

Original Article

A Method for Isolation and Cultivation of Adult Schwann Cells for Nerve Conduit

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Background: It has been found that one of the methods to repair peripheral nervous system or even central nervous system injury is to use Schwann cells as nerve regeneration promoters. Therefore, it seems necessary to look for a way to obtain activated Schwann cells, with a sufficient amount of numbers and purity, in a short time for clinical applications. However, the previous methods using mitogens are not much clinically acceptable, and other methods that do not require mitogens, fail to isolate adult Schwann cells effectively or require a long period of time.

Methods: In this study, Schwann cells were isolated from predegenerated sciatic nerves of adult rat (one to three nerves per primary culture) and subcultured two times in a week with the 10% fetal bovine serum supplementation. Thereafter, Dulbecco's Modified Eagle's Medium media supplemented with 10%, 5%, 2.5%, 1.25%, and 0.625% fetal bovine serum were employed to determine their influence on the density and purity of Schwann cells after a 10-day period of cultivation.

Results: The concentrations of fetal bovine serum less than 10% immediately stimulated some morphological changes to happen in Schwann cells but not fibroblasts. Finally, Schwann cells acquired their normal shape on day 6 when fibroblasts just began to alter and die.

Conclusion: Our results demonstrated that total cell density was highly significant ($P < 0.05$) in the medium supplemented with 10% fetal bovine serum (950 cells/mm²) while purity was significant ($P < 0.05$) in the medium supplemented with 2.5% fetal bovine serum (97%) in comparison with other concentrations of fetal bovine serum.

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Introduction

One of the promising methods for nerve repair in peripheral nervous system (PNS)¹ or even central nervous system (CNS)² is to use the Schwann cells (SCs) potential for the induction of nerve regeneration. These cells are able to proliferate after nerve transection, especially in the proximal portion of the severed nerve and immigrate to fill the gap produced

between this part and the distal stump. These are also able to promote the growth of axon buds across the gap and conduct these to the distal stump of the transected nerve.

Many studies have confirmed that these cells can promote the nerve regeneration through three ways: 1) enhancement of the synthesis of surface cell adhesion molecules,^{3, 4} 2) production of a basement membrane which is composed of extracellular matrix proteins such as collagen⁵, and 3) increase in the synthesis of neurotrophic factors and their receptors.⁶⁻⁸

Although autologous transplantation of nerves to bridge the gap is the method of choice, but outcome after grafting surgery is poor. That is why many researchers have recently considered the promoting effect of SCs and a variety of artificial

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nerve conduits to stimulate nerve regeneration.⁹⁻¹¹ However, the main prerequisite for performing this operation is to obtain a large population of SCs in a short time span.

An important note in SC preparation is to reduce the accompanied fibroblasts to increase SC purity. Many investigations have been conducted to achieve these purposes.¹²⁻¹⁶

In the path to increase SC yield, Cajal was the first who described the method of in situ nerve predegeneration, during which the nerve was sectioned in situ to allow wallerian degeneration to take place.¹³ Based on this method, it was determined that the best time period for nerve predegeneration to happen, would be seven days.¹⁴ Some investigators used this method of predegeneration to improve expansion of SCs.¹³

It had been known for a long time that 10% fetal bovine serum (FBS) is suitable for proliferation of both SCs and fibroblasts. Some researchers have reported that the serum-free media increase the purity of primary cultures of SC.¹²

Verdu et al worked on serum-containing medium and obtained the best results with a medium supplemented with 1% fetal calf serum (FCS).¹⁵ Komiyama et al did a research on the effect of FBS concentrations from 0% to 10% to induce SC migration and proliferation selectively, while suppressing fibroblast overgrowth in an explant method. They finally determined that 2.5% FBS is the optimum concentration for both SC proliferation and fibroblast suppression.¹⁶

In this research, we applied Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.625%, 1.25%, 2.5%, 5%, and 10% FBS on SC culture to assess the effect of these various FBS concentrations on SCs and fibroblasts.

