325±0.035 on day 9, and 0.570±0.015 on day 11. In the improved passage group, it was 0.411±0.002 on day 1, 0.430±0.003 on day 3, 0.488±0.008 on day 5, 0.612±0.010 on day 7, 1.270 ±0.065 on day 9, and 0.551±0.030 on day 11 (Figure 4).

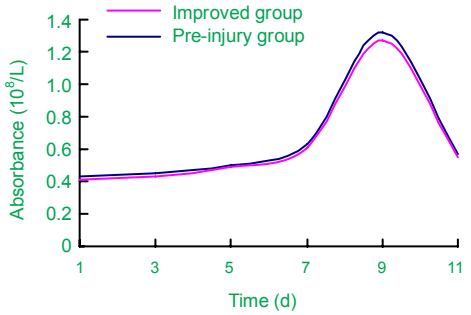
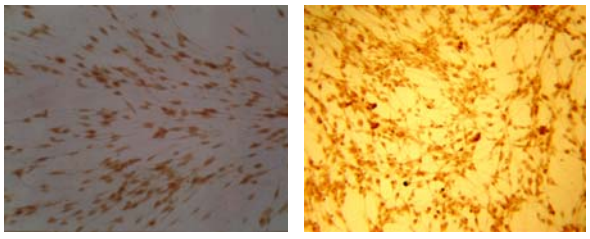


Figure 4 MTT assay for the activity of Schwann cells

Schwann cells identified by SABC immunohistochemical staining (Figure 5)



a: Pre-injury method b: Improved passage method

Figure 5 S-100 protein labeled Schwann cells (Inverted microscope, ×100)

The positive cells were stained yellow in cytoplasm and lightly in nuclei. Under inverted microscope, we observed that Schwann cells showed a clear boundary, spindle-shaped body and process protruding at both ends, while fibroblasts exhibited negative reactions. The morphology of S-100 protein labeled

Schwann cells cultured with the pre-injury and improved subculture methods is shown in Figures 5a–b.

DISCUSSION

Subsequent to the peripheral nerve injury, the axons at distal end degenerate and swallow, while Schwann cells can self-proliferate and connected into a flake, thus promoting axonal regeneration, while its secretion and synthesis functions are greater than before injury, so cells can produce more neurotrophic factors to promote neural regeneration. Therefore, the pre-injury method is an important access to obtain Schwann cells of high purity and high quality *in vitro* as a seed cell for nerve tissue.

Schwann cells' culture methods include tissue culture method, single enzyme digestion method, double enzymes digestion method, specifically adhesion method and immune selection method[2-6]. The commonly used methods have improved certain steps or liquid ingredients based on Brockes method. Briefly, nerve segments are isolated and epineurium is removed; cellular components are extracted for primary culture; cells are subcultured for purification and amplification. The key link is to inhibit fibroblast growth and to maintain Schwann cell's proliferation within a long time. Traditional culture methods, such as Brockes utilized Ara-C to selectively inhibit fibroblasts, ultimately the purity of Schwann cells can reach 99% and survival period is up to 150 days. This method is relatively complex and prone to induce toxicity of Ara-C although it obtains high purity and long-term survival of Schwann cells. Thus, we have improved the methods for Schwann cells' culture and purification[7-8] and compared with the pre-injury method, summarized as follows:

First, the cells were derived from sciatic nerve of Sprague-Dawley 3-day-old rats, previous experiments have showed that Schwann cells in neonatal rat have strong proliferative capacity. There were no exogenous substances, especially Ara-C added to remove fibroblasts during the primary culture of Schwann cells with these two methods, to avoid the Ara-C toxic effects on Schwann cells.

Because of sciatic nerve injury, Schwann cells obtained with pre-injury method are shown to fully activate secretion functions, cells grow more vigorously and cell activity is stronger compared with the traditional culture methods. While improved subculture method adopts double-enzyme digestion and single-enzyme digestion methods for the primary culture of Schwann cells, 5–7 days later cells were subcultured through single enzyme digestion and centrifugation, thus obtaining high-purity Schwann cells. However, this group of cells contacted with digestive enzymes for a period slightly longer than that in pre-injury group, and slightly shorter than that in traditional culture group, so the activity of Schwann cells was slightly inferior to that of pre-injury group and slightly stronger than that in traditional culture group, but the number of cells obtained was significantly higher that of traditional culture method.

When Schwann cells were cultured with pre-injury method, nerve tissue fragments cost shorter time to contact with digestive enzymes, differential adhesion method adopted to purify Schwann cells in primary culture and subculture process can achieve higher content of fibroblasts compared with traditional culture method; improved passage culture utilized single enzyme digestion and centrifugation to purify cells, and achieved fewer contents of fibroblasts compared with traditional culture methods, thus improving Schwann cells' purity. By a comparison, these two methods are satisfactory due to Schwann cells' purity reached 95%.

