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Original Research

Modified PLGA nanofibers as a nerve regenerator with Schwann cells

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Abstract: Polylactide-co-glycolide acid (PLGA) is known as a biodegradable and biocompatible polymer. This polymer has been highly used in tissue engineering. In this study, the biological behavior of Schwann cells (Rat) was investigated in co-culture with L lysine/gelatine coated PLGA nano-fiber. In this study, PLGA was dissolved in a hexafluoro propanol based solvent and nanofiber prepared by an electronic method. They were coated with gelatin and poly-L-lysine individually. These polymer properties were investigated by Scanning Electron Microscopy (SEM) analysis and contact angle measurement. After extraction of rat Schwann cells, the cells were cultured in three groups of nano-fiber; nano-fiber PLGA, nano-fiber gelatine coated PLGA and nano-fiber poly-L-lysine coated PLGA. Cell death and Cell proliferation were evaluated by Acridine orange staining (living cell with a green nucleus and dead cell with an orange nucleus) and morphology was investigated by SEM in 2, 4 and 6 days. The diameter of electronic nanofiber PLGA was between 270 to 700 nm. Average contact angles of PLGA, PLGA coated with gelatine, coated with poly-L-lysine and PLGA were 40.12, 64.58 and 107.66degrees, respectively. The findings showed a significant reduction of cell proliferation in PLGA nanofiber (it was important than PLGA without nano-fiber ($P < 0.05$)). But, this amount was increased in nanofiber which coated with poly-L-lysine and gelatine. PLGA nanofiber-poly-L-lysine was more biocompatible than PLGA nanofiber-gelatine and this comparison was done with rat Schwann cells.

Key words: Nano-fiber; Schwann cells; Poly-L-lysine; Gelatine.

Introduction

Central and peripheral nervous system diseases cause the loss of myelin and the trauma causes major functional disorders in patients. But, the capacity for regeneration in PNS axons is more than The CNS axons (1). Studies show that there is a significant difference between the peripheral and central nervous system. The peripheral nervous system has a high capacity for axon repairing because the central nervous system has inhibitory factors and axon regeneration will be stopped after this defect (2). The overall frequency of peripheral nerve injuries was 2.8% of our population of patients with multiple injuries. Peripheral nerve injuries were defined as an injury to a major nerve in the upper or lower limb. Peripheral nerve injuries are increasingly being recognized with developed trauma services and also, are not uncommon in trauma. Primary identification of peripheral nerve injuries is a significant step, because untreated peripheral nerve injuries may cause serious disabilities in later life (3).

PNS axons are repaired in an appropriate environment. In-vivo and in-vitro studies indicate that among components, Schwann cells are essential for axonal regeneration. Schwann cells are started to proliferate when they are facing the injuries. This situation provides an environment which stimulates nerve regeneration (4, 5). On the other hand, these cells accelerate the reconstruction process to remove wastes, including degraded myelin and transfer them to the macrophages in the early stages of injury (6). The use of alternative scaffolds to repair damaged tissue is one of the things which is very important in tissue engineering. There are some problems in transplantation such as tissue transplantation from one person to another due to immunological responses and rejection by the host immune system and tissue harvested from ones of own and transplants to another part of the body. Hence, tissue engineering and production of natural and synthetic polymer scaffold are more crucial than ever (7-12).

Tissue engineering scaffolds are highly regarded such as biodegradable drug delivery for applications in medical engineering, wound healing, tissue regeneration, replacing organs in the past decade. Technologies such as electronic biodegradable polymers such as PLA, PGA, chitosan copolymer such as PLGA used for cell culture in tissue engineering. In addition, the scaffold of natural human proteins (collagen and elastin) has been investigated as biological materials for creating the tissue scaffolds. These materials have been known as biocompatible, flexibility, cell adhesion materials (13-20). Surface modification method is done by secondary

molecule mixed with a biodegradable polymer to increase the absorption properties of proteins, modifying the surface polarity or change the surface hydrophobicity of the fiber (19, 21). PLGA composite is one of the synthetic biocompatible and biodegradable copolymers. PLGA composite has advantages include its biodegradability, that would be changed from months to years and this feature makes it suitable for studying the regenerative nerve and Schwann cell culture in in-vivo or in-vitro. But, this polymer does not have hydrophobic properties and also, it has a lack of cellular connectivity sites on this polymer and this polymer can have a negative effect on the growth and cellular connectivity (22, 23). Therefore, a lot of studies have been done to solve this problem such as surface modification of PLGA, fibronectin, hyaluronic acid and poly-lysine (21, 24, 25). The comparison between the adhesions of the cells on modified nanofibers with poly-lysine or gelatin has not been taken. In this study, in the case of Schwann cells of rat, the biocompatibility of PLGA was investigated to find the best nanofiber for treating the peripheral nerve injuries. In this study, the biological behavior of Schwann cells (Rat) was investigated in PLGA coated with poly-L-lysine and gelatine.

