The most appropriate antimitotic treatment of Ara-C in Schwann cell-enriched culture from dorsal root ganglia of new born rat

Soung-Min Kim, Jeongwon Jahng[1], Jong-Ho Lee[2]

Department of Oral &Maxillofacial Surgery, College of Dentistry, Kangnung National University,
[1]Department of Pharmacology, Yonsei Medical School, [2]Department of Oral &Maxillofacial Surgery,
College of Dentistry, Seoul National University

Abstract

Schwann cell, one of important components of peripheral nervous system, interact with neurons to mutually support the growth and replication of embryonal nerves and to maintain the different functions of adult nerves. The Ara-C, known as an antimitotic agent, have been used to have high effectiveness in eliminating fibroblasts during Schwann cell culture period. This enrichment effect is also known to be cummulative with each successive pulse of Ara-C applied and is due to a progressive loss of fibroblasts. But the cytotoxicity by Ara-C is also cummulative and noticeable over the period.

To determine the most effective application time and interval of Ara-C in the Schwann cell culture, we observed the Schwann cell purity and density with the Ara-C treatment in plain and three-dimensional culture from dorsal root ganglion of new born rat. By culturing dispersed dorsal root ganglia, we can repeatedly generate homogenous Schwann cells, and cellular morphology and cell count with mean percentages were evaluated in the plain culture dishes and in the immunostainings of S-100 and GFAP in the three-dimensional culture.

The Ara-C treated cultures showed a higher Schwann cell percentage (31.0%±8.09% in P4 group to 65.5%±24.08% in P2 group), compared with that obtained in the abscence of Ara-C (17.6%±6.03%) in the plain culture after 2 weeks. And in the three-dimensional culture, S-100 positive cells increased to 56.22%±0.67% and GFAP positive cells to 66.46%±1.83% in G2 group (p<0.05), higher yield than other groups with Ara-C application.

Therefore, we concluded that the Ara-C treatment is effective for the proliferation of Schwann cells contrast to the fibroblasts *in vitro* culture, and the first application after 24 hours from cell harvesting and subsequent 2 pulse treatment (P2 group in plain culture and G2 group in three-dimensional culture) was more effective than other application protocols.

Key words
Schwann cells, Dorsal root ganglia, S-100, GFAP, Ara-C

INTRODUCTION

Schwann cells are known to be the chief supporting factor in the peripheral nerve regeneration. Culturing of Schwann cells from the adult peripheral nerve is a very difficult since these cells are greatly differentiated and proliferate poorly. Obtaining abundant and viable Schwann cells *in vitro* culture studies depends on many factors, i.e numerous enzymatic and mechanical dissociation culture techniques[1-3].

Among these, one of the main techniques is based on the application of mitogens like heregulin and adenylate cyclase activators[4], pituitary extract[5,6], lymphokins secreted by activated killer lymphocytes[7] and axolemmal fragments[8]. These mitogens have multiple effects on cells so their use should be continuously evaluated through suc-

* Corresponding author

Jong-Ho Lee
Department of Oral & Maxillofacial Surgery,
College of Dentistry, Seoul National University
28 Yeongun-dong, Jongro-gu, Seoul, 110-768, South Korea
Tel: +82-2-760-2630, 3813 Fax: +82-2-766-4948
E-mail: leejongh@plaza.snu.ac.kr

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cessive passages, paying an attention to possible changes which may alter Schwann cell characteristics.

The use of substances known to enrich the Schwann cells *in vitro* culture by reducing the proliferation of other cells is another important technique. The substances most commonly used to reduce proliferation of other cells are adenylate cyclase (cholera toxin, forskolin, isobutyl-methyxanthine and 8-bromo-cAMP), all of which are very effective in the inhibition of fibroblast proliferation at the low concentrations[9]. Antimitotics, such as Ara-C and 5-fluouracil, have been used as they show high effectiveness in eliminating fibroblasts, and this enrichment effect is cummulative with each successive pulse of Ara-C applied and is due to a progressive loss of fibroblasts. But, the cytotoxicity by cytosine arabinoside is also cummulative and noticeable over the period.

The purpose of this study is to obtain Schwann cell-enriched cultures from dorsal root ganglion of new born rat with most appropriate Ara-C application[3,10-12], so we used a enzymatic dissociation culture technique combining different Ara-C application method in plain and three-dimensional cultures *in vitro*.

