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Full Length Research Paper

A simple and efficient method that uses low concentration fetal bovine serum to culture and purify Schwann cells

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To develop a simple and efficient method for the isolation of highly purified Schwann cells (SCs) from the sciatic nerves of newborn rats, cells were initially cultured in 10% FBS for 6 days, and then cultured in 2% FBS for 6 days. And we used the immunocytochemical characterization, flow cytometry, RT-PCR, to observe the condition of SCs. After the 12-day treatment protocol, we obtained a high yield of SCs with an average purity of 96.9%. Furthermore, the condition of SCs had no change. In our purification protocol, we had shown that our procedures did not alter the cell cycle and neurokines' secretion. Our simple and efficient method yielded pure and healthy SCs and did not require treatment with anti-mitotic agents or any special equipment.

Key words: Schwann cells, cell culture, low fetal bovine serum concentration, tissue engineering, fibroblast.

INTRODUCTION

Tissue engineering, the use of cells and engineering methods to modify biological functions, has already been applied to diverse clinical fields. In neurology, tissue engineering appears to have certain advantages over nerve cell transplantation. There are three critical meterials of tissue engineering: (i) biodegradable materials; (ii) trophic factors; and (iii) cells, typically autologous cells that do not cause antigenic reactions (Mackinnon and Dellon, 1990; Vacanti et al., 2001). In peripheral nerve tissue engineering, it is critical to rapidly obtain large numbers of pure Schwann cells (SCs) (Fansa and Keilhoff, 2004). SCs are the principal supporting cells of the peripheral nervous system, and are crucially involved in the functional recovery of injured peripheral nerves (Bunge, 1993). SCs play an important role in nerve regeneration and are at the core of peripheral nerve tissue engineering, a procedure that requires a large number of cells. Diverse methods have been proposed for the purification of SCs: antimitotic agent and antibody-mediated cytolysis methods (Brockes et al., 1979); repeated explantation methods (Oda et al., 1989); the cold jet technique (Jirsová et al., 1997); immunoselective methods (Calderon-Martinez et al., 2002); *in vivo* or *in vitro* predegeneration methods (Komiyama et al., 2003; Mauritz et al., 2004); differential adhesion methods (Pannuzio et al., 2005); differential detachment methods (Jin et al., 2008); and a combination of the *in vitro* predegeneration and cold jet technique (Haastert et al., 2007; 2009). These methods can yield highly purified SCs with various efficiencies, but all require special equipments, complicated procedures and/or high costs.

Preparation of SCs for tissue engineering requires a safe, rapid, easy and high yield methodology for purification. We aimed to develop an economical method besides those requirements from 7 days old Sprague Dawley (SD) rats by use of low concentration FBS.

MATERIALS AND METHODS

Animals

Postnatal SD rats (7 days old, 11.3±0.5 g) were obtained from the Dalian Medical University Animal Center (Dalian, China). All animal

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protocols were approved by the Animal Experiment and Care Committee of Dalian Medical University.

Primary culture

Rats were sacrificed by decapitation, and sciatic nerve segments (10 to 15 mm in length) were harvested aseptically under a dissecting microscope and were temporarily maintained in 60 mm culture plates (Corning, USA). Then, 2 ml of ice-cold D-Hanks solution (Sigma USA) was added, and the sciatic nerve segments were removed from the epineurium and cut into pieces (1 mm^3) under a dissecting microscope. Finally, nerve fragments were placed in 15 mL conical centrifuge tubes (Corning, USA) and centrifuged (140 g × 5 mins). The supernatant was discarded. 1 mL each of 0.25% trypsogen (Gibco, USA) and collagen 0.2% type- II (Gibco, USA) were added, then incubated in a cell incubator (Thermo, USA) with 5% carbon dioxide at 37°C for 30 min and heat shocked once every 5 mins. Addition of 2 mL of Dulbecco's Minimum Eagle's Medium (DMEM) (Hyclone, USA) that contained 10% FBS (Gibco, USA). The mixture was centrifuged again (140 g × 5 min), and the supernatant was again discarded. Removed nerve fragments to 60 mm culture plates. Then, 1.5 mL DME/10%FBS containing 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco, USA) was added, and all plates were maintained in a cell incubator within a humidified atmosphere with 5% CO_2 at 37°C. The growth medium was changed every 48 h.

Purification of SCs

Cells isolated from nerve segments after the first 6 days were divided into three groups: Group A (control) were fed every 2 days using DMEM with 10% FBS; Group B were fed every 2 days using DMEM with 2% FBS and cells were collected on day 6; Group C were fed every 2 days using serum-free DMEM, and cells were collected on day 6.

Immunocytochemical characterization of SCs and SC purity

Immunohistochemical staining was accomplished in the culture dish after 12 days. Cells were marked with the Strept Avidin-Biotin Complex (SABC) kit and rabbit anti-S100 protein antibody (Boster, China) according to the manufacturer's instructions. Then, cells were washed two times with PBS (pH 7.2) (Gibco, USA), fixed for 90 mins in 4% paraformaldehyde, and then immersed in a mixture of 30% H_2O_2 and pure methanol (1:50) for 30 mins. After that, 5% BSA (confining liquid) was added drop-wise at room temperature in 20 mins. BSA was shaken out of the dish. Then anti-S100 protein antibody was added drop-wise at 37°C for 1 h, washed 3 times with PBS, incubated with secondary antibodies at 37°C for 20 mins, washed 2 times with PBS. Then, SABC was added drop-wise at 37°C for 20 mins, washed 4 times with PBS. Finally, cells were stained by DBA (Hsu and Ree, 1980; Klosen, 1989) using a kit (Boster, China), at room temperature for 8 min, then post-stained with hematoxylin and examined under a microscope. Cells with a bipolar or tripolar shapes were identified as SCs, whereas flat or polygonal cells were identified as fibroblasts (Pannuzio et al., 2005).

SC purity was expressed as the percentage of SCs (SC purity = [number of SCs]/[number of SCs + number of fibroblasts]) from samples of ten visual fields at 200 × magnification.

Flow cytometry

Cells from Groups A and B were digested with 0.25% trypsogen and 0.02% EDTA, treated into the single cell suspension, centrifuged (314 g × 5 min) and washed with PBS. Then, centrifuged again, and the precipitate were resuspended and fixed in 2 mL of cold 80% reagent grade ethanol and left overnight at 4°C. Then, centrifuged and used 0.01% RNAase (Sigma, USA) to resuspend the precipitate and heat shocked at 37°C for 10 min. The cells were centrifuged and0.5%PI (propidium iodide) (Sigma, USA) was added to the precipitate and cell concentration was adjusted to 1×10^6 per mL.

Finally, the cells were stained in darkness for 30 min and detected by flow cytometry.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted separately from Groups A and B after 12 days and cDNA was synthesized from the total RNA using a RNA PCR Kit (AMV) Ver.3.0 (Takara, China) according to the instructions. The sequences of the primers for NGFb (Yan et al., 2007), BDNF (Yan et al., 2007), β-actin are depicted in Table 1. The PCR conditions were 94°C for 30s (denaturation), 56°C for 30 s (annealing) and 72°C for 1 min (extension) for a total of 30 cycles (NGFb, BDNF), or 55°C for 30 s (annealing) for β-actin. And a DL2000 DNA-ladder (TaKaRa, Japan) was run in parallel to the samples. Amplification products were separated by 2.0% agarose gel electrophoresis. After the gels were scanned, the relative intensity of NGFb and BDNF bands was determined by using Gel-pro Analyzer software (Media Cybernetics, USA).

Statistics

SC purity and cell yields are presented as means ± SDs. Values were subjected to a Student's t-test, with a P value less than 0.05 considered to be statistically significant.

RESULTS

Primary culture and purification of SCs

During the first 48 h of the primary culture, two distinct types of cells split off from the nerve fragments (Figure 1a). Phase-contrast microscopy (× 200) indicated that most of the cells attached to the bottom of the culture dishes could be classified as SCs (spindle-shaped, bipolar, and sometimes tripolar, with a small cytoplasm-to-nucleus ratio) or as fibroblasts (flat polygonal shaped, with a prominent ovoid nucleus and abundant cytoplasm). Most of the fibroblasts appeared to be scattered among the SCs, but some were beneath the SCs. During the first 48 h, SCs proliferated faster than fibroblasts. However, 96 h later, fibroblasts gradually became the dominant cell type, and SCs stopped growing and migrated onto fibroblasts. SCs and fibroblasts were both growing rapidly after 4 days.

