


Original Article



Sub-threshold electrical stimulation improves wingless-type3 and eukaryotic initiation factor-2 α expression in the presence of exogenous astrocyte in the rat model of spinal cord injury

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Abstract

Introduction: Spinal cord injury (SCI) is a condition which can lead to permanent loss of neurons, glial and precursor cells. According to the positive influences of electrical stimulation in the neurogenesis, we hypothesized that sub-threshold electrical stimulation in the presence of exogenous astrocyte may trigger the differential regulation of wingless-type3 (Wnt-3) and eukaryotic initiation factor-2 α (eIF2 α) mediators in spinal cord injured rats.

Methods: Forty male Wistar rats (weighing 250-280g) were randomly divided into four groups: sham, SCI, SCI+astrocyte and SCI+astrocyte which followed by electrical stimulation. We evaluated the glial fibrillary acidic protein (GFAP), doublecortin, Wnt-3 and eIF2 α proteins by immunofluorescence and immunoblotting techniques.

Results: The results show that expression of Wnt-3 and eIF2 α proteins significantly enhanced after 14 days in the electrical stimulation+ SCI+astrocyte group in comparison with SCI and SCI+astrocyte groups. Also, the expression of GFAP cells was significantly increased after 14 days by electrical stimulation compared with other groups. Electrical stimulation had no effect on expression of doublecortin after 14 days.

Conclusion: This survey demonstrates that sub-threshold electrical stimulation up-regulates Wnt-3 and eIF2 α mediators. Also, GFAP marker expression has been increased in animals subjected to electrical stimulation. But there are no evidences based on doublecortin expression as a neurogenesis biomarker.

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Keywords:

Spinal cord injury;
Electrical stimulation;
GFAP;
Doublecortin;
Wingless-type3

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Introduction

Spinal cord injury (SCI) leads to permanent loss of

different cells including neurons, oligodendrocytes, astrocytes and precursors resulting in sensory and motor dysfunctions, urinary infections and mental

disorders (Bradbury & McMahon, 2006; Horky *et al.*, 2006; Thuret *et al.*, 2006b). Approximately, 90 million people live with different severities of SCI and more than 130000 new cases have been reported per year in the world (Jackson *et al.*, 2004; Thuret *et al.*, 2006b). Following SCI, regeneration of neurons is a tremendous challenge (Su *et al.*, 2014b). There are some general therapeutic interventions for SCI such as cellular, molecular and rehabilitative treatments (Thuret *et al.*, 2006a).

The cellular transplantation aims to replace lost cells, create a new guidance structure and a favourable condition for axon regeneration and trophic factors secretion (Thuret *et al.*, 2006b). However, following SCI, the capability to produce new neurons is restricted (Horner *et al.*, 2000). Investigations have shown that in the presence of some transcription factors, differentiated cells can be reprogrammed to induced pluripotent stem cell (iPSC) (Takahashi & Yamanaka, 2006; Takahashi *et al.*, 2007). Production of iPSCs is one of the big achievements throughout the history of stem cell biology which may be useful in regenerative medicine (Takahashi & Yamanaka, 2006). Recent studies demonstrated that conversion of astrocyte, as differentiated cell, to the neuroblast and neurons can be considered for SCI treatment (Su *et al.*, 2014b). Given that glial cells can be reprogrammed into functional neurons in the spinal cord and brain, so, these cells are ideal targets for *in vivo* neuronal conversion after neural injury in adult central nervous system (CNS) (Heinrich *et al.*, 2010; Torper *et al.*, 2013; Guo *et al.*, 2014). However, the mechanisms of reprogramming are approximately unclear (Niu *et al.*, 2015). *In vitro* studies showed that astrocytes, as non-neuronal cells, can be converted to the stem-like cells and neurons directly by expressing a single transcription factor (Heins *et al.*, 2002; Pfisterer *et al.*, 2011). *In vivo* study has been identified that when the SOX2 gene is expressed, conversion of adult astrocytes to neuroblast occurs. This conversion confirmed by tracing of doublecortin (DCX) protein as neuroblast and immature neuron markers which is apparently detectable during neuronal development process (Heinrich *et al.*, 2014). Furthermore, eukaryotic initiation factor-2 α (eIF2 α)-phosphorylation mediates SOX2 up-regulation in pancreatic tumor cell repopulation following irradiation (Yu *et al.*, 2016). On the other hand, wingless-type3 (Wnt-3) as one of the initial

regulators, activates Wnt/GSK3bb signaling for neurogenesis in the neuronal developmental process (Berwick and Harvey, 2012). This signaling controls self-renewal, cell proliferation and elevates by inducing of exogenous electrical stimulation (ES) *in vitro* (Liu *et al.*, 2015b).

Electrical stimulation is a target to repair the CNS degeneration (Huang *et al.*, 2015). According to performed study, deep brain stimulation (DBS) increases glial fibrillary acidic protein (GFAP) expression, so, it has corroborated that the DBS could markedly promote astrocyte proliferation (Vedam-Mai *et al.*, 2012). Functional electrical stimulation is able to increase the formation of newborn cells after SCI in animals expressing neural progenitor cells-associated markers (Becker *et al.*, 2010). In addition, an investigation conducted on both in patients with schizophrenia and depression demonstrated the electrical stimuli, kind of electroconvulsive therapy, increases the transcription factors, such as OCT4, SOX2, cMyc and Klf4 in iPSCs, therefore, promoting the cellular reprogramming (Nishiguchi *et al.*, 2015).