MATERIALS AND METHODS

1. Cell culture from dorsal root ganglia (DRG) of new born rat

DRG were harvested from new born Sprague Dawley (Sam:TacN (SD) BR) rat under a dissecting microscope. (Fig. 1, 2) The main culture medium (M-10) was consisted of MEM (Eagle's Minimum Essential Medium) 93 ml, horse serum 5 ml, 50% glucose and 2 mg/ml gentamicin 1 ml.

DRG were harvested and cut into small pieces under the dissection microscope, and enzymatically dissociated using 0.25% collagenase (Worthington collagenase type I, USA) 1 ml and 0.2% DNase (Gibco, USA) 0.1 ml in phosphate-buffered solution (PBS, pH 7.4, Gibco, USA), and incubated at 37℃ for 70 minutes. After centrifugation (800 rpm, 5 minutes), 0.125% trypsine-EDTA (Gibco, USA) 2 ml was added, and incubated at 37℃ for 10 minutes. DRG were dispersed with 5 ml of M-10 media by gentle pipetting. The dispersed cells were collected by centrifugation (1000 rpm, 5 minutes), washed 3 times with M-10, and plated on the Polyethylemine coated dish (Sigma & Corning, USA). The cells were purified with a differential antimitotic agent (Cytosine β-D-Arabino-Furanoside, Sigma, USA) to remove fibroblasts according to this experiment conditions. The other culture protocol was followed as our previously published method (Lee's culture technique)[11,12].

2. Three-dimensional cell culture on Matrigel®

ECM gel of EHS mouse tumor (GFR Matrigel®, BD Biosciences, USA) preparations were placed on sterile 13 mm diameter glass coverslips and then covered with 20-30 μl of GFR Matrigel®, part of which was immediately removed with a pipette leaving only a thin film of fluid. DRG was gently and directly plated on Matrigel® (Fig. 3-a, b). A coverslip (in 35-mm Petri dishes) was placed in a humidified incubator at 37℃, and gassed with 95% air / 5% CO_2 for 15 minutes to allow the Matrigel® to solidify, and was cultured in rafting fashion with 2 ml of M-10

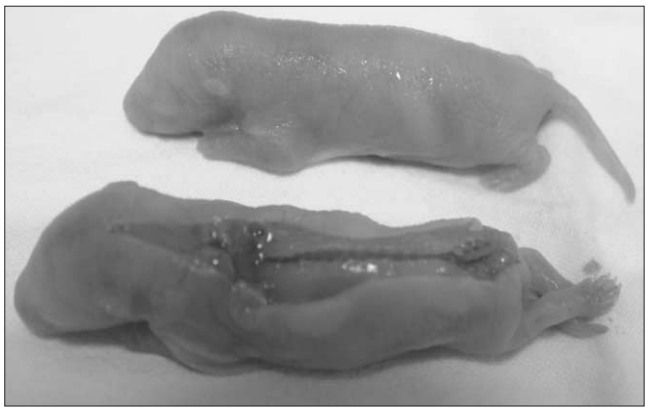


Fig. 1. Sprague Dawley 1 day rat.

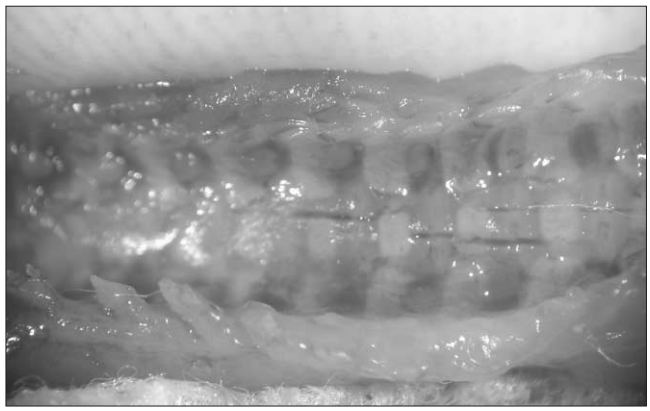


Fig. 2. Dissected spine for dorsal root ganglia harvesting under a dissecting microscope.

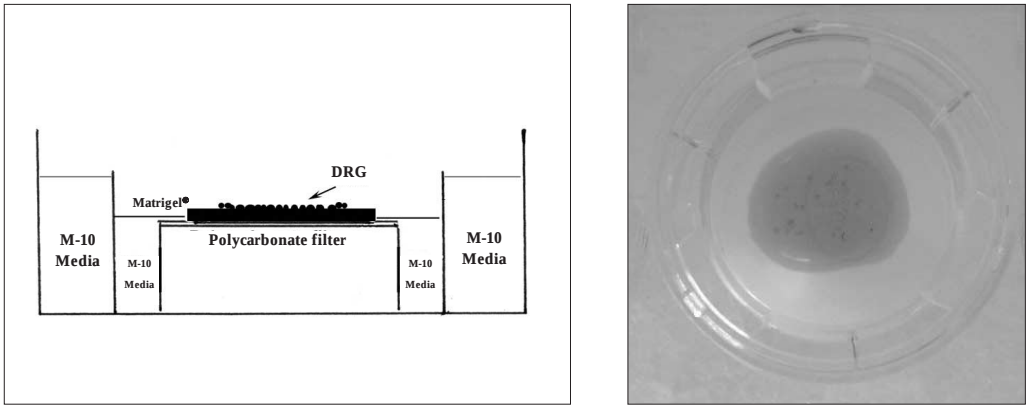


Fig. 3. Dorsal root ganglia cells plated on Matrigel®.

Fig. 4. Schem of experimental conditions applied to individual groups.

Exp. Group	Each pulses (Ara-C 1㎕/ml)									
P 1 (G 1)	1st		2nd		3rd					
P 2 (G 2)		1st		2nd		3rd				
P 3 (G 3)			1st		2nd		3rd			
P 4 (G 4)				1st		2nd		3rd		
Days	0	1	2	3	4	5	6	7	(2 weeks)	Eval.

0 : Initial culture day, Eval. : Evaluation, P 1-4 : Plate group 1-4, G 1-4 : Three-dimensional culture group 1-4

medium. The coverslip was maintained in a humidified incubator at 37℃ and gassed with 95% air / 5% CO_2 for up to 2 weeks by changing M-10 media according to Lee's culture technique[11-14].

3. Different experimental conditions

The cell cultures were divided into four groups as Fig. 4. Cytosine arabinoside (Ara-C, 1㎕/ml, 5 mM) was applied to the plain cultured group on the harvesting day (P1), and another group was divided after 24 hour (P2), 48 hour (P3), 72 hour (P4) application, individually. Each experimental group was replaced by new culture media (D-10) including 5 mM Ara-C in each other day by two times (total three pulses). And the three-dimensional cultures were also divided as G1, G2, G3, G4 by the same method and period. GDNF (0.2 ㎍/ml) was also used by two pulses with Ara-C application.

The control group in the plain cultured group (P-C) was not treated with Ara-C at all.

4. Immunostaining of S-100 and GFAP on three-dimensional culture groups

To evaluate the Schwann cell purity and density according to three-dimensional culture groups (G1-G4), three-dimensionally cultured cover slips were prepared 3 ㎛-sections after 2 week, and stained (H&E staining and immunohistochemical staining of S-100 and GFAP [Glial fibril acidic protein]). The procedure of immunohistochemical stain was followed as indirect PAP method; briefly, after rehydration in PBS (pH 7.4), the microsections were incubated with 0.6% hydrogen peroxide for 30 minutes to inactivate endogenous peroxide, incubated with normal swine serum (1:50) in PBS for blocking of nonspecific background, and followed by incubation of primary antibody, rabbit polyclonal S-100 antibody (1:100, Dako, Denmark) or rabbit polyclonal GFAP antibody (1:100, Pharmingen, USA) for 1 hour. The horseradish peroxidase-conjugated secondary antibody was then applied for 1 hour. Finally, all sections

were incubated with 3,3'-diaminobenzidine tetrahydrochloride chromogen (DAB, Dako, Denmark) substrate up to 10 minutes and counterstained with 10-fold diluted hematoxylin solution for 8 minutes[5].

5. Cell count

The cellular count on culture dishes was assessed with inverted microscope (Leica Cambridge Ltd., UK) at 2 weeks. Each counting was carried out by hemocytometer three times and calculated as mean±SD. Total number of cells and the number of Schwann cells were counted in six random fields (×100, 1 mm^2) on the basis of cell soma and nuclear morphology. To reduce the counting error, we counted the cells lying within 1 mm^2 area, using the subdivisions, and ruled out the undistributed cell or highly grouping cells. The cell in the grid lines was calculated three times and averaged, and total cell count within 1 ml was calculated by 10,000 multiplication.[12,15,16]

The number of positive cell in the immunostaings was counted using ImageTool® (Version 2.0 Alpha 3 [patch level 1]) computer analysis image system. In each experimental group, serial sections were also evaluated at least three times, and expressed as mean±SD.

6. Statistical Analysis

All numerical data acquired from culture dishes and three-dimensional cultures were tested for significant differences using Student's t-test. And also to test for semiquantitative differences among groups, Mann-Whitney U-test was used to determine the Ara-C effects on each group. The significance level was 5% ($P<0.05$), which was considered to be significant in all cases.

RESULTS

1. Evaluation of cells in plain cultured dishes

After 2 weeks of culture period, cell yield and proliferation were measured by dividing into two types as Fig. 5. Schwann cells usually had phenotypically long bi- or tripolar processes with an oval nucleus, and spindle-shaped morphology. But fibroblasts had a flattened polygonal shape with a large round nucleus and blunt cytoplasmic processes as usual. And so, Schwann cell purity was evaluated only as cellular morphology and the mean percentage of Schwann cells, respect to the total number of cells was counted as table 1 and Fig. 9.

2. Evaluation of three-dimensionally cultured cells on Matrigel®

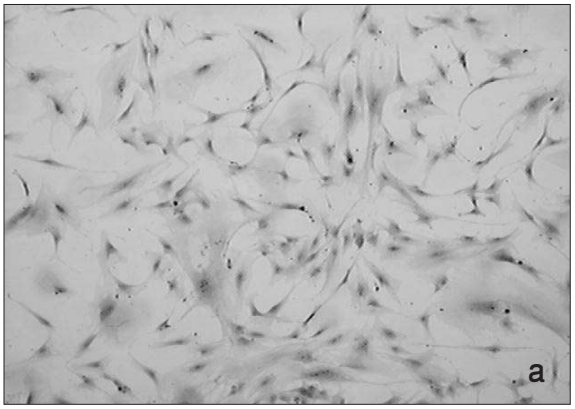
The total number of cells were counted using ImageTool® computer analysis image (×400) system, and in each experimental group, serial sections were also evaluated at least three times, and expressed as mean±SD (Table 2 and Fig. 7).

In the immunohistochemical staining of S-100, the G2

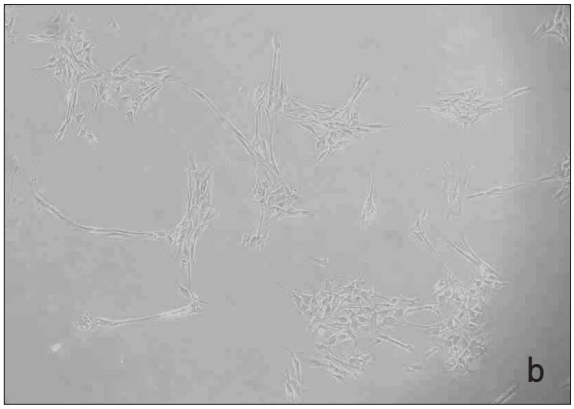
Table 1. Cell number by cellular morphology & mean percentage

	Spindle-shaped, bi-polar	Mean (%)
P-C	$7.30\pm1.52\times10^4$	17.6%±6.03%
P 1 *	$3.02\pm0.43\times10^5$	32.0%±4.55%
P 2 *	$6.80\pm2.50\times10^5$	65.5%±24.08%
P 3 *	$3.56\pm1.58\times10^5$	45.3%±20.10%
P 4 *	$1.80\pm0.47\times10^5$	31.0%±8.09%