After 6 days of primary culture of Group A cells (DMEM with 10% FBS), most of the fibroblasts growing among the SCs were easily detached from the culture plates, whereas those growing under or interacting with SCs could not be removed (Figure 1b). For group B cells (DMEM with 2% FBS), the fibroblasts started to die after 48 h, quite a number of cell were gone after 96 h of

Table 1. The sequences of the primers for NGFb, BDNF, β-actin.

	Forward primer	Reverse primer	Ta (°C)	Amplicon length (bp)
NGFb	5'-GGCCACTCTGAGGTGCATAG-3'	5'-CATGGGCCTGGAAGTCTAAA-3'	56	349
BDNF	5'-AAACCATAAGGACGCGGACT-3'	5'-GATTGGGTAGTTCGGCATTG-3'	56	393
β-actin	5'-TCTACGAGGGCTATGCTCTCC-3'	5-GGATGCCACAGGATTCCATAC-3'	55	320

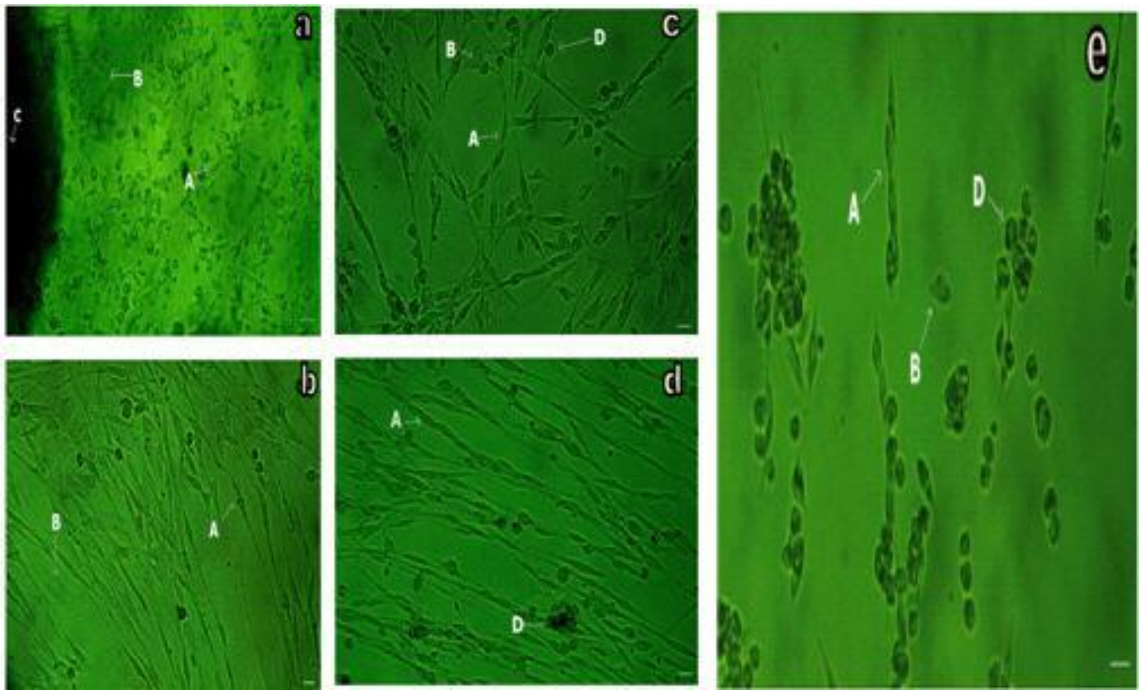

Figure 1. Observe the results of the growing cells with inverted microscope. A as SC, B as fibroblast, C as nerve segment and D as dead cells. During the 2nd day of the primary culture, two distinct types of cells emerged from the nerve fragments (a). And, at the 6th day of the primary culture, SCs and fibroblasts were growing fast (b). However, used DMEM with 2% FBS, numerous fibroblasts died within 96 h; (c) and almost all of the fibroblasts were cleared away by the 6th day and SCs morphology was not changed with 2% FBS (d). But after 6 days barely any cells survived at the culture plate in Group C. scale bar: 100 um).

culturing (Figure 1c), and almost all fibroblasts were gone after 6 days of culturing. The morphology of SCs did not change during this time (Figure 1d). For group C cells (serum-free DMEM), a few cells died after 48 h, but after 96 h, both types of cells had died in significant numbers.