Generally, the positive role of low intensity of electrical stimulation in escalating neuronal regeneration after nerve injury has been determined and suggests that low intensity of electrical stimulation could increase levels of myelin protein zero mRNA and protein, extending regeneration at injured site (Zhang *et al.*, 2013). In addition, significant effect of sub-threshold electrical stimulation has been confirmed to accelerate neuronal repair in rats-subjected to SCI (Gad *et al.*, 2013).

Therefore, the SCI model in Wistar male rats was conducted and the green fluorescent protein (GFP⁺) labelled human astrocytes injected into the lesion site and subsequently electrical stimulation was applied. But, choosing the best kind of ES for the stimulation was a challenge, and to prevent any adverse effects, sub-threshold ES applied at injured site. Astrocyte proliferation and neurogenesis were traced by the expression of DCX, GFAP, Wnt-3, and eIF-2 α factors.

Materials and methods

Antibodies and reagents

Antibodies were purchased from Abcam (Abcam Inc., Cambridge, MA, UK): GFAP antibody, DCX antibody, goat anti-rabbit IgG H&L Alexa Fluor 594, Wnt3 α

antibody, p-eIF-2 α , total eIF-2 α and β -actin antibody. Other chemical and reagents were purchased from Sigma (Sigma-Aldrich Co, Missouri, US).

Animals

Forty male wistar rats (weighing 250-280g) were obtained from Tabriz University Laboratory animal care centre (Tabriz, Iran) and exposed to 12h light/dark cycle. Water and standard pellet food *ad libitum* were available under the temperature-controlled condition 25 \pm 2°C. All experimental procedures and protocols were approved by the veterinary ethics committee of Tabriz University of Medical Sciences, Iran (approval number: 1394.1116) and animal's care was performed according to the National Institutes of Health Guide (NIH Publications No. 8023, revised 1978).

Study design

Animals were randomly divided into four experimental groups (n=10 rats per group) including, 1) sham group: laminectomy surgery without contusion injury as a control group; 2) SCI group: moderate contusion at 10 thoracic segments; 3) SCI+AS (astrocyte) group: SCI and astrocyte cell injection and 4) SCI+AS+ES group: received sub-threshold electrical stimulation following the SCI and astrocyte cell injection. The immune system of rats in all experimental groups was suppressed by daily intraperitoneal injection of 10mg/kg cyclosporine A (Novartis) for 7 days prior to interventions up to the end of study (Li et al., 2015).

Interventions and SCI model

To induce SCI model, rats' surgery was performed under anesthesia with isoflurane (4% for induction and 2.5% for maintenance) (Girgis et al., 2007) and laminectomy procedure was performed at T10 segment. To some up, after removing paravertebral muscles overlying at T9 and T11, transverse processes of the vertebra column were stabilized by device clamps. Moderate contusion injury was induced by an impactor using a 2.5mm diameter of the tip and a 150-kilodynes force (Ghorbani et al., 2018). Following the surgery, in the third and fourth groups, 300,000 human astrocytes suspended in 3 μ l of DMEM (Dulbecco's Modified Eagle Medium) and were immediately injected around the damaged area at a depth of 1.3mm from the dorsal surface using a

5 μ l Hamilton syringe held in an electronic syringe pump (New Era Pump Systems, Inc). Before injection of human astrocyte, the cell counting was performed by trypan-blue staining and neobar slide. For delivering the beneficial number of astrocytes and to prevent reflux from the injection site, the needle was maintained over 5 minutes and then withdrawn. Following injection, incised muscles, subcutaneous tissues and skin were sutured in layers. After surgical procedures, animals were kept in a separate cage and bladder or bowel dysfunction was checked twice a day until detecting normal function of the urinary system. All animals were monitored for any pain and health condition every day.

Sub-threshold electrical stimulation protocol

In the group 4, rats received moderate SCI and human astrocytes have injected. Immediately, unipolar electrodes were implanted carefully in the upper injured site of the spinal cord. Then, the wire electrodes sutured into the muscles, adjacent to the paravertebrate muscles and wires socket was stayed out of the skin. Twenty-four hour later, threshold of neuronal excitability in the spinal cord was recorded from each rat (by revealing to muscle reflex in lower limbs). The sub-threshold electrical stimulation protocol has been applied from 10:00am by a 2-channel battery-powered electrical stimulator (WPI; A320) every day (Badri et al., 2017). The electrical parameters of sub-threshold electrical stimulation followed by an amplitude between 0.3-0.6 (mA), pulse width: 0.1 (msec) and pulse rate delivered at 100Hz in the upper site of the injured location (Sadighi et al., 2013; Nejad et al., 2015; Badri et al., 2017).

Human astrocyte culture

Human astrocytes were obtained (line 1321N1) (Brown & Simoni, 1995) and cultured on poly-L-lysine (Sigma-Aldrich)-coated flasks which described in pervious study (Ghasemi-Kasman et al., 2015). The culture medium composed of DMEM (Invitrogen), supplemented with 10% fetal calf serum (Invitrogen) and 1X penicillin/streptomycin (Invitrogen) which the culture medium substituted twice a week. Two days after culture process, astrocytes prepared to transplant into rat injured site of the spinal cord in the groups 3 and 4.