Materials and Methods

Prepare a polymer solution

PLGA (LA/GA 75/25, Mw=66000-107000),. Gelatin, poly-L-lysine and Hexafluoro Bis-propanol were purchased from Sigma Aldrich USA. PLGA solution (7%) was prepared with Hexafluoro Bis-propanol in a balloon and then, closed with the Teflon and stirred for 5 hours. Then, it was centrifuged for removing air bubbles (5 minutes, 5000 rpm) (26, 27).

PLGA electrospinning

PLGA solution was loaded into a 2 mL syringe and was connected to a high voltage (12 kV) power supplier for electrospinning. The polymer solution was fed at a rate of 1 mL/h using a syringe pump (Model 100 series; KDS). A rotating drum (6 cm in diameter and 1 cm in width) was horizontally located at 10 cm from the needle tip. Then, they were coated with poly-l-lysine or gelatin. Finally, three scaffolds were made includes; PLGA coated with gelatin, poly-L-lysine and PLGA nanofibers.

Prepare a scaffold from nanofibers PLGA

Nanofibers were cut and put in 6 well plate and then, they were sterilized by alcohol 70% and UV for 1 hour. They were fixed in PBS buffer for one day.

Schwan cell extraction

In this study, two methods were used for extracting of Schwann cells. Newborn rats (4-6 days) were anesthetized with chloroform. Their sciatic nerves were obtained and put in dishes containing penicillin, streptomycin and PBS. In the first method, they were cut into pieces and transferred in DMEM-F12 (Gibco-England) containing 0.01% collagenase (Sigma) and 0.25% trypsin (Sigma) for 4 to 6 hours in an incubator with O_2, CO_2 and humidity. They were centrifuged at 2000 rpm for 5 minutes and the supernatant was discarded. Then, precipitation was transferred into a flask (28-30). After enzyme digestion, they were transferred into flasks containing DMEM-F12 culture medium with 10% of FBS and incubated for 4 days in 37ºC and 5%. The culture medium was replaced with fresh medium containing 2.5% FBS for removing debris and dead cells and they were incubated in an incubator for achieving a maximum number of cells. In the second method, we did not use FBS in DMEM-F12 after enzyme digestion and incubated for 4 days in 37ºC and 5% and Then, the culture medium was replaced with fresh medium with 2.5 % FBS for removing debris and dead cells and they were incubated in an incubator for achieving a maximum number of cells. The culture medium was replaced every 48 hours.

Cell culture on PLGA scaffold

Schwann cells prepared from cell suspension in the third passage. The number of 5×10^3 cells were added to each cell culture plates and were placed in an incubator (Co_2 5%, 37 ° C). Cell culture groups: first group; without polymer scaffolds (control group), second group; polymer nanofibers PLGA scaffold, third group; polymeric PLGA nanofibers scaffold coated with gelatin and fourth group; PLGA nanofibers scaffolds coated with poly-L-lysine.

Evaluation of growth and proliferation

To check the growth and proliferation of cells, each well were observed with an inverted microscope for 2, 4 and 6 days. Cells growth on polymer scaffolds were not so visible and countable. Therefore, Acridine Orange staining was used for counting cells.

Cell death

Acridine Orange staining was used to determine cell death at the end of 6 days. In briefly, 100 ml of color Acridine Orange were added in each of the squares on 6 days, 100 cells were counted and taken photos with a fluorescence microscope. Those cells were dead with Orange and red color in the cytoplasm and nucleus but the live cell was green in the nucleus and cytoplasm.

The purity of Schwann cells

4 microscopic fields were counted to obtain purity, and the number of Schwann cells (which were stained with antibodies) were calculated as a percent of total cells. There are some markers for the difference between fibroblast and Schwann cells and the most important of them is S100 and or P75 which fibroblasts are negative and Schwann cells are positive (31-33).

Statistical Analysis

A one-way ANOVA was used to compare the means of different data sets, and $P< 0.05$ was considered as an indication of a significant difference.

Results

SEM and Invert images

PLGA nanofibers electrospinning were formed with different thicknesses in different directions. The diameter of porous was estimated between 270 to 700 nm in SEM and Invert images (Figure 1). Invert images, shown the fibers stretched and smoothly (Figure 2-A)

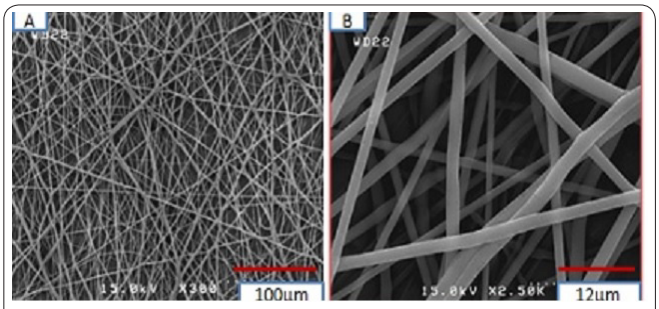


Figure 1. Nanofibers coated with poly-L-lysine 300× (A) and 2500× (B) (SEM Images). The diameter of the fiber porous was estimated between 270 to 700 nm.

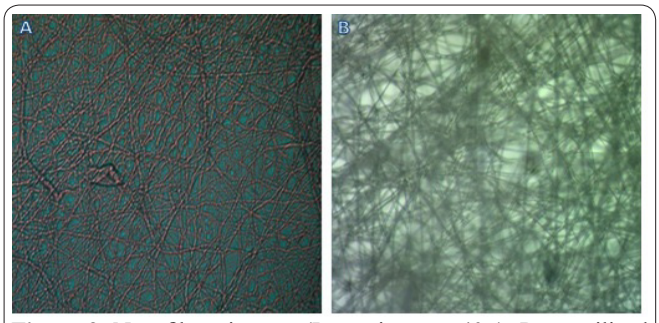


Figure 2. Nanofibers images (Invert images, 40×). Pre-sterilized with alcohol fibers that appears smooth and stretched (A). After sterilization with 70% alcohol which they are shrinkage mode (B).

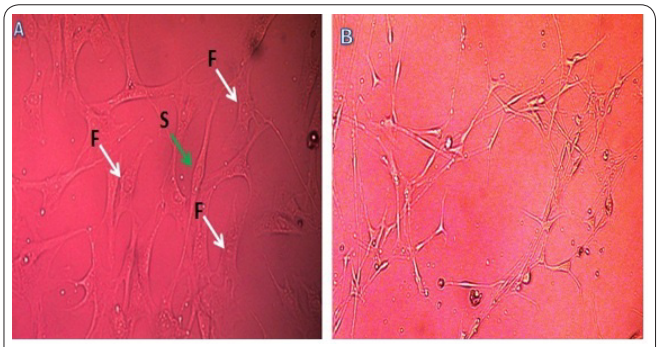


Figure 3. Schwann cells were cultured in a DMEM-F12 medium under an inverted microscope (40×) (A), a Schwann cell is shown (S) among fibroblast cells (F). Image of Schwann cells is visible as bipolar and tripolar cells (20×) (B).

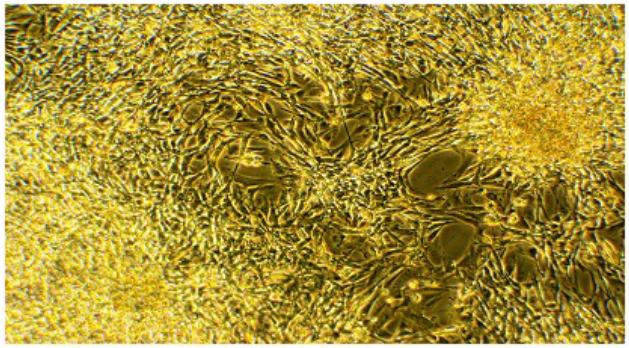


Figure 4. Phase contrast microscope images. Rat sciatic nerve cells were cultured in DMEM-F12 medium (Second week).

and after sterilization with 70% alcohol the fibers formed in shrinkage mode (Figure2- B).

Schwann cells morphology

In the case of Microscopic study bipolar cells among of Schwann cells (spindle-shaped) or three-pole (star-shaped) elongated oval cells were observed, while fibroblasts were shown as wide cells with round nuclei and large (Figure 3). These cells were distinguishable under a phase contrast microscope (Figure 4).