The death rate continued to increase, and after 6 days of culturing, almost all cells had died (Figure 1e).

Immunocytochemical characterization of SC and SC purity

S-100 is a well-established sensitive and specific marker for SCs (Raff et al., 1979). We observed S-100 staining in the SCs body and along the processes (Figure 2). Our purified SCs had typical bi- or tri-polar morphology and oval nuclei. In contrast, the contaminating fibroblasts were negative for S-100 staining. Because our immunological staining was performed in culture dishes, we had acquired very high yield and high-quality cells. Since so many of the Group C cells died, we could not collect enough cells for immunocytochemical staining. Table 2 shows the percentage of cells positive for S-100. SC purity in Group B was significantly better than in Group A ($P < 0.01$). However, the total numbers of SCs were too little to discriminate Group A from B ($p > 0.05$).

Flow cytometry

To further provide qualitative evidences of the characters of our SCs, we compare the SC cycle of Groups A and B cells (Figure 3). As shown in Table 3, there was no significant difference in the cells cycles between Groups

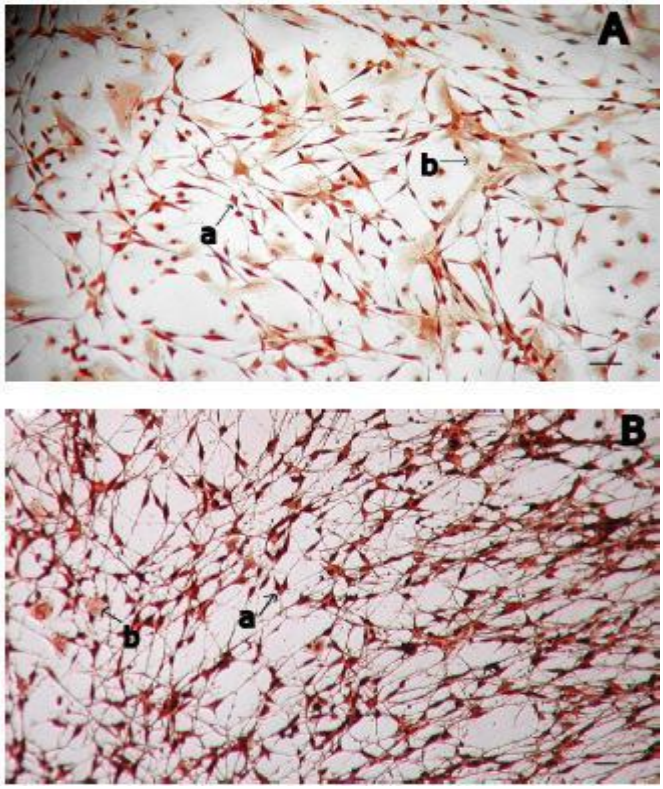

Figure 2. Immunocytochemical characterization of SCs with S100 showed that the purified SCs had typical bi- or tri-polar morphology and oval nuclei. (a) In contrast, the contaminating fibroblasts were negative for S-100 staining (b). Used DMEM with 10% FBS, numerous fibroblasts are shown at (A). Used DMEM with 2% FBS (B), by contrast, cells were very purified (scale bar: 100 um).

Table 2. Summary of results in the present method for SCs preparation.

View	Number of S100 protein positive cells/total number of counted cells	
	Group A	Group B
1	646*/753 (0.857902)**	779*/824 (0.945388)**
2	988*/1173 (0.842285)**	1045*/1094(0.955210)**
3	1111*(/12340.900324)**	1368*/1415(0.966784)**
4	845*/978 (0.864008)**	722*/746 (0.967828)**
5	750*/849 (0.883392)**	901*/936 (0.962607)**
6	731*/862 (0.848028)**	1711*/1752(0.976598)**
7	1130*/1270(0.889764)**	749*/762 (0.982940)**
8	1083*/1210(0.895041)**	939*/956 (0.982218)**
9	1092*/1206(0.905473)**	839*/860 (0.975581)**
10	592*/738 (0.802168)**	836*/856 (0.976636)**

* There was an insignificant difference between SCs total at Groups A and B ($p>0.05$); **There was a significant difference between SC purity at Groups A and B ($p<0.01$).

A and B cells ($p > 0.05$).

Reverse transcriptase polymerase chain reaction

RT-PCR analysis demonstrated mRNA expression of Schwann-cell-associated molecules, NGFb, BDNF in SC cells of Groups A and B (Figure 4) which displayed high levels of cell secretion activity. These results indicate the cell secretion ability of Schwann cells from Group B compare with Group A that do not have obvious alteration.

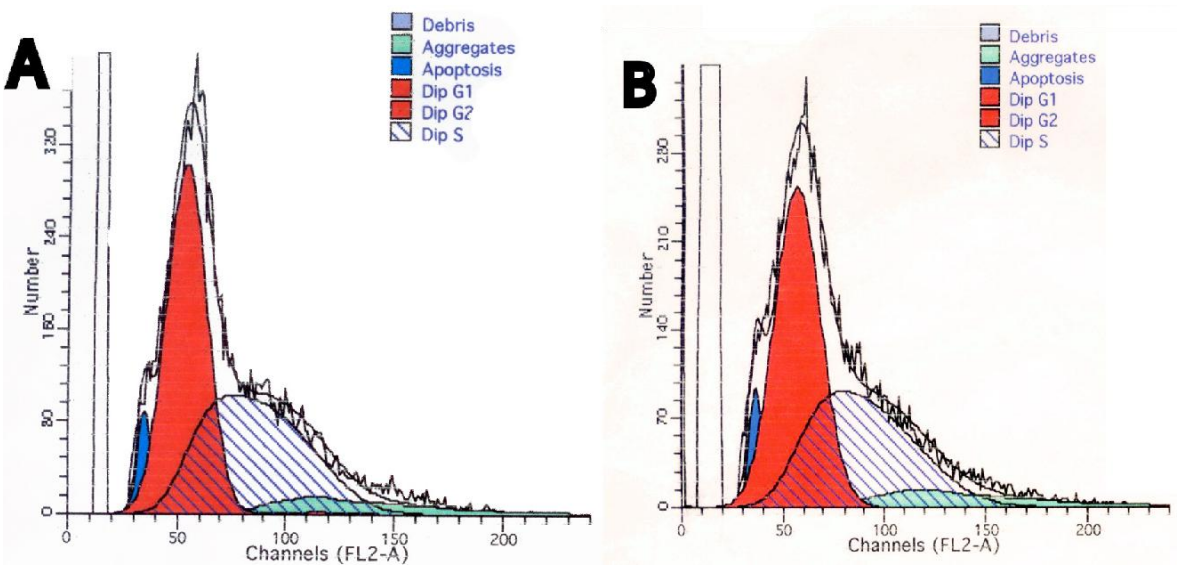

Figure 3. The cell cycle of SCs detected by flow cytometer showed that used DMEM with 10%FBS(A). DIP G_2:0.53%, S:47.37%, and used DMEM with 2%FBS(B) DIP G_2:0.10%, S:44.92%.

Table 3. Summary of results in the present method for SCs cycle.

Cell cycle	Group A		Group B	
	G_2[a] (%)	S[b] (%)	G_2[a] (%)	S[b] (%)
	0.53	47.37	0.10	44.92
	0.37	45.44	0.68	48.23
	0.13	44.80	0.42	46.04

a There was a insignificant difference between G_2 percentage at Groups A and B **(**p>0.05); b There was a insignificant difference between S percentage at Groups A and B **(**p>0.05).

DISCUSSION

Main findings

Our method of culturing the sciatic nerves of newborn SD rats in DMEM with 10% FBS allowed SCs and fibroblasts to split off from intact nerve fragments. Once SCs have migrated in high densities, they proliferate actively due to the production of autocrine growth factors, IGF-2, NT-3 and PDGF-BB (Jessen and Mirsky, 1999), regardless of being cultured in a medium with low FBS concentration. However, cells in serum-free DMEM (Group C) did not have adequate nutrition for growth and proliferation. Thus, although there were few fibroblasts in Group C, the growth of SCs was also greatly affected. There was no significant difference in the number of SCs in Groups A and B, but the SC purity was significantly greater in Group B. Our results indicate that culturing of nerve cells in DMEM with 2% FBS can gradually reduce the percentage of fibroblasts, while having no effect on SC differentiation, morphology or character.

The immuno staining results indicated that staining was evident in both SC cell body and processes during the culture period. The flow cytometry results indicated that the number of cells in S period and G_2 period were similar in Groups A and B. NGFb and BDNF are the main growth factors secreted by SCs, which are known to have neurotrophic effects on nerve regeneration (Schicho et al., 1999; Serpe et al., 2005).

Semi-quantitative analysis of NGF mRNA and BDNF mRNA by RT–PCR showed that SCs of Group B had similar productive function of secretion the normal cultured SCs. Thus, we conclude that our culturing protocol is a simple and efficient method to purify SCs that also preserves the function and morphology of SCs.

Comparison with the existing literature

Gross dissection of nerve cells may lead to contamination of the desired cells (Jessen and Mirsky, 1999) and peripheral nerve regeneration with tissue-engineered cells requires highly purified SCs for the construction of artificial nerve grafts. Many previous studies have

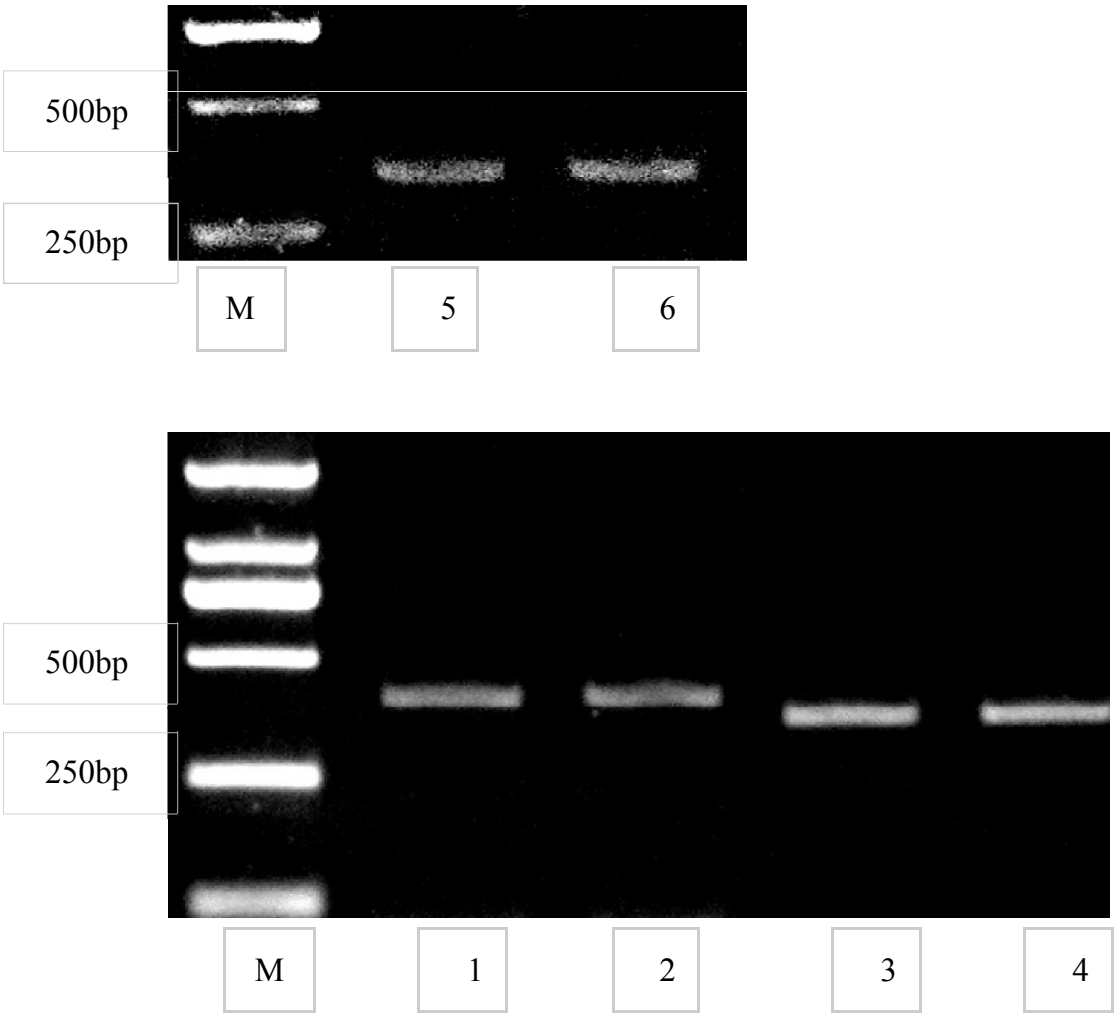

Figure 4. Total RNA was analyzed for the expression of NGFb and BDNF by RT–PCR at 12 days.1: BDNF (Group A); 2 BDNF (Group B); 3: NGFb (Group A); 4::NGFb (Group B); 5: β-actin(Group A);6: β-actin(Group B); M: Marker.

attempted to reduce the fibroblast contamination and increase SC purity by use of: (i) antimitotic treatment (Schicho et al., 1999); (ii) a combination of antimitotic treatment and antibody-mediated cytolysis with complements (Brockes et al., 1979); (iii) repeated explantation methods 6 (Serpe et al., 2005; Smith and Greenfield, 2003); (iv) differential adhesion methods (Pannuzio et al., 2005; Wood, 1976); and (v) immunoselective methods (Manent et al., 2003; Askanas et al., 1980; Wrathall et al., 1981). Those previously proposed methods have been shown to enrich SCs with various efficiencies, but all have certain limitations. In particular, antimitotic agents are harmful to SC function (Serpe et al., 2005; Assouline et al., 1983) and can reduce SC yield because of their non-specific antimitotic effects. Antibodies and complements are expensive for large-scale SC preparations, so do not provide an economical approach to SC purification. Repeated explantation and differential adhesion can be comparatively complicated and time-consuming procedures, and may lead to a loss of SCs and delay of therapy. Immunoselection is a good method for achieving high purity, but requires expensive antibodies and special facilities.

In a previous study, Needham et al. (1987), described the use of serum-free medium (S4) that allowed for optimal SC proliferation (up to 90% purity after 10 days), and suppressed fibroblast overgrowth in primary cultures of neonatal rat sciatic nerves (Assouline et al., 1983). But this serum-free media, though suitable for selective SC migration, was not as conducive for SC proliferation. However, use of a growth medium with 10% FBS supported not SC proliferation but an explosive fibroblast overgrowth. Komiyama et al. (2003) described the use of low FBS concentration for the purification of SCs, and obtained an average purity of 98% after 8 days by using DMEM for the first 6 days and changing to DMEM with 2.5% FBS on day 6. However, this method yielded low cell counts, and the resulting SC purity was only 88.0%.

Another method described by Komiyama et al. (2003) cultured cells in DMEM with 10% FBS for the first 3 days, then in serum-free DMEM for 3 days, then, on day 6 in DMEM with 2.5% FBS. The SC purity was 93.6% at 14 days, but was reduced to 89.5% at 21 days. Thus, regardless of method, the total cell counts were increasing, but the percentage of SCs decreased over time.

This phenomenon was also noted in our study.

IMPLICATIONS FOR FUTURE RESEARCH

The protocol described here is a simple, economical, rapid and efficient method for the purification of SCs. Our method has a high yields and results in highly pure cells. We suggest that our method might be useful for the development of tissue-engineered nerves and for other studies related to peripheral nerve injuries.

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