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Efficacy of optimized *in vitro* predegeneration period on the cell count and purity of canine Schwann cell cultures

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ABSTRACT

Objective(s): Predegeneration is a standard technique to obtain mitotically activated and enriched cultures of Schwann cells (SCs). This study, for the first time, evaluated the impact of various duration of predegeneration on cell yield and enrichment of SCs from dog peripheral nerve.
Materials and Methods: Dog sural nerves were subjected to 5, 10, 15 day-long *in vitro* predegeneration. The total cell yield and the purity of SCs were evaluated in each group on the first and seventh day after plating.
Results: The maximum and minimum numbers of cells were counted in 15 day-long predegeneration and control groups which underwent no predegeneration. The 10 day-long *in vitro* predegeneration group with 80±0.5% SCs enrichment had the best purity after plating day and could maintain its purity with elapsing on cultures.
Conclusion: 10 day-long predegeneration results in the higher cell number and the better and prolonged purity of SCs in culture.

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Introduction

Following peripheral nerve injury such as total section, a series of cellular and molecular changes, called as Wallerian degeneration, starts in the distal stump of the nerve. Denervated Schwann cells (SCs) lose their myelin and go one step differentially back, start proliferation and launch Bungner's band which supports nerve regenerations (1). Head to head alignment of promptly torn peripheral nerves may recover almost completely. However, SCs potential to help axonal regeneration is limited when considerable gap exists between proximal and distal nerve stumps (2). Thus, predegenerated nerves or biodegradable conduits containing SCs are much interested to bridge the gap. The later one needs mass preparation of pure SCs within a rationale period of time (3). In addition, it has been shown that, the predegenerated peripheral nerve graft could protect transected retinal ganglionic cell (RGC) and hippocampal neurons from axotomy induced death and allow far distance axonal growth comparing to fresh nerve implants (4).

Peripheral nerve predegeneration, which could be implemented *in vitro* or *in vivo*, takes advantage of Wallerian degeneration to obtain more and activated SCs comparing to fresh nerve culture (3). *In vitro* predegeneration prevails on *in vivo* counterpart; given the required twice surgical operation to prepare the *in vivo* predegenerated nerve. *In vitro* predegeneration helps fibroblasts (FBs) migration from the cultured explants and thus, reduces following FBs contamination which is the major impediment in SC cultivation (5). Intriguingly, the various durations of *in vitro* predegeneration were evaluated by Kraus and colleagues and seven day-long *in vitro* predegeneration was recommended to obtain the more cell count and purity of rat SCs in culture (5).

Traditionally, the dog as a large animal model provides a useful platform for studying mechanisms and examination of new therapies in a variety of human diseases including cardiovascular disease, diabetes, solid organ transplantation, stem cell therapy, and spinal cord injury (6-8). Similarly, the peripheral nerves of canines could be lesioned by traffic accidents, biting and shooting wounds as well

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as iatrogenic reasons (9). The time span of *in vitro* predegeneration has variously been reported in articles which implemented large animals for SC culture (9, 10). To our knowledge; for the first time, this manuscript addresses the impact of different duration's of *in vitro* predegeneration on the total cell yield and the purity of SCs provided from dog peripheral nerve in primary and extended cultures.

Materials and Methods

A total of 6 male and female 50-60 day old domestic dogs from Iran (2.5 to 3 Kg) were used in this study. All Animal works was done in strict compliance with the approval of Institutional Animal Ethics Committee of the Ardabil University of Medical Sciences, which follows the NIH guidelines for care and use of experimental animals.

Nerve harvesting and predegeneration

Before entry to the experiment, all animals were carefully observed of any neurological problems. Pups were anaesthetized with intravenous pentobarbital (25 mg/kg) administration. After preparation and drape, two incisions, 6 centimeters apart, were made on the path of the right sural nerve of animals (11). The sural nerve was harvested and transferred to the cell culture bench in a sealed container within ice cold PBS. Animals were followed a week after surgery and then returned to their holders. Nerves, in a sterile environment, were weighed and washed three times with 2% penicillin/streptomycin supplemented PBS and the epineurium was stripped off under stereomicroscope. A sterile scalpel was utilized to cut the tissues into short segments of 2–4 mm in length and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (3). Explants were randomly assigned into 5, 10, 15 day-long predegeneration groups. Explants with no *in vitro* predegeneration assumed as control group. Peripheral explants were incubated at 37˚C and 5% CO_2. Medium was refreshed every 3-4 days and petri dishes were weekly changed.

Dissociation and primary plating

After being washed with PBS, explants were transferred into a new dish and incubated with dissociation solution at 37˚C and 5% CO_2 for 20 hr. Dissociation medium consisted of DMEM, 15% FBS and an enzyme mixture including 0.125% collagenase Type IV and 1.25U dispase/per ml (3). Nerve fragments were intermittently agitated to disperse explants. Finally, cells were collected and centrifuged at 1800 rpm for 10 minutes at room temperature. The pellets were resuspended in DMEM supplemented with 10% FBS and plated onto poly –L- ornithine (15 µg/ml) and laminin (15 µg/ml) coated petri dishes at 75,000 cells/cm^2. After 24 hr, the medium was changed to DMEM supplemented with 2.5 % FBS plus 2 µM Forskolin (FSK) and 10nM Recombinant human neuregulin1-b1/heregulin-b1 epidermal growth factor (EGF) domain (rhuHRG) and cultured for six days (seven days after primary plating).

Characterization of SCs

Refinement of SCs was morphologically evaluated and verified by immunofluorescent staining and flow-cytometry analyses.

Morphology

Under inverted microscope, SCs were referred as phase bright, bi-, tri- or multipolar cells with a small cytoplasm to nucleus ratio; while fibroblasts were identified by a much more flattened polymorphic shape with larger rounded nuclei (3). Pictures were prepared using an inverted microscope equipped with a digital camera (Olympus DP 71). The total cell numbers and number of SCs were counted from six random fields (magnification 10X) by two independent investigators.

Immunofluorescent staining

SCs were plated on laminin and poly L-ornithine coated 12 well plates. The cells were fixed with 4% paraformaldehyde for 30 minutes. Fixed cells were washed twice with PBS and incubated with a primary antibody against p75 low affinity NGF receptor ($P75^{LNGFR}$, 10 ng/ml, Abcam; ab8877) diluted in blocking buffer at 37°C for 2 hr. Blocking buffer consisted of 10% goat serum and 1 mg/ml bovine serum albumin (BSA) in PBS. For secondary antibody, goat anti-mouse IgG conjugated-fluorescein isothiocyanate (FITC, 1:50, Chemicon; AP124F), were utilized for one hour and then counterstained with DAPI (Sigma, D9542) for 2 minutes at room temperature. Images were visualized with a fluorescent microscope equipped with digital camera (Olympus DP 71). For negative controls, the primary antibody was excluded. The percentage of putative Schwann cells among the total cells was obtained based on three independent experiments.

Flow-cytometry analysis

After antibody incubation, cells were analyzed on a FACS Calibur flow-cytometer (Becton-Dickinson, Germany) and Cell Quest Pro™ software. At least 10,000 events were recorded for each sample and were processed with WinMDI 2.8 software. The fraction of $P75^{LNGFR}$ positive cells in the fluorescence intensity dot plot was compared to the total amount of intact cells to determine the purity of SCs and histogram was subsequently prepared.

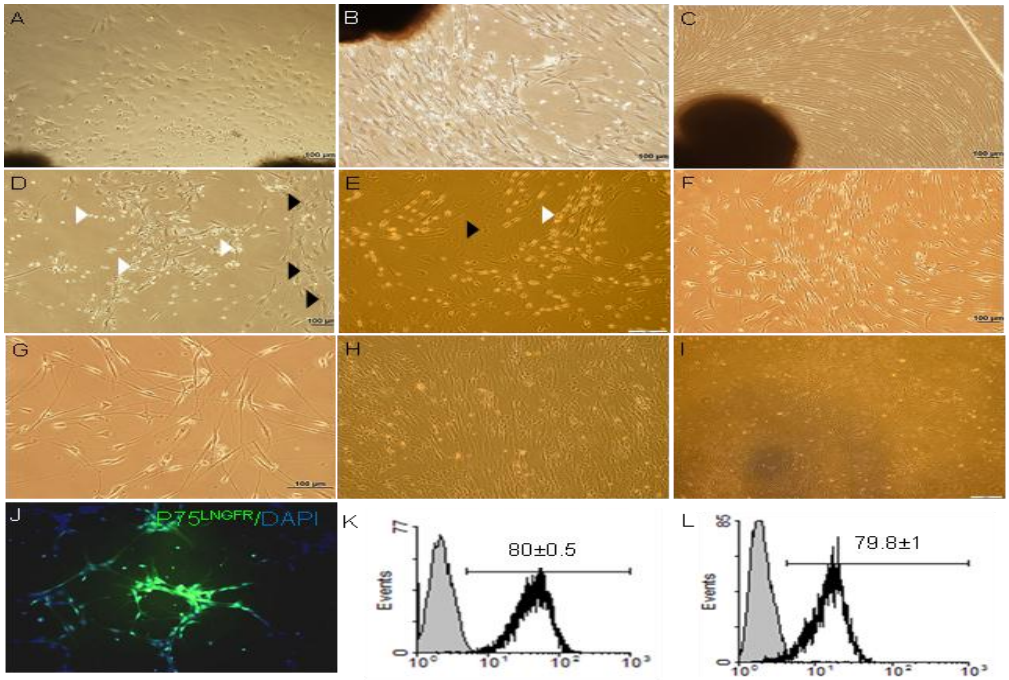

Figure 1. Photomicrographs of the sural explants, primary culture of dissociated nerves and SCs characterization via immunostaining and Flow cytometry for $P75^{LNGFR}$. The sural explants were shown after 5 (A), 10 (B) and 15 (C) day-long *in vitro* predegeneration. First day of primary cultures in control group, 5, 10 and 15 day-long *in vitro* predegenerated nerve were displayed in D- F, respectively. Higher magnification of F was represented in part G. Some SCs were indicated by white arrows head in part D and F, which could be morphologically identified from FBs marked with black arrow head. Samples of for 5 and 15 day-long predegenerated groups after seven days were shown on H and I, respectively. After seven days of primary culture, SCs were immunostained against $P75^{LNGFR}$ as a specific marker for SCs (J) and counterstained with DAPI for nucleus. SCs purity 10 day-long predegenerated group (K) has been illustrated via flow-cytometry histogram. Purity of SCs in 10 day-long predegeneration group after seven days of enzymatic dissociation and culture was shown in part (L) plot

Statistical analysis

Data are expressed as mean±SD. The one-way ANOVA followed by Tukey's post hoc test was performed to determine statistical differences between cultures. All statistical analyses were performed on standard statistics computer software (SPSS 19 for Windows). *P*-value<0.05 was considered statistically significant.

Results

The sural nerves with 5-6 cm length were excised from animals. The average weight of nerve after peeling the epineurium off was 18±2 mg. Nerve fragments were cultivated in different durations of *in vitro* predegeneration. Following elapsing time on predegeneration, FBs migrated out of explants, proliferated and covered containers. In addition, some SCs were identified, but SCs to FBs ratio was very low, particularly when predegeneration time was extended (Figure 1A-C).

Enzymatically digested explants were plated onto PLO/L coated petri dishes and total cell yield and percentage of SC purity were documented at first and seventh day after primary plating (Table 1). The cumulative number of cells and purity of SCs in all groups were significantly improved comparing to control (Figure 1D-G) (*P*<0.001). Highest and lowest number of cells were counted in 15 day-long predegeneration and control groups which were 15.2±0.7 and 10±0.3 million cells/per nerve, respectively (Table 1). The 10 day-long predegeneration group with 80±0.5% SCs purity was a head of others in first day after plating (Fig 1F, G and K) (Table 1) (*P*<0.001). Seven days after primary plating, the cumulative number of cells increased in all groups. The number of cells in 10 day-long predegeneration group (35.1±0.3 million cells/per nerve) was significantly more than 5 day and 15 day-long predegeneration groups (Table 1) (*P*<0.01). Meantime, the purity of SCs was reduced in all groups (Table 1). SCs purity decreased to 51.1±0.8 in 15 day-long predegeneration group which indicated significant drop in comparison to 5 and 10 day-long predegeneration groups (at least *P*<0.001). Interestingly, 10 day-long predegeneration group was almost able to sustain SCs purity through cultivation period at least for next six days (Table 1) (Figure 1L).