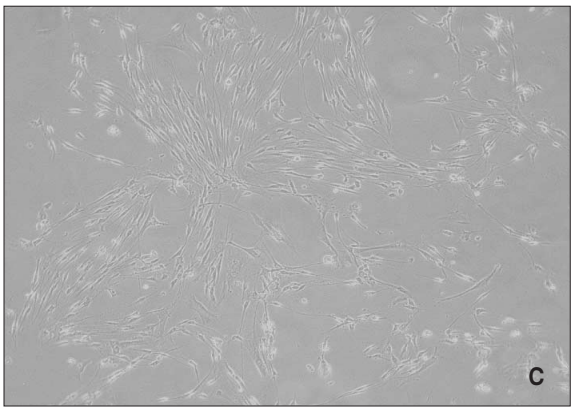
Each data were counted using a hemocytometer and * means significant data ($p<0.05$)
Mean value (%) was the ratio of spindle-shaped cells to total counted cells
P-C : cytosine arabinoside was not applied during two weeks
P1 group : cytosine arabinoside (Ara-C, 1㎕ / ml, 5 mM) was applied on the harvesting day
P2 group : Ara-C was applied after 24 hour
P3 group : Ara-C was applied after 48 hour
P4 group : Ara-C was applied after 72 hour



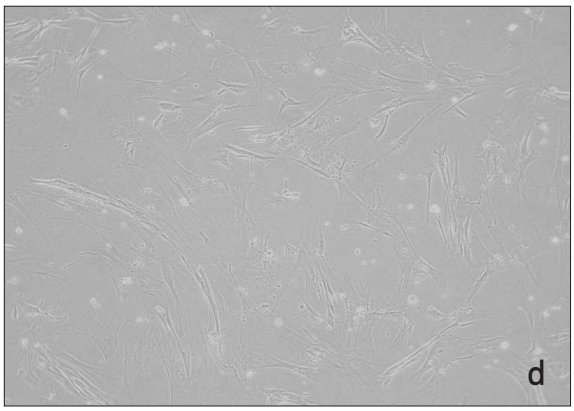
a. Control group showing flattened polygonal shape with a large round nucleus and blunt cytoplasmic processes (H&E stain, ×100)



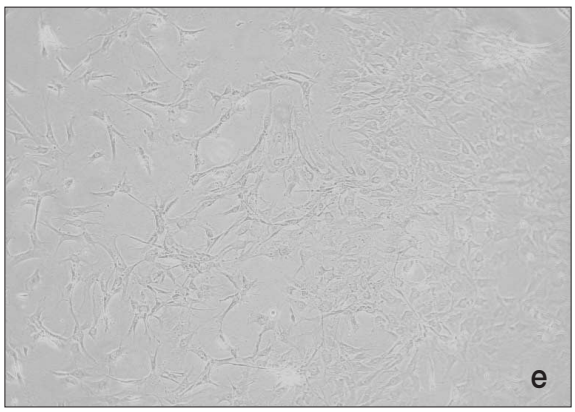
b. P1 group after 2 week culture (Phase contrast, ×200)



c. P2 group showing abundant cells with multi-polar processes and spindle-shaped morphology (Phase contrast, ×100)



d. P3 group (Phase contrast, ×200)



e. P4 group showing fibroblast-like cells with polygonal shape and blunt cytoplasmic processes (Phase contrast, ×200)

Fig. 5. Photomicrographs of plain cultured cells.

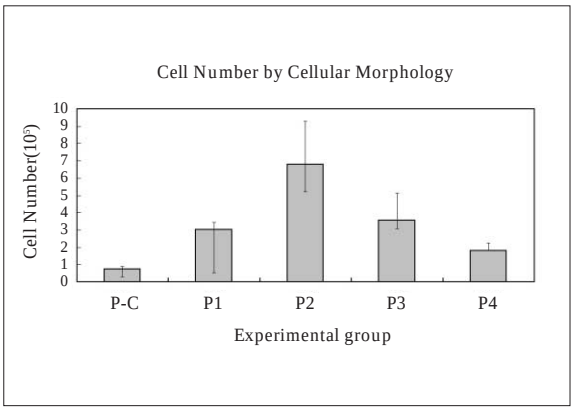


Fig. 6. Cell number comparisons by cellular morphology.
P-C : cytosine arabinoside was not applied during two weeks
P1 group : cytosine arabinoside (Ara-C, 1㎕ / ml, 5 mM) was applied on the harvesting day
P2 group : Ara-C was applied after 24 hour
P3 group : Ara-C was applied after 48 hour
P4 group : Ara-C was applied after 72 hour

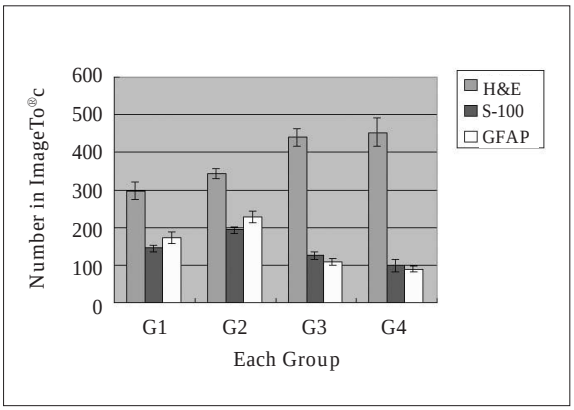


Fig. 7. Cell number comparisons in three-dimensional culture after 2 weeks.
G1 group : cytosine arabinoside (Ara-C, 1㎕ / ml, 5 mM) was applied on the harvesting day
G2 group : Ara-C was applied after 24 hour
G3 group : Ara-C was applied after 48 hour
G4 group : Ara-C was applied after 72 hour

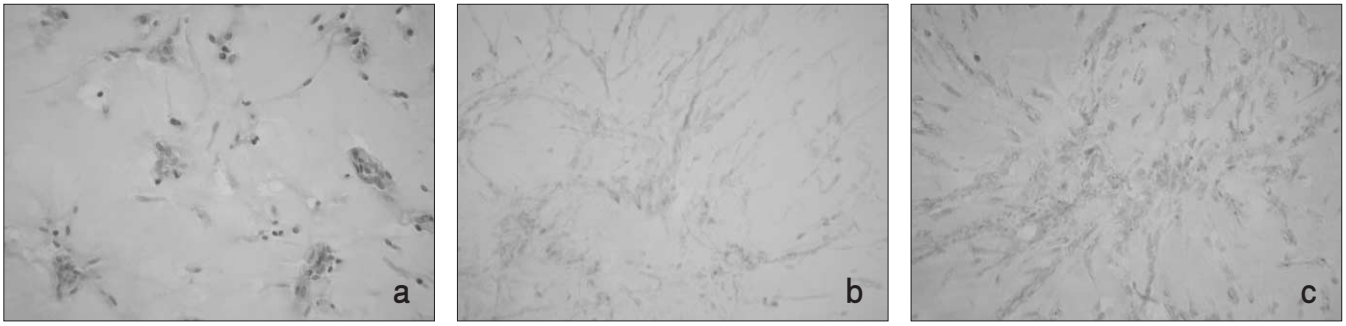
Table 2. Cell number and relative percentage (%) respect to the H&E stained cells in three-dimensional culture after 2 weeks

	H&E	S-100	GFAP
G 1	297.02±23.4	144.80±8.8 (48.75%±1.75%)	173.21±16.2 (58.31%±0.81%)
G 2	343.62±12.5	193.21±9.4 (56.22%±0.67%)	228.4±14.7 (66.46%±1.83%)
G 3	438.72±22.5	125.93±8.9 (28.70%±0.53%)	108.93±10.0 (24.82%±0.96%)
G 4	452.96±36.7	98.83±17.2 (21.81%±1.88%)	89.32±7.4 (19.72%±0.03%)

Each data were counted using ImageTool® computer analysis image system and all data was significant ($p<0.05$).
G1 group : cytosine arabinoside (Ara-C, 1㎕ / ml, 5 mM) was applied on the harvesting day
G2 group : Ara-C was applied after 24 hour
G3 group : Ara-C was applied after 48 hour
G4 group : Ara-C was applied after 72 hour

group showed the most increased positive cells with large amount of extracellular collagen matrix, followed G1 group. The typical oval shaped cell body with a prominent nucleus and bipolar extensions, most likely Schwann cells were abundant in G2 group. In the immunohistochemical staining of GFAP, astrocyte and astroglial precursor cells are also abundant in G2 and G1 group (Fig. 8-c, 9-c).

And we calculated the relative percentage of positive cells (S-100 or GFAP positive cells) respect to the total cells (H&E stained cells) in Table 2.