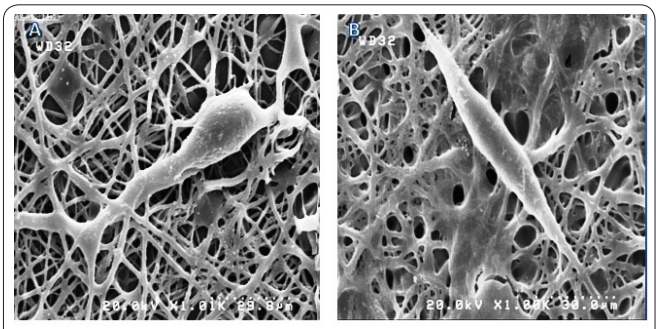


Figure 5. SEM images of cultured Schwann Cells on PLGA fibers. The PLGA fibers coated with poly-lysine (A). PLGA fibers coated with gelatin (B).

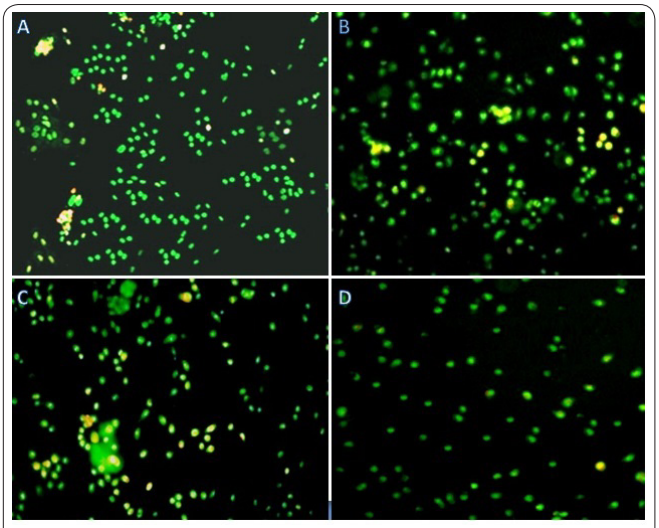


Figure 6. Acridine Orange staining of Schwann cell on 6 days. PLGA nanofibers as a control group (A), PLGA coated with poly-L-lysine (B), PLGA coated with gelatin(C) and PLGA without coating (D).

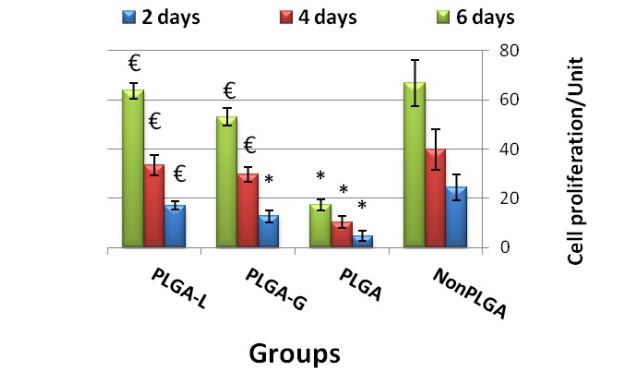


Figure 7. Schwann cells proliferation during 2, 4 and 6 days ($P < 0.05$).

Percentage of Schwann cells purity

Cells morphology and S100 antibodies methods showed that Schwann cells purity were 96% in the first method and 97% in the second method. There was no significant difference between the two methods.

Culturing Schwann cells on PLGA scaffolds

In the electron microscope pictures, Schwann cells were observed on the nanofibers and they were detectable from fibroblasts significantly (Figure 5).

Schwann cells proliferation on nanofiber

Schwann cell proliferation showed a significant

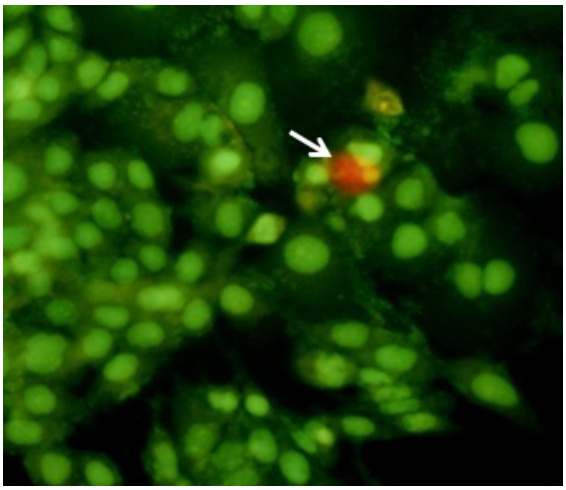


Figure 8. Acridine Orange staining (40×). After 6 days, the lives cells were shown in green with a nucleus in the cytoplasm and a significant difference was not observed between the groups.

decrease compared to the control group in cell culture on PLGA. Figure 6 and 7 are shown the proliferation of Schwann cells in PLGA coated with poly-L-lysine, PLGA coated with gelatin, PLGA and PLGA without coating. The proliferation of PLGA coated with poly-L-lysine and PLGA coated with gelatin were more than PLGA and PLGA without coating.