Materials and Methods

Cultivation of Schwann cells

Adult male Wistar rat (10 weeks old) was deeply anesthetized with a combination of ketamine (50 mg/kg) and xylazine (2.6 mg/kg) (Nasr, Iran). The animal's left sciatic nerve was exposed through a dorsal incision of the thigh and transected at the greater sciatic notch to allow the nerve predegeneration to take place. Seven days later, the animal was sacrificed with overdoses of anesthetics and the distal segment of transected nerve, about 25 mm in length, was resected and maintained in a dish containing DMEM (Sigma,

USA). The epineurium was stripped off with a pair of fine forceps using dissecting microscope under sterile conditions. After dissection, nerve fascicles were briefly washed with phosphate-buffered saline (PBS, pH 7.4; Sigma, USA) and transferred to a dish containing 10% FBS (Gibco, Scotland, UK) and an enzyme mixture consisting of 1.25 IU/mL dispase (Gibco, Scotland, UK), 0.05% collagenase type IA (Sigma, USA), and 0.1% hyaluronidase type I (Sigma, USA). Then, the nerve fascicles were teased by fine needles and incubated for three hours while they mechanically dissociated hourly by triturating through a glass Pasteur pipette. After incubation, the cell mixture was recovered by centrifugation at 1200 rpm for five minutes, the supernatant was removed, and the pellet was washed by the medium and centrifuged again. Then, the pellet was resuspended again in DMEM containing 10% FBS, 100 IU/mL penicillin (sigma, USA), and 100 µg/mL streptomycin (Sigma, USA). After counting cell suspension with an hemocytometer, its aliquots were seeded onto 35 mm Petri dishes coated with 10 µg/mL poly-L-lysine (Sigma, USA) at a final density of 2×10^5 cells/cm² and was incubated in 5% CO₂ at 37°C in complete medium supplemented with 10% FBS. After two days of incubation, tissue debris and dead cells were discarded by changing the entire medium while adherent cells remained. They were allowed to reach to confluent state.

Passaging of confluent cultures

After reaching the cultures to confluence, the supernatant medium was replaced with 0.25% trypsin (Gibco, Grand Island, NY). During trypsinization, cell dissociation from the substrate had to be monitored continuously under inverted microscope (Olympus IX70, Japan) until the maximum amount of cells were dissociated. The resultant cell suspension was transferred into the 15 mL centrifuge tube and centrifuged after adding 10 mL PBS. The centrifuged supernatant was removed and replaced with complete medium and was mixed with the pellet using a Pasteur pipette. In general, the cells were subcultured two times within a week and were finally counted by hemocytometer and seeded into poly-L-lysine-coated culture dishes at a final density of 1.2×10^5 cells per dish.

Cell culture groups

Each dish represented one group. There were

five groups in which the media, at the first 24 hours after plating, were supplemented with 10% FBS. This continued to the last day (day 10) in group 1 whereas, on day 2, serum concentrations were decreased in groups 2, 3, 4, and 5 to 5%, 2.5%, 1.25%, and 0.625%, respectively, and maintained to the last day.

Characterization of Schwann cells

The total cell numbers and the number of SCs were counted from six random fields (magnification 40 \times) blindly by two independent investigators on the basis of immunocytochemistry and cell morphology using phase contrast microscopy (Olympus BX 51, Japan). At first, immunocytochemistry was performed to identify the SCs by labeling these cells against S-100 protein. For this purpose the cells were fixed with 4% paraformaldehyde in PBS for 30 minutes, washed with 0.1 M PBS (pH 7.4), and incubated with anti-S-100 antibody (1:200, rabbit; Sigma, USA) at 4 $^{\circ}$ C overnight. The following day, after washing with PBS, cells were incubated with

fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (1:40; Sigma) for 60 minutes at room temperature. After final washes with PBS, cells were observed under an Olympus microscope equipped with epifluorescence using appropriate filter and photographed. The cultures were then stained with hematoxylin to distinguish better both the nuclear shapes and the cell boundaries of each two types of cells under an inverted microscope (Figure 1A). In general, nuclei of SCs were smaller and oval than the big round nuclei of fibroblasts. SCs bodies had multi- and tripolar morphology but fibroblasts had a flat, polymorphic cytoplasm, very larger than SCs. The purity of SCs was evaluated on the basis of the mean percentage of counted SCs with respect to the total numbers of cells^{16,17} All the stages of the study were performed in duplicate and sample quantities were presented in the text as mean \pm standard error of the mean. For comparison of quantitative measures, the values were subjected to statistical analysis using one way ANOVA, considered significant at $P < 0.05$.

