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Effects of low level laser therapy on proliferation and neurotrophic factor gene expression of human schwann cells in vitro

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ABSTRACT

Previous studies have been proposed that proliferation and release of certain growth factors by different types of cells can be modulated by low level laser therapy. We aimed to demonstrate the effect of laser irradiation on human schwann cell proliferation and neurotrophic factor gene expression in vitro. Human schwann cells (SCs) were harvested from sural nerve that was obtained from organ donor followed by treatment with an 810 nm, 50 mW diode laser (two different energies: 1 J/cm^2 and 4 J/cm^2) in three consecutive days. SC proliferation was measured, after first irradiation on days 1, 4 and 7 by the MTT assay. Real time PCR analysis was utilized on days 5 and 20 to evaluate the expression of key genes involved in nerve regeneration consist of NGF, BDNF and GDNF. Evaluation of cellular proliferation following one day after laser treatment revealed significant decrease in cell proliferation compared to control group. However on day 7, significant increase in proliferation was found in both the irradiated groups in comparison with the control group. No significant difference was found between the laser treated groups. Treatment of SCs with laser resulted in significant increase in NGF gene expression on day 20. Difference between two treated groups and control group was not significant for BDNF and GDNF gene expression. Our results demonstrate that low level laser therapy stimulate human schwann cell proliferation and NGF gene expression in vitro.

1. Introduction

During last decade, low level laser therapy (LLLT) has attracted many attentions [1]. In this technique, optical waves are used with specific wave length so no heat is produced. LLLT was first used in 30 years ago for treating sluggish ulcers and burn wounds [2].

Albeit the exact mechanism of its action remains obscure, there are some experiments reporting the positive effects of this method both in vitro and in vivo. In vitro studies have shown that the application of LLL could have an influence on cellular process including altering DNA synthesis and gene expression [3–6], protein production [7,8], biomodulation in cytoskeletal organization [9] and stimulating cellular proliferation [10–13] and differentiation [8,14,15].

There has been some reports showing results of LLL on different aspects of neural biology, including significant improvement of neurite outgrowth [16–18] ATP production [19], gene expression and secretion of neural factors [3]. One of the current usages of LLL is in central and peripheral neural regeneration [20–24].

Schwann cells are actually glial cells on peripheral neural system (PNS). These cells remyelinate demyelinated axons and are responsible for guiding regenerating axons into central tracts [25]. In addition, several neurotrophic factors are secreted by them including nerve growth factor (NGF); brain-derived neurotrophic factor (BDNF); glial cell line-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF). Also, they can produce some extracellular matrix components like laminin which helps injured axons to attach and extend [26].

In this experiment, the effects of LLL gallium–aluminum–arsenic (Ga–Al–As) on human schwann cell proliferation and neurotrophic factor gene expression were studied for the first time.

2. Material and methods

2.1. Cell culture

Human schwann cells were harvested from sural nerve that obtained from organ donor (age from 20 to 25, $n = 3$). All protocols

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of human tissue handling were approved by ethical committee of Stem Cell Technology Research Center, Tehran, Iran. Isolation and culture of schwann cells were performed according to the previously described method by Calderón-Martínez et al. using cytosine arabinoside (Ara-C) for highly purification of cells [27]. In brief, after 14 h remaining of 1–2 mm segments of peripheral nerve in medium (DMEM supplemented with FBS) for wallerian degeneration, the enzymatic dissociation medium containing DMEM (Gibco, USA), supplemented with 1300 U/mL collagenase IV, 10 U/mL dispase (both from Sigma, USA) and 100 mg/mL streptomycin and 100 U/mL penicillin (Gibco, USA) was added to cells for 3 h in 37 °C until most of the cells were dissociated from explants. The isolated cells were cultured in the 25 cm^2 flasks, four pulses of 10 μM Ara-C were added every 4 d with beginning on day 4 in culture medium.

On day 16, after fixation of the isolated cells in 4% paraformaldehyde, they were processed for immunofluorescence staining. In brief, after fixation, cells were permeabilized with 0.1% Triton X-100 for intracellular antigen staining, and then blocked with 5% goat serum. Cells were then incubated at 4 °C overnight with the anti-S100 (Chemicon, USA) antibody. Cells were then incubated with appropriate FITC-conjugated secondary antibody (Abcam, USA) for 3 h at room temperature. The cells nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:1000) and visualized by fluorescent microscope (TE2000-S; Nikon-Eclipse, Tokyo, Japan). Images were merged using Adobe Photoshop CS4, version 11.

2.2. Low level laser irradiation

Infrared laser irradiation was obtained using an 810-nm-emitting gallium–aluminium–arsenic (Ga–Al–As) diode laser (Advanced, Australia) in continuous mode and a maximal output power of 50 mW. First, 5000 cells were cultured on each well of 24 well plates. The laser beam irradiated the wells from a distance of 5 mm. The output laser beam was directed at the center of the wells and created 0.4 cm^2 spot size through medium culture. The laser was irradiated at two different energies (1 J/cm^2 and 4 J/cm^2). In order to deliver an energy density of 1 J/cm^2 to the cultured cells, irradiation time of 8 s was required. In order to deliver an energy density of 4 J/cm^2, required irradiation time was 32 s. The laser protocol was started 6 h after cell plating and the irradiation was repeated for three days. The irradiation was done on a clean bench through the medium culture. The control plates received no laser irradiation but were removed from the incubator for the same duration as laser treated plates.

2.3. MTT assay

On days 1 (immediately after the first day laser treatment), 4 and 7 in culture, proliferation of the cells was assessed using 3-(4,5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide (MTT) solution assay, according to the manufacturer's instructions (Sigma, USA). Briefly, MTT solution in an amount equal to 10% of the culture medium volume was added to each well. After 3 h the media were removed and MTT solvent (50 ml, 0.1 N HCl in anhydrous isopropanol) was added to dissolve the resulting MTT formazan crystals. Absorbance was measured at a wavelength of 570 nm. All experiments were done in triplicate.

2.4. Real-time PCR

On day 21, total RNA was extracted using the Qiazol (Qiagen, Germany) and cDNA synthesis was performed using Revert Aid kit (Fermentase, Burlington, Canada). Next, the cDNA products were used for standard RT or Real time PCR. Real time PCR reactions were performed using Maxima™ SYBR/ROX qPCR Master Mix (Fermentas, Canada) and monitored in Rotor-gene Q real-time analyzer (Corbet, Australia). The presence or absence of expression of some neural growth factors consist of NGF, BDNF and GDNF were evaluated using RT PCR (data not shown) and alteration of the level of expression was evaluated with real-time PCR in triplicate and then the average threshold cycle was estimated and normalized by GAPDH. The fold change of each target gene was calculated with ΔΔCT method. The used primers were designed with primer 3 programs with complete cDNA sequences obtained from the NIH Gene Bank Entrez program.

2.5. Statistical analysis

Statistical comparisons between experimental groups were performed using repeated measures analysis of variances (ANOVA) with SPSS version 13. Data are shown as mean ±SD.

3. Results

After harvesting human schwann cells from sural nerve and adding Ara-C for increasing purification of them, we stained schwann cells with S-100 antibody. Using Image J free software, merged images showed that purification of human schwann cells was >95% (Fig. 1).

SC proliferation was measured by the MTT assay, which assesses cellular proliferation as a function of mitochondrial activity. The starting number of cells within the culture was the same across treatment groups. Evaluation of cellular proliferation following 24 h after first day of laser treatment revealed that the average absorption was 0.235 ± 0.042 for the control group, 0.094 ± 0.022 and 0.135 ± 0.024 for 1 J/cm^2 and 4 J/cm^2 irradiation groups respectively. Significant decrease in proliferation was found in both irradiated groups in comparison with control group (P value <0.05; Fig. 2). On day 4, one day after final laser treatment, data showed that the average absorption was 0.263 ± 0.011 for the control group, 0.164 ± 0.003 for 1 J/cm^2 and 0.143 ± 0.018 for 4 J/cm^2

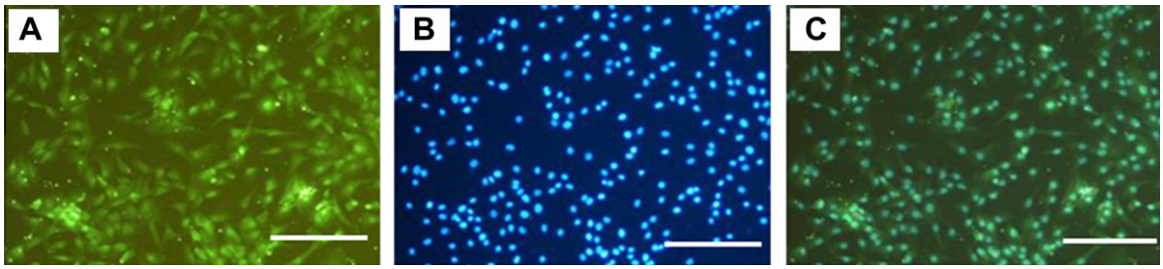

Fig. 1. Immunostaining of harvested schwann cells. Cells were stained with S-100 antibody to check the purification quality. (A) Staining with S-100 antibody. (B) Nuclear staining with DAPI. (C) Merged pictures. Scale bar is 50 μm.