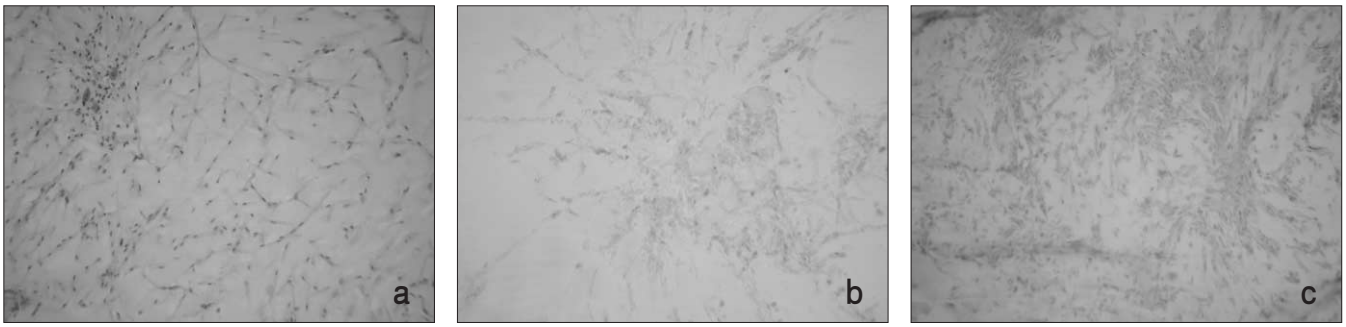


a. H&E stain

b. S-100 immunocytostain showing the typical oval shaped cell body with a prominent nucleus and bipolar extensions, overall spindle shape, most likely Schwann cells

c. GFAP immunocytostain, showing abundant astrocyte proliferation

Fig. 8. Representative photomicrographs of three-dimensional culture in Group 1 (Phase contrast ×400).



a. H&E stain (Phase contrast ×200), showing multiple neurite projections

b. S-100 immunocytostain (Phase contrast ×400), showing bipolar or some tripolar cells were found

c. GFAP immunocytostain (Phase contrast ×200), with abundant astrocyte and astroglial precursor cells

Fig. 9. Representative photomicrographs of three-dimensional culture in Group 2.

a. H&E stain

b. S-100 immunocytostain

c. GFAP immunocytostain

Fig. 10. Representative photomicrographs of three-dimensional culture in Group 3 (Phase contrast ×400).

a. H&E stain b. S-100 immunocytostain c. GFAP immunocytostain

Fig 11. Representative photomicrographs of three-dimensional culture in Group 4 (Phase contrast ×400).

DISCUSSION

Schwann cell proliferates under pathological conditions such as peripheral nerve injury, demyelination and tumorigenesis. Schwann cell secretes a variety of neurotrophic factors, cell adhesion molecules and basement membrane components that are known to promote axonal regeneration[1-3]. Schwann cell studies in rat sciatic nerve have shown that the proliferation of Schwann cell could be induced by several mitogenic factors. But many mitogens that are effective for rat Schwann cell proliferation are also effective in human Schwann cell proliferation. It has also been known that Ara-C that can be applied to human peripheral nerve cultures without toxic effects is crucial for obtaining good results[7-11]. In this study, we used the proliferation-stimulatory activity on Schwann cell derived from rat dorsal root ganglion (DRG). In order to enrich the Schwann cell in the culture, the Schwann cells have to proliferate actively and the other non-relevant cells, i.e., fibroblasts, should be eliminated in the culture. Ara-C seems to be beneficial to the proliferation of Schwann cell, since its treatment produced more increased Schwann cells in the culture[4-6].

The use of substances known to enrich the Schwann cells *in vitro* culture by reducing the proliferation of other cells is required and proved to be effective in adenylate cyclase (cholera toxin, forskolin, isobutyl-methyxanthine and 8-bromo-cAMP), all of which are very effective in the inhibition of fibroblast proliferation at the low concentrations[9]. Antimitotics, such as Ara-C and 5-fluouracil, have been used as they show high effectiveness in eliminating fibroblasts. It has also been known that Ara-C that can be applied to human peripheral nerve cultures without toxic effects is crucial for obtaining good results[7-11]. This enrichment effect is cummulative with each successive pulse of Ara-C applied and is due to a progressive loss of fibroblasts. But, the cytotoxicity by cytosine arabinoside is also cummulative and noticeable over the period.