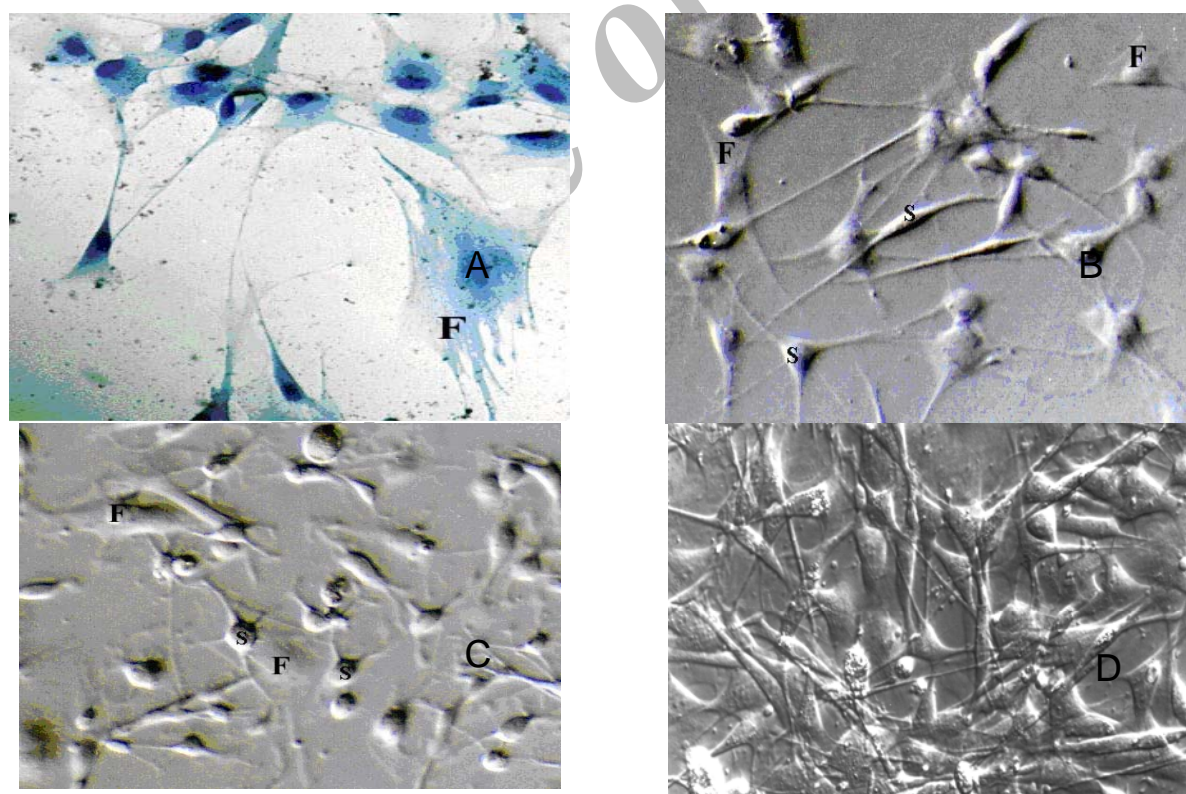


Figure 1. Micrograph of cultured Cs with different time intervals and various FBS concentration.

(A) First day of culture (40 \times). A fibroblast (F) with a flat, polymorphic cytoplasm and some multipolar and tripolar SCs (S) with oval nuclei are seen in the photograph. (B) Day 3 with 0.625% FBS (20 \times). Following to the reduction of FBS concentration, SCs became round and star-like but fibroblasts (F) maintained their morphology. (C) Day 8 with 1.25% FBS (20 \times). SCs (S) acquired their typical morphology with long slender processes but fibroblasts (F) went smaller than before. (D) Confluent SC culture at day 10 (40 \times). All micrographs were taken with inverted microscopy (Olympus IX 70, Japan).

Results

SCs attached to the bottom of the dish in less than 24 hours in primary culture while displaying the typical bipolar and tripolar morphology. They proliferated extensively during the next days. Fibroblasts appeared among SCs, but SCs proliferated more considerable than fibroblasts. Both SCs and fibroblasts attached to the bottom of the dish immediately when plated and left for the final 10 days of culture (third subcultivation) in less than five hours, the majority of the SCs adopted a multipolar and tripolar and less bipolar morphology. Fibroblasts spread their cytoplasm and became flat over which some SCs attach (Figure 1A). On days 2 and 3, a series of morphological changes took place in the SCs following to the reduction of FBS concentration to 5%, 2.5%, 1.25%, and 0.625% in groups 2 to 5. These cells became star-like or round while their processes shortened (Figure 1B). But at the same time, fibroblasts maintained their morphology and proliferated considerably.

On day 4, some cells detached from the bottom of the dish, floated in the media, and finally discarded with the changing of the media on the following days. On day 6, in group 2, fibroblasts proliferated but much less than SCs. In groups 3 and 4, fibroblast numbers decreased more than before but SCs proliferated fairly well. In group 5, a few fibroblasts were observed at day 6 and SCs proliferation was less than groups 1, 2, 3, and 4. There were few dead cells, floating in the media, on days 6, 7, and 8. At these days, SCs acquired

their normal morphology as a multipolar, tripolar, and bipolar cells with slender and smooth processes (Figure 1C).