In 1988, Scarpini[9] have proposed that, Schwann cells can be identified by morphological characteristics through fluorescent labeling. Therefore, this experiment observed the morphology of Schwann cells under phase contrast microscopy and found that: Schwann cells cultured *in vitro* with both methods were closely arranged, fibroblasts were less and the adherent Schwann cells were nested, with bright halo and good activity. In addition, SABC immunohistochemical staining and counting results have revealed that, both of two methods achieved more than 95% purity of Schwann cells *in vitro*. Results from the MTT test found that, Schwann cells can proliferate and survive for 3–4 weeks, and the peak of proliferation occurred at 8–10 days after the culture.

In summary, both pre-injury and improved passage methods are ideal to obtain high-quality Schwann cells *in vitro*. Researchers can make a choice according to their own experimental conditions and requirements, we believe that these two methods will lay a solid theoretical and experimental basis for the construction of nerve bridges.

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预损伤与改良传代体外培养许旺细胞的比较★◆

杨　俊[1]，秦建强[2]，裴国献[3]，郭新辉[1]，叶汉深[1]，陈大军[1]，洪加津[1]，蔡伟斌[1]([1]解放军第四二一医院骨科，广东省广州市　510310；[2]南方医科大学临床解剖学研究所广东省组织构建与检测重点实验室，广东省广州市　510515；[3]解放军第四军医大学附属西京骨科医院，陕西省西安市　710032)

杨俊★，男，1977年生，辽宁省辽阳市人，汉族，2005年解放军第一军医大学毕业，硕士，主治医师，主要从事创伤骨科、脊柱外科的临床诊治、周围神经损伤及军事训练伤的科学研究。
通讯作者：秦建强，博士，教授，南方医科大学临床解剖学研究所广东省组织构建与检测重点实验室，广东省广州市　510515

摘要

背景：通过组织工程方法构建神经桥接体修复神经损伤，需要大量纯化体外培养的许旺细胞。

目的：对比观察预损伤法和改良传代法获取许旺细胞的纯度与质量。

方法：①预损伤法：预损伤SD乳鼠坐骨神经，3 d后取出坐骨神经，分离神经外膜，用胰酶、胶原酶消化，差速贴壁除去成纤维细胞，接种培养。②改良传代法：直接获取SD乳鼠坐骨神经，分离神经外膜，运用双酶消化法结合单酶消化法进行许旺细胞原代培养，5~7 d后采用单酶快速消化离心法行传代培养，同时纯化许旺细胞。

结果与结论：预损伤法和改良传代法体外培养的许旺细胞纯度均达95%以上，两种方法获得的许旺细胞纯度差异无显著性意义($P > 0.05$)。两种方法获取的许旺细胞形态正常，数量及纯度高，增殖旺盛，说明预损伤法和改良传代法都是体外获取高质量与高纯度许旺细胞的理想方法。

关键词：许旺细胞；细胞培养；组织工程；预损伤法；改良传代法
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本文创新性：许旺细胞传统的体外培养和扩增方法均存在神经组织接触消化酶时间长致使提取的种子细胞活性较弱、数量有限等不足。实验比较了预损伤法和改良传代法获取许旺细胞的纯度与质量，发现两种方法都是体外获取高质量许旺细胞的理想方法。

外国专家修饰的医学英语句型：(本刊英文部)

中文	英文修饰后	英文修饰前
面神经核内TNF-α(+)神经元的表达逐渐增加，同时运动神经元丧失数量也逐渐增多。	TNF-α expression **continued to increase concurrently with** facial motor **neuron loss**.	TNF-α expression kept increasing **while** facial motor neurons kept losing.
以探讨FK506在组织工程化人工神经中应用的可能性	In addition, the **suitability** of FK506 application as tissue engineered artificial nerves **was considered**.	to explore the **possibility** of FK506 application to tissue engineered artificial nerves.
本实验采用FK506在体外对新生SD大鼠许旺细胞进行直接干预，动态观察不同浓度FK506作用下许旺细胞生长、活性及分泌功能状态。	The present study **administered** FK506 to Schwann cells **harvested** from neonatal Sprague Dawley rats and observed the growth, activity, and secretion following treatment with various concentrations of FK506.	This study used FK506 to directly **intervene** Schwann cells **of** neonatal Sprague-Dawley rats in vitro, and dynamically observed the growth, activity and secretion of Schwann cells following various concentrations of FK506 treatment.