Table 1. Total cell yield and purity of canine schwann-cell are influenced by various durations of *in vitro* predegeneration

Duration of *in vitro* predegeneration	Cell yield a day after plating (million cells/nerve)	Purity a day after plating (%)	Cell yield seven days after plating (million cells/nerve)	Purity seven days after plating (%)
Control	10±0.3	54.8±0.7	20±0.9	46.6±0.9
5	12.8±1	63.3±0.5	27.8±0.7	57.5±0.1
10	14.1±0.6	80±0.5	35.1±0.3	79.8±1
15	15.2±0.7	64±1.7	31.9± 0.6	51.1±0.8

Data represent the mean of results from n = three experiments (±SD)

Discussion

In 1981, Albert Aguayo and associates recognized axonal elongation through a bridge of predegenerated peripheral nerve implemented across the transected site of central nervous system (12). Later on, *in vivo* predegeneration was introduced as a standard technique to obtain mitotically activated and enriched cultures of SCs (13). These purified SCs could be used in preparing SC containing nerve conduits (14) or transplantation into central nervous lesions. However, performing double surgical operation and lower cell proliferating potential after first passage are two main drawbacks which confines clinical usage of *in vivo* predegeneration and fosters it's *in vitro* counterpart instead (5). Interestingly, transplantation of seven day-long dissociated *in vivo* predegenerated nerve into spinal cord injury resulted in almost the same level of anatomical functional recovery as *in vitro* predegenerated derived SCs (15).

Since the canine model provides a large animal system which achieved advances may directly adjustable to human (8), the duration of *in vitro* predegeneration and its impact on dog SCs culture would be of interest. The cumulative cell counting showed that although the highest cell yield was obtained in the longest predegenerated group, 10 day-long predegeneration group had significantly the higher SCs purity compared to other groups in primary plating. This finding supports articles which implemented ten days of *in vitro* predegeneration for SCs cultures (9). In 2010, Kraus and colleagues evaluated the various durations of *in vitro* predegeneration on total cell yield and SCs purity on rat model. they showed that seven day-long *in vitro* predegeneration has considerable impact on SCs yield and purity in culture condition (5). Although melanocyte growth medium (MGM) was utilized for rat SCs culture, the results of Kraus et al, and our findings have displayed an augmentation of SCs number and reduction of FBs propagation up to 10 day-long of *in vitro* predegeneration.

In the second step, the primary plated cells were cultured for six more days. Although the cumulative cell number increased twice or more with culture extension, the purity of SCs declined in all groups. Interestingly, 10 day-long predegeneration group could sustain the purity of the primary plating for six more days and considerable loss of purity was observed in 15 day-long group. This finding indirectly implies that predegeneration for more than 10 days, may result in more proliferative FBs. Although, it remains to understand the mechanisms related to this finding, review of literatures indicates that the alteration of retinoic acid (RA) and some of cytokines at lesioned spot may relate with our findings. It has been shown that the activity of retinaldehyde dehydrogenase 2 (RALDH2: RA synthesizing enzyme) is increased following peripheral nerve injury. Intriguingly, RA peaks at seven days after the injury. Furthermore, the expression of neuregulin receptor ErbB3 on SCs is up regulated in response to RA (16). Besides, it is known that ATRA administration is helpful in epidural fibrosis relief after laminectomy (17). Thus, it could be hypothesized that RA may reduce nerve fibroblasts proliferation.

Following peripheral nerve insult, cytokines like IL-1, IL-6 and TNFα are released and reach their maximum level by 14 days after nerve injury. It has been proved that IL-1 and TNFα enhance FBs growth and proliferation (5). Accordingly, our results showed that 15 day-long predegeneration has amplified fibroblast proliferation potential. However, the exact mechanism(s) involved remains to be addressed in future investigations.

Conclusion

Our findings demonstrate that the 10 day-long *in vitro* predegeneration results in the higher cell number and the better and prolonged purity for SCs and therefore 10-day long *in vitro* predegenerated may recommended as nerve conduit grafts.

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Conflict of interest

The authors have declared that no competing interests exist.

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