On the other hand, fibroblasts progressively went smaller than before with the continuation of reduced concentrations of FBS during the following days; as this was clearly evident in groups 4 and 5. During the first three days in group 1, fibroblasts proliferated considerably and immigrated to all areas of the dish.

On day 4, the rate of SC proliferation increased. These cells proliferated while immigrating on fibroblasts and even attached over them. Eventually, cells of this group reached to confluency on day 8 (Figure 1D).

Finally, the total cell density of SCs in groups 1, 2, 3, 4, and 5 increased from the initial number of 150 cells/mm² on day 1 to 950, 575, 537, 500, and 475 cells/mm² on day 10. The purity of SCs in different groups was about 94.32%, 92.25%, 97.12%, 92.57%, and 92.35% (SD±2.07), respectively (Figure 2).

Immunostaining for S-100 protein was positive in all groups. The staining was evident in both SCs body and its processes. SCs demonstrated typical multi-, tri-, or bipolar morphology *in vitro*. They had oval nuclei and long, slender processes, and brightly stained for S-100 antibody (Figure 3).

Discussion

Nerve predegeneration, so-called wallerian degeneration, is a process which occurs in the peripheral nerve following its injury. It causes

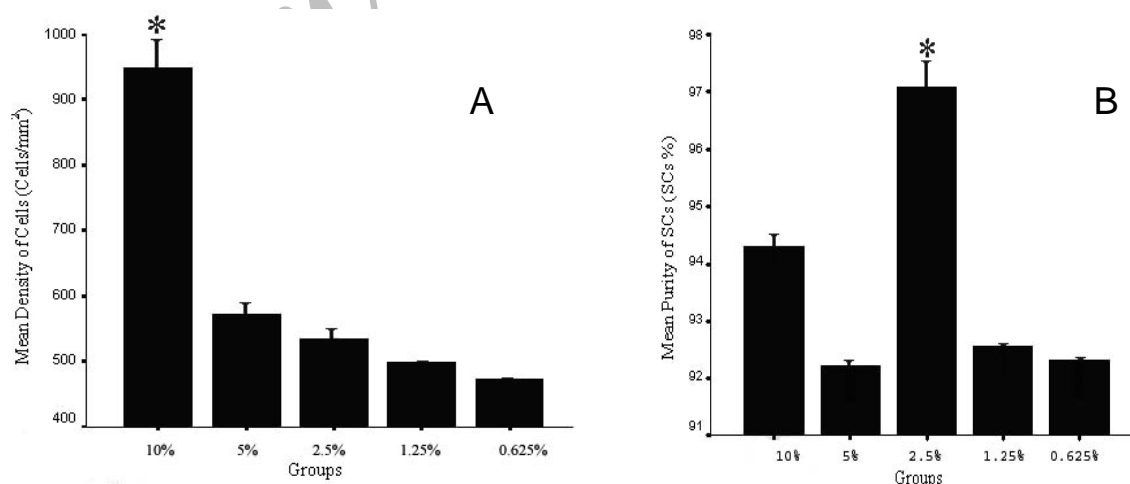


Figure 2. Density and purity of adult cultured SCs. (A) Density (cells/mm²) of SCs was significantly high (* $P < 0.05$) in group 1 when reached to confluency on day 8 compared with the other groups. (B) Purity (SC%) on the final day (day 10) was significant ($P < 0.05$) in group 3.

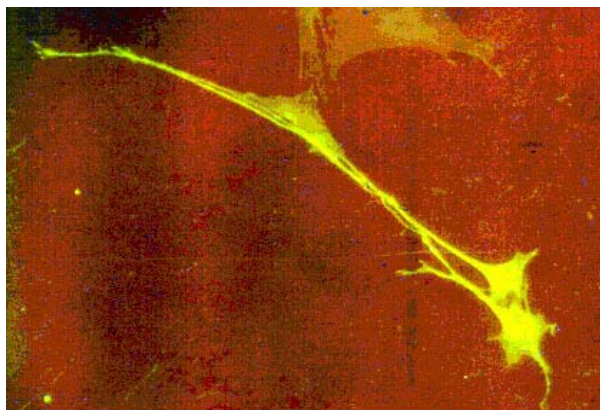


Figure 3. Fluorescent micrograph of adult SCs immunostained against S100 protein (40x). SCs frequently display a typical multipolar and tripolar morphology after subcultivation.

axons to become fragmented, and macrophages and fibroblasts to infiltrate into the predegenerating nerve. Up to now, many studies have shown that these cells are supportive cells for SCs, promoting them to proliferate by the production of their mitogenic factors,¹⁸⁻²¹ and inducing neural growth factor (NGF) expression by interleukin-1 secretion.²²