In the absence of Ara-C, Schwann cell number remained constant during the first one or two weeks but fibroblasts proliferated very rapidly, in result, Schwann cell did not survive at all due to the contact inhibition by the fibroblasts.[17] In this study, we observed the 2 weeks results from the initial harvesting. Casella et al[18]. and Ansselin et al[19]. demonstrated that 2 weeks period (14 days) of *in vitro* Wallerian degeneration process is adequate to obtain an increased Schwann cell yield. But, these results were concluded from the human nerve explants with heregulin and forskolin during the process of *in vitro* Wallerian degeneration, which 1 cm nerve is around 9.9±0.6 mg of dry fascicles, providing approximately 10^6 cells[17-19]. In contrast, we used only M-10 medium and our enzymatic dissociation technique only to know the Ara-C effects after 2 weeks. Ara-C treated cultures showed a higher Schwann cell percentage (31.0%± 8.09% in P4 group to 65.5%±24.08% in P2 group), compared with that obtained in the abscence of Ara-C (17.6% ±6.03%) in the plain culture after 2 weeks.

Fibroblasts also played an important role in the regeneration of nerve by stimulating the deposition of Schwann cell in the extracellular matrix, that was known to be dominant during the Wallerian degeneration and axonal regeneration. They also affect myelin removal by producing granulocyte macrophage-colony stimulating factor (GM-CSF), which induces macrophages and

Schwann cell to upregulate their surface expression of MAC-2 (a galactose-specific lectin), which is involved in mediating myelin phagocytosis and activating macrophages. Fibroblasts are also essential for providing mechanical, nutritional, and protective structures in peripheral nerve regeneration. It was also found that the perineurium mainly composed of fibroblasts could bridge the nerve gap, providing a guide for the susbsequent cellular elements, such as Schwann cell and new axons. However, the overgrowth of fibroblast may produce the fibrosis in the peripheral nerves and also induce the scar formation at suture sites, which may induce an adverse effect on nerve regeneration[20,21]. So it is presumed that the minimum number of fibroblasts and the maximum number of Schwann cell may provide a favourable environment for the nerve regeneration.

The peripheral nerve regeneration requires an interaction of extracellular matrix, neurotrophic factors, and cellular components. Peripheral nerves are composed primarily of two kinds of cells, the neuronal cell and the Schwann cell. Schwann cells are main, supporting factor in the development of nerve regeneration and appear to play a critical role in creating a permissive climate for regrowth of injured nerves, with mitosis in response to nerve trauma and in nerve gaps following axotomy in the early cellular phase of nerve regeneration[1-3], and schwann cells also release a variety of neurotrophic factors during the Wallerian degeneration, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), cell adhesion molecules L1/Ng-CAM, N-cadherin, integrins, and extracellular matrix components such as laminin. These neurotrophic factors are also regarded as axonal growth and promoting factors. In addition, the Schwann cell membrane also serves as a suitable substrate for axonal outgrowth[4,20]. In three-dimensional culture, we used the GFR Matrigel® obtained from the Engelbreth-Holm-Swarm (EHS) mouse. This basement membrane matrix may work as a solubilized basement membrane and its major component is laminin (61%), followed by collagen IV (30%), entactin (7%), and heparin sulfate proteoglycan. It also contains TGF-β, fibroblast growth factor (FGF), Tissue plasminogen activator (TPA) and others[13,22,23]. Therefore, these Matrigel® components must be very favorable environment for the Schwann cell *in vitro* culture for the short time. The transformed anchorage within Matrigel® was also beneficial for the three-dimensional culture of the neurons and other anchorage dependent cells. And more, the protein and proteoglycan macromolecules of the Matrigel® are also known to be highly regulated and are thought to be organized to provide permissive and non-permissive three-dimensional pathways for the neural cell migration and axonal guidance[20,22,23].

The antigenic profile of the three-dimensional Schwann cell culture *in vitro* was studied via immunohistochemistry using GFAP and S-100. The proportion of S-100 positive cells in the three dimensional cultures was consistently 56.22%±0.67%, and the early glial marker, GFAP, was also more abundant in G2 group, up to 66.46%± 1.83% ($p<0.05$), higher yield than other groups with Ara-C application. Because the immunoreaction of GFAP was barely detectable in adult human Schwann cell culture *in vitro*, we presumed that as the Schwann cell differentiated into the mature state, they gradually down-regulated the GFAP expression[18,24,25].

Therefore, we concluded that the Ara-C treatment is effective for the proliferation of Schwann cells contrast to the fibroblasts *in vitro* culture, and the first application after 24 hours from cell harvesting and subsequent 2 pulse treatment was more effective than other application protocols.

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