Immunohistochemistry investigations

For fluorescence immunostaining on the spinal cord tissue, animals were euthanized by overdose of ketamine (100mg/kg) and (5mg/kg) xylazine after 2 weeks and sequentially fixed by intracardial perfusion with 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS). The animal's spinal cord tissues were dissected carefully and collected with 4% PFA overnight at 4°C. The next day, post-fixed spinal cord immersed for 24-48h in 30% sucrose solution. The tissues were cryoprotected at -22°C and sectioned longitudinally on a cryostat embedding at 8µm thickness by a cryostat instrument (Histo-Line Laboratories, Italy). Then, sections rinsed with 0.2% Triton X-100 and blocked with 10% normal goat serum for 1h at room temperature. Blocked sections incubated with primary antibodies at least 24h at 4°C followed by extensive washing with PBS buffer. Then sections were subsequently incubated with appropriate fluorescent labelled secondary antibodies for over 2h at room temperature and after that washed again (Ghasemi-Kasman et al., 2015). Counted cells were obtained from 15 random sections from ten rats in each experimental group. Images for immunohistochemistry analysis were taken by fluorescence microscope (Olympus BX51) and captured using a DP-72 camera.

Immunoblotting assay

The spinal cord tissues were used to measure the phosphorylation of phospho eIF-2α and Wnt-3. All tissues were subjected to homogenization in radioimmunoprecipitation assay buffer (RIPA buffer) [20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na₂EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM b-glycerophosphate, 1mM Na₃VO₄ and 1µg/ml leupeptin including protease inhibitor cocktail (Sigma)] and followed by centrifuge at 12000g, 4°C, for 20min. Then, the supernatant was collected and quantified to protein concentration assay using a commercial Bradford kit (Sigma). Total protein (30mg) was separated on 10% denaturing acrylamide gels. Subsequently, protein bands were transferred to polyvinylidene difluoride (PVDF) membranes (Lie et al., 2005). The blots probed with primary antibodies (Abcam Inc., Cambridge, MA) for phosphorylated or total eIF-2α and Wnt-3 overnight at 4°C. The PVDF membranes were incubated with the horseradish

peroxidase-conjugated secondary anti-rabbit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted at 1:7000 for 1h at room temperature. Enhanced chemiluminescence detection kit (Bio Rad) was used to visualize the immunocomplexes. The density of bands was quantified using Image J (NIH, Bethesda, MD, USA). β-actin was used as internal control. The phospho eIF-2α normalized against total eIF-2α. All data were expressed as mean±SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey as post-hoc test and *P* values less than 0.05 considered as statistically significant.

Results

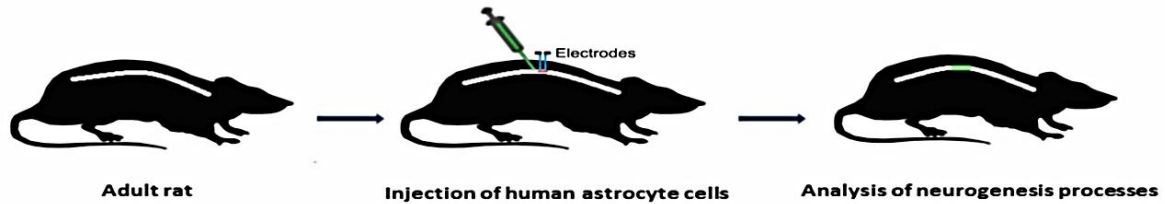
The effect of electrical stimulation on phosphorylation of eIF2-α and expression of Wnt-3 in rat models of spinal cord injury

Overall process of the study has shown (Fig. 1A). The results of immunoblotting investigations showed that electrical stimulation had a significant effect on the wnt3 protein expression (Fig. 1B). In the animals-exposed to ES following the injury and astrocyte cell injection, the expression level of Wnt3 protein was significantly enhanced versus SCI+AS group (*P*=0.00088). In the other groups, the mean differences of Wnt3 proteins were not statistically significant (Fig. 1C). Moreover, ES significantly increased phosphorylation of eIF-2α protein in the SCI+AS+ES group compared to SCI+AS group (*P*=0.025). There were no significant changes in the level of eIF-2α phosphorylation in those groups which did not received any ES protocol (Fig. 1D).

The effect of electrical stimulation on induction of astrocyte proliferation in rat models of spinal cord injury

To investigate the effect of electrical stimulation on the proliferation of astrocytes, expression of GFAP marker was evaluated at the end of the second week at the injured site of the spinal cord (Fig. 2). As shown in Figures 2A and a, SCI had no significant effect on the astrocyte proliferation compared to the sham group. Predictably, in the SCI+AS group, there was a significant increase (*P*=0.011) in fluorescent intensity compared to the SCI group. But after electrical stimulation induction for two weeks, a significant augmentation was seen in the expression of GFAP marker (red) versus SCI+AS group

A



B

ES	-	-	-	+
AS	-	-	+	+
SCI	-	+	+	+

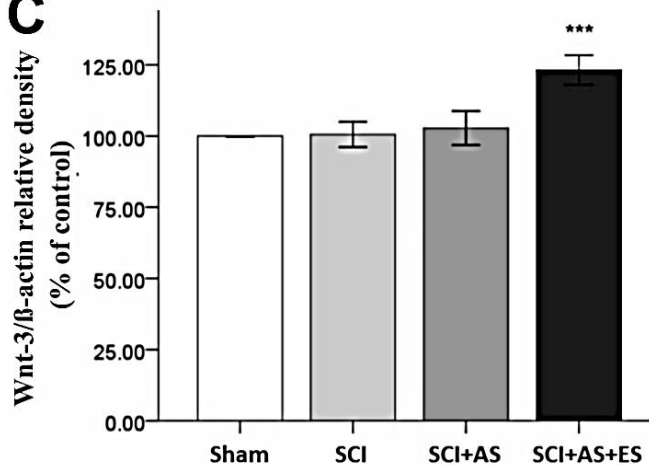
Wnt-3 39 kDa

β -actin 42 kDa

Phospho eIf-2 α 65 kDa

Total eIf-2 α 65 kDa

C



D

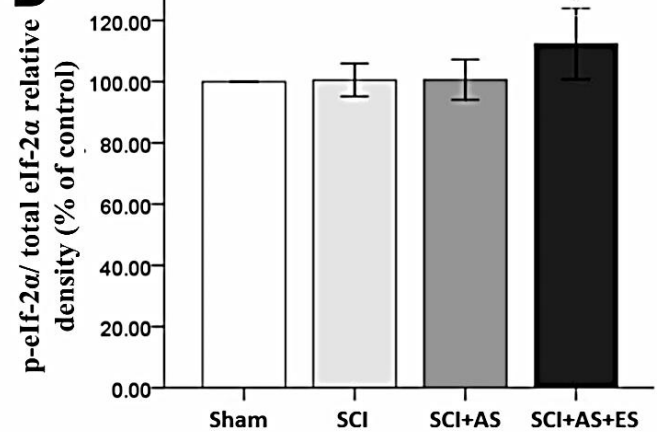


Fig.1. Western blotting analysis of phosphorylation of eukaryotic initiation factor 2 (eIf-2 α) and expression of Wingless-type3 (Wnt-3) proteins levels after 14 days in four experimental groups. (A) Experimental scheme. (B) Immunoblotting images of expression of Wnt-3 protein against β -actin and phospho-eIf-2 α (p-eIf-2 α) against total eIf-2 α . (C) Bar chart represents the quantified Wnt-3 protein bands in all experimental groups. The statistical one-way analysis of variance (ANOVA) of data showed the significantly elevated levels of Wnt-3 protein expression in the sub-threshold electrical stimulation (ES) group after 14 days. (D) Bar chart represents fold change of p-eIf-2 α in all experimental groups. Analysis of results also, showed that the levels of eIf-2 α phosphorylation in the sub-threshold ES group increased significantly after 14 days. * $P=0.025$ and *** $P=0.00088$ compared with the SCI+AS group (n= 10 rats per group).

($P=0.002$) by immunofluorescence staining targeting GFAP protein.

In order to confirm the astrocyte proliferation, GFAP expression was also investigated by the

immunoblotting assay (Figs. 2B and b). The western blotting analysis showed that induction of electrical stimulation protocol in the injured area of the spinal cord led to over-expression of GFAP marker at the

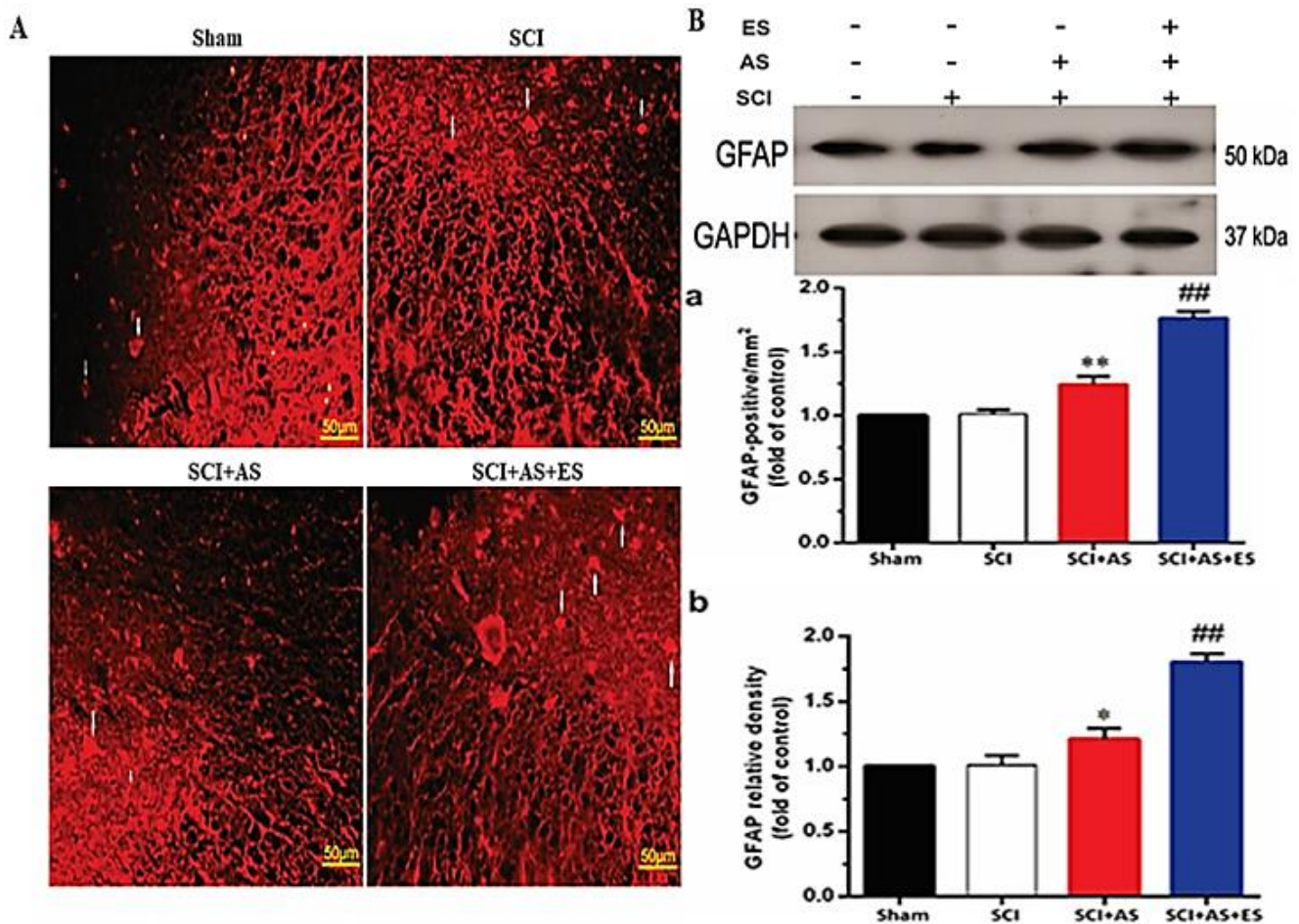


Fig.2. Glial fibrillary acidic protein (GFAP) expression after two weeks in four experimental groups in immunoblotting and immunohistochemical studies. (A) The fluorescent intensity of GFAP labeled cells after applying the sub-threshold electrical stimulation (ES) protocol in all experimental groups. Bar=50 microns. (B) Immunoblotting images of GFAP protein in the rat spinal cord for all experimental groups. (a) Quantified analysis of immunohistochemical images showed that in animals which received two weeks' period of ES, expression of GFAP marker significantly increased compared with spinal cord injury (SCI)+astrocyte (AS) cell group. (b) Bars illustrate fold change of GFAP protein normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard. As shown, the expression levels of GFAP protein in animals that received two weeks' period of sub-threshold ES was significantly enhanced versus SCI+AS group. * $P=0.037$ and ** $P=0.011$ compared with the SCI group. *** $P=0.002$ compared with the SCI+AS (n=10 rats per group). Magnification $\times 40$.

end of two weeks' period of ES in comparison with SCI+AS group ($P=0.037$).

The effect of electrical stimulation on astrocyte reprogramming into neural progenitors in rat models of spinal cord injury

Spinal cord sections were examined to detect the co-expression of DCX and GFP⁺ cells (Fig. 3) and possibility of astrocyte cells conversion to neuroblast by staining against DCX. According to Figure 3, the levels of co-expression of DCX⁺ (red) with GFP⁺ cells (green) around the injected site was not statistically significant in manipulated by electrical stimulation protocol animals after two weeks in comparison with group 3 ($P=0.834$). Also, expression of DCX marker

in group 3 versus group 2 was not significant ($P=0.991$). To approve these data, the possibility of cellular reprogramming was checked by tracing of DCX protein expression in immunoblotting studies. In the Figure 4B, ANOVA analysis revealed no significant changes in expression levels of DCX marker among different groups such that group 3 in comparison with group 2 and group 4 versus group 3 had no significant differences ($P=0.999$ and $P=0.999$, respectively).

Discussion

In this study, immunofluorescence and immunoblotting results demonstrated that electrical stimulation in rat model of spinal cord injury after two

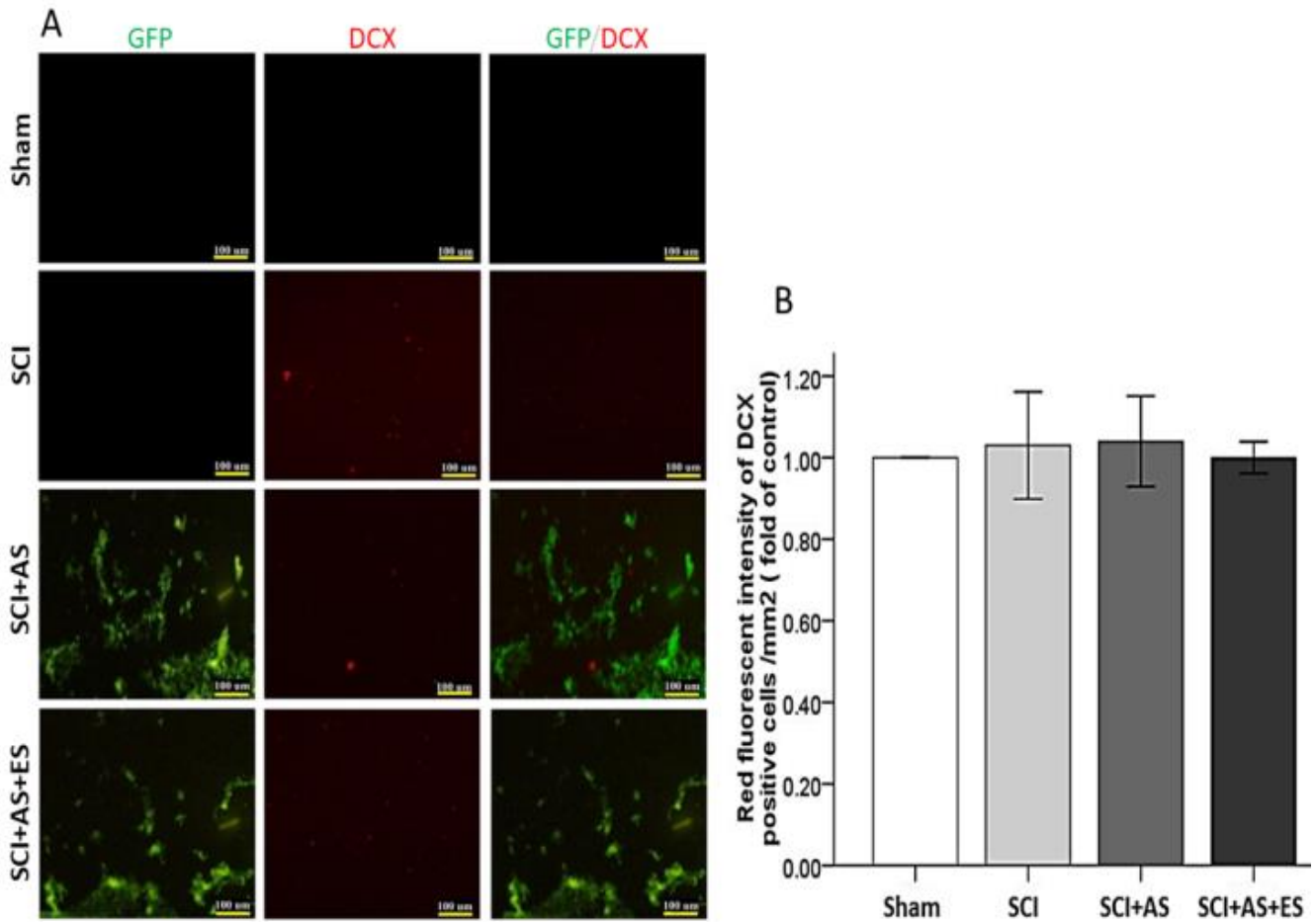


Fig.3. Immunohistochemical analysis of doublecortin (DCX) marker after two weeks in all experimental groups. (A) Doublecortin⁺ (DCX⁺) cells were not detected after 14 days in animal which received sub-threshold electrical stimulation (ES) as well as other groups Bar=100 microns. (B) Quantification analysis of DCX marker showed that there was no significant change in expression of DCX marker in all groups (n=10 rats per group). Magnification ×40.

weeks induced translational regulations and cell differentiation modification as well as astrocyte proliferation without any evidences in reprogramming into neuroblast.

Astrocytes as non-neural cells have a potential role in the cellular reprogramming process. Generation of the neurons from the astrocyte cells in adult spinal cord is considered as a new therapeutic window for SCI treatment (Su *et al.*, 2014a). Vedam-Mai and colleagues examined the role of DBS on the astrocyte cell and proved that DBS could increase the number of neural progenitor cells as well as the proliferation of neurogenic astrocytes in the anterior thalamic nucleus of rat (Vedam-Mai *et al.*, 2012). It is revealed that direct electrical stimulation not only effects on neurons but also has a significant proliferative effect on glial cells (McIntyre *et al.*, 2004; Giaume *et al.*, 2010). Also, astrocyte could be directly stimulated by high frequency stimulation (Pekny &

Nilsson, 2005; Sofroniew, 2009; DiLorenzo *et al.*, 2010). Remarkably, previous studies have shown that a subset of astrocyte acts as a neural stem cell in the adult CNS (Doetsch *et al.*, 1999; Van Den Berge *et al.*, 2010), and have stem cell features in the brain injury (Buffo *et al.*, 2008). Moreover, previous reports revealed that astrocyte-like neural stem cells are able to enhance several neurogenesis aspects in response to high frequency stimulation (Malatesta *et al.*, 2000; Hartfuss *et al.*, 2001; Götz *et al.*, 2002). The results of the present study indicated that the sub-threshold electrical stimulation induced GFAP⁺ cells as compared to SCI+AS group. This augmentation of GFAP marker was also seen in SCI+AS group in comparison with SCI group. Therefore, it has been shown that electrical stimulation had positive role to increase injected human astrocyte proliferation in the injured site after two weeks.

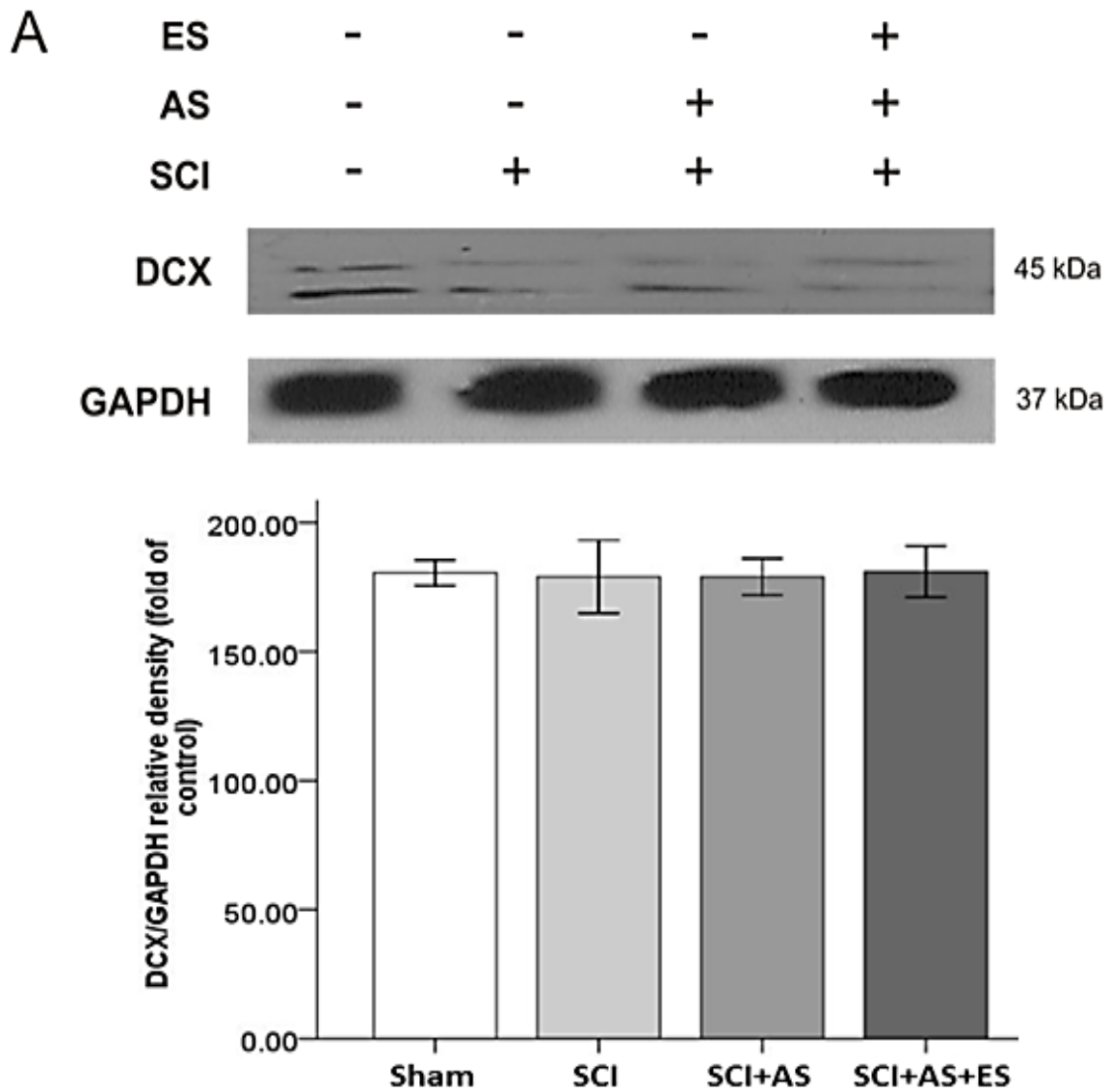


Fig.4. Effect of sub-threshold electrical stimulation (ES) on expression of doublecortin (DCX) protein in the spinal cord after 14 days in all experimental groups. (A) Immunoblotting images of DCX protein expression against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which considered as an internal standard. (B) Bars represent fold change of DCX protein (n= 10 rats per group).

Investigations have demonstrated that production of GFAP, vimentin, nestin and synemin proteins are promoted in response to electrical stimulation (Stock et al., 1979; Pilitsis et al., 2008) and accordingly this outcome showed in an *in vitro* study (Jun et al., 2007). Also, other studies have demonstrated that neural progenitors or neurogenic astrocytes are enhanced by DBS (Steindler & Laywell, 2003; Middeldorp *et al.*, 2010; Khaindrava *et al.*, 2011). Hence, it apparently proved that ES could significantly increase astrocyte proliferation *in vitro* and *in vivo* conditions which in our study examined. Recent evidence showed that the cellular pluripotency is modulated by several complex signalling networks involved in the regulation of gene

expression, translation and even post-translational modifications (Cai et al., 2012; Wang et al., 2014). Also, it has been reported that neurodevelopmental events are associated modification in mRNA translation, protein synthesis and neocortical development (Wang et al., 2014). Phosphorylation of the alpha subunit eIF2 is one of this differentiation-related post-translational modifications which could be catalyzed by a various activated kinases such as PERK (protein kinase R [PKR]-like endoplasmic reticulum kinase) in stress response to some cellular, heat shock, nutrient deprivation, changes in intracellular calcium, accumulation of misfolded or denatured proteins, iron deficiency and induction of apoptosis (Roffé et al., 2013). The exogenous

electrical stimulation as an inducer of the neural activity in the nervous system has a positive influence on the different neurogenesis aspects at the cellular level which is able to promote the neuronal regeneration.

A recent study showed that ES induced phosphorylation of eIF-2 α as a marker of continuous protein synthesis and expression of Wnt-3 as an instructive neurogenesis factor which could be secreted by astrocyte cells (Wakabayashi et al., 2016). So, in the present study, we examined phosphorylation of eIF-2 α and Wnt-3 level in the presence of human astrocyte after applying electrical stimulation protocol. Phosphorylated eIF2 inhibits the eIF2B and thus prevents effective protein synthesis. Hyper phosphorylation of eIF-2 α along with strong blocking of eIF2B activity led to negative regulation of protein synthesis, whereas promote the selected translation of preferential transcripts, such as stress response related activating transcription factor 4 (ATF4) (Gu et al., 2017). Stabilized ATF4 mutants show diminished β -TrCP (beta-transducin repeat containing) degra phosphorylation, β -TrCP interaction and ubiquitination, as well as provoke early G1 arrest. Given that expression of stabilized ATF4 also promote the developing neocortex (Frank et al., 2010). Phosphorylated eIF2 modulates bradyzoite differentiation through epigenetic modifications, promoter-based cis-elements and protein translation (Sullivan Jr et al., 2009). In line with our findings, stimulation of HeLa (Henrietta Lacks) S3 cells by nanosecond pulsed electric fields (nsPEFs) quickly induced activation of eIF-2 α upstream stress-responsive kinases PERK, phosphorylation of eIF-2 α and general control non-depressible 2 (GCN2) as well as translational suppression (Morotomi-Yano et al., 2012).

Additionally, Wnt signalling controls and impacts on the diverse neurogenesis processes, including cell proliferation and self-renewal (Valvezan & Klein, 2012). During neurogenesis, the Wnt signalling pathway has various important roles in the nervous system by regulating the cell division, migration of neural progenitor cells and proliferation process (Berwick & Harvey, 2012). Studies suggest, this pathway could be promoted by exogenous ES (Liu et al., 2015b). Likewise, *in vivo* study emphasized that neurogenesis can be triggered by astrocyte-derived Wnt in the hippocampus. Thus, according to the

effect of electrical stimulation on the molecular signalling, we also examined the expression of the Wnt as an early event before cellular reprogramming. Our results revealed that expression of Wnt was significantly increased in animals-received ES 14-days (in group 4) compared to other groups. In line with our results, Liu et al. showed that functional electrical stimulation after 14 days increases the expression of Wnt-3 protein that plays a key role in neurogenesis promotion in the ipsilateral subventricular zone (SVZ) in rats with ischemia (Liu et al., 2013). It has been reported that over-expression of Wnt-3 protein promotes functional recovery after focal ischemia and this event could relate to increase immature neurons in the striatum and SVZ regions (Shruster et al., 2012).

Finally, despite some obvious findings related to pro-regenerative events such as Wnt3 signaling up-regulation and eIF-2 α hyper phosphorylation which support the changes in astrocyte phenotype driven by neural activity, significant enhance in GFAP+ cell could not be addressed as reactive gliogenesis or injected astrocyte proliferation. According the analysis of immunofluorescence and immunoblotting outcomes, we did not find any significant modifications in expression of DCX marker as revealing conversion of human astrocyte to neuroblast in the fourth group after two weeks. However, in 2015, a study showed that electrical stimulation of ventromedial prefrontal cortex increased the expression of NeuN, DCX, Angpt2 and S100a4 genes in the neurogenic zone (Liu et al., 2015a). Evaluating expression of DCX has been established to reflect changes in adult neurogenesis and nowadays used as a common immunohistochemical marker to detect newborn neurons in brain tissues (Horner et al., 2000; Horoky et al., 2006). DCX is expressed in dividing neuronal precursor cells and is stable for approximately one month until the cells integration into the granular cell layer be stopped. Nonetheless, DCX-expression and localization to various parts of brain tissues differs depending on type of applied DCX-antibodies and confirmation methods (Kremer et al., 2013). Moreover, recently an evidence for a noticeable "non-neurogenic" DCX-protein pool suggests caution for the interpretation of DCX+/- finding in rodent brain (Takahashi et al., 2007).

Conclusion

Undeniably, these data are not enough to clarify the effect of electrical stimulation intervention on a direct neurogenesis, so we need comprehensive analyses to investigate the other neurogenesis biomarkers like NeuN, Angpt2 and S100a4. Also, we need other investigations to correct interpretations of increased GFAP+cells. Of course, electrical stimulation protocol including, severity and frequency of ES and even adjunctive agents can be improved in the future. However, Wnt-3 signaling up-regulation and eIF-2 α hyper-phosphorylation findings are notable and deserve more attentions for CNS regeneration.

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Conflict of interest

The authors declare no conflict of interest.

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