In our study, fibroblasts maintained their normal morphology and proliferated following the reduction of serum concentration during the first four days. They covered the bottom of the plate, while SCs had been deformed under these conditions and could not proliferate as much as the fibroblasts. Acquiring their normal shape from the day 5 on, SCs proliferated rapidly, immigrated to all areas of the plate, and filled the vacant spaces among fibroblasts. This may be partly due to the large population of fibroblasts, which had been proliferated during the past days and the secretion of SCs growth factors of SCs by them. At the same time, fibroblast numbers decreased, possibly due to the fast proliferation of SCs or apoptosis. Since the clinical application of SCs is to use them in nerve conduit as nerve regeneration promoter, one of the essential prerequisites to achieve this aim is to obtain a sufficient amount of mitotically active and fully functional SCs, especially from adult donors in a very short time span. Some researchers add substances like cholera toxin or forskolin as SCs mitogens to the culture medium.²³⁻²⁵ But, an additional pharmacological treatment with possible side effect should be prevented for a clinical application in human.

Another way to enrich cultures for SCs is the depletion of fibroblast by the addition of

genotoxins like arabinoside.²⁶⁻²⁸ There is controversy about using cytosine arabinoside because it has been noted to block the signaling pathway of nerve growth factor and decrease neurite outgrowth.²⁹ To obtain a high yield of SCs, Morrissey et al recommended the subcultivation.³⁰ Preparation of SCs cultures from predegenerated nerve has also been suggested.¹⁴⁻¹⁶

Our aim was to obtain a high yield of SCs with maximal purity. Therefore, we considered two factors to achieve these goals. Serum as a cheap and accessible cocktail containing a lot of growth factors and mitogens with different time intervals. This let us to manage the growth of both cells successfully. On the other hand, we did not apply any pharmacological compound with possible side effect as previous study. However, it was very important to determine when and what FBS concentration needs to be added to the media to achieve this objective. In our study, 10% FBS was necessary for establishing SCs culture on day 1, facilitating attachment of these cells to biosubstrate. On day 2, morphological changes occurred in SCs due to the reduction of FBS concentration to 5%, 2.5%, 1.25%, and 0.625%. It shows that this reduction has a tremendous impact on SCs during the first hours but not on fibroblasts. However, fibroblast suppression or even death occurred on the following days with the continuation of these concentrations. This situation was much evident in 1.25% and 0.625% FBS, while SCs proliferated normally. In a series of experiments, we showed that the reduction of serum concentration (from 10% to 0.625%) caused total cell numbers to decrease. On the other hand, the purity decreased along with the serum reduction, except in group 3. Reaching purity to an almost similar level in groups 4 and 5 indicated that SCs and fibroblasts death has accomplished in a constant and definite proportion. In general, decrease of total cell numbers and purity from group 1 to 5 demonstrates permanent SCs death throughout the 10 days of culture and that, fibroblast death accelerates during the last days in groups 4 and 5 (Figure 2).

The other factor that we considered was to employ the abilities of fibroblasts as SCs supporter and to remove them later at an appropriate time to increase the purity of cultured SCs. Therefore, we did not use any materials such as genotoxins, and depleted fibroblasts by manipulating the concentration of serum and the time which was to

be supplemented to the culture media.

The results of our study are in agreements with the finding of Komiyama et al who demonstrated in an explant method that the concentration of 2.5% FBS was suitable for both SCs proliferation and fibroblasts suppression. They obtained SCs with an average purity of $93.0 \pm 0.58\%$ at 21st days *in vitro*.¹⁶ But, we used different concentrations of serum and added these in certain times to the culture media in order to stimulate the proliferation of fibroblasts, use their potential as SCs supporters, and deplete them at an appropriate time. Eventually, the purity of our cultured SCs was 97.12% with the concentration of 2.5% FBS in less than 18 days.

In conclusion, concentration of 10% FBS is a suitable one for obtaining SCs with maximal numbers and a rather appropriate purity while concentration of 2.5% FBS is good for purifying SCs cultures. The most important advantage of our research is to decrease the time of SCs cultivation from 21 days in previous studies to less than 18 days. The other ones are: to use a cheap and accessible material (FBS), which is not harmful like pharmacological treatments for human and animal SCs, to purify SCs cultures by manipulating concentrations of FBS in different time intervals, and to employ fibroblasts as SCs supporters to induce proliferation in these cells.

Our method is a simple one, which may be useful for SCs cultivation and purification in the further studies.

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