Cell death

The cells were dead with Orange and red color in the cytoplasm and nucleus but the live cell was green in the nucleus and cytoplasm. The majority of the cells were alive after 6 days and a small percentage of cells were dead and there was no significant difference between groups (Figure 8).

Contact Angle

The contact angle studies indicated that the hydrophobicity of PLGA coated with poly-L-lysine significantly increased (contact angle was increased about 55%). The contact angle of PLGA coated gelatin was more than PLGA coated with Poly-L-lysine (Table 1). Comparison between of them showed the large increase in Poly L lysine coated with PLGA ($P< 0.05$).

Discussion

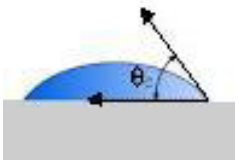
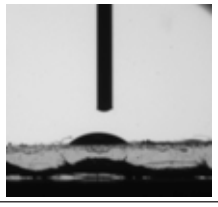
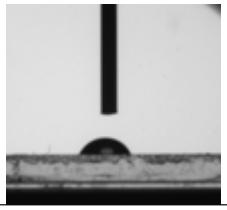
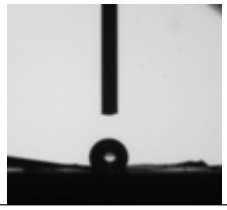
Cell therapy is one of the ways for nerve regeneration and Schwann cells have been investigated in Iran and other countries as a stimulator in nerve regeneration (34, 35). Results of this study showed that the traditional methods such as cultured Schwann cells on an appropriate scaffold can be modified to reduce risks and without reducing the number and purity of Schwann cells simultaneously. There are different methods for increasing the Schwann cells purification and reducing the fibroblasts purification by adding Jenotoxin such as Arabinoside (which is clinically used as an anti-cancer drug) in cell culture (36).

In this study, drugs were not used but, the amount of FBS serum was changed to achieve maximum efficiency and purity of Schwann cells as a cheap and available method. Two methods were used for the proliferating of Schwann cells and we found that the second method had a better result. DMEM-F12 without FBS was used after enzyme digestion and the culture medium was replaced after 4 days with fresh medium containing 2.5 % FBS for removing debris and dead cells. Then, they were incubated in an incubator for achieving a maximum number of cells. Verdu and colleagues studied the culture medium containing different concentrations of fetal calf serum (FCS) serum and the best results obtained from FCS serum in 1% concentrations (37). Hedayatpour and colleagues studied Schwann cells which were isolated from sciatic nerves of the adult rat. They used supplemented DMEM media with 10%, 5%, 2.5%, 1.25%, and 0.625% FBS which were used to determine their effect on the density and purity of Schwann cells after 10 days and they found that purity was significant in the supplemented medium with 2.5% FBS.

Komiyama and colleagues studied on different concentrations of FBS serum from 0 to 10% to stimulate the migration and proliferation of Schwann cells which 2.5% FBS was determined as the amount of serum to stimulate proliferation Schwann cells and inhibition of fibroblast proliferation (38). Pansri and colleagues studied on the different concentrations of PLGA solution from 2% to 7% in HFIP solvent voltage and found that the best concentration was 7% (voltage 12 kV, distance 10 cm) (39). In this study, images of SEM showed that diameter porous of the scaffold were from 270 to 700 nm and cell had a good attachment to nanofibers. Contact angle indicated that PLGA coated with poly-L-lysine and gelatin are the hydrophilic but hydrophilic property of poly-L-lysine coated PLGA were more than gelatin coated PLGA. And also, PLGA groups which were coated with gelatin showed a reduction for 2 days. in the case of PLGA coated with poly-L-lysine in comparison to uncoated PLGA Schwann cells proliferation was significantly increased in all days, this amount was determined in a group of gelatin coated PLGA after 4 and 6 days.

Results showed that in the case of cell proliferation, a significant reduction was determined in nanofibers

Table 1. Contact Angle of PLGA coated with poly-L-lysine and gelatin, without coating.

Nanofibers	PLGA coated with poly-L-lysine	PLGA coated with gelatin	PLGA without coating
Contact Angle	40.12	64.58	107.66
			

PLGA in comparison with other groups (the PLGA coated with gelatine, the poly-L-lysine and nanofiber), but cell proliferation was increased in PLGA coated with poly-L-lysine and gelatin groups.

Acknowledgment

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Interest conflict

There is no conflict of interest.

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