

The comparative effects of estrogen and tacrolimus on crushed sciatic nerve regeneration in male mice: functional and histopathological evaluation

Fereshteh Khomejani Farahani¹, Hamidreza Fattahian^{1*}, Ahmad Asghari¹, Pejman Mortazavi²

¹ Department of Clinical Science, Faculty of Specialized Veterinary Science, Science and Research Branch, Islamic Azad University, Tehran, Iran; ² Department of Pathobiology, Faculty of Specialized Veterinary Science, Science and Research Branch, Islamic Azad University, Tehran, Iran.

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Abstract

Some pharmacological agents can be effective for peripheral nerve injuries treatments. Present study was aimed to apply different agents and to compare the nerve regenerative effects following crushed sciatic nerve injuries. Twenty-four adult male mice were conducted in this study. Standard unilateral left side sciatic nerve crush was performed with 2.00 mm width mosquito hemostat forceps. The mice were randomly divided into four groups with the same numbers in each group which received subcutaneously, estrogen (group I), tacrolimus (group II), the combination of estrogen and tacrolimus (group III), and saline 0.90%. Functional recovery, histopathology, and immunohistochemistry (IHC) were performed on days 14th and 28th. Walking track analysis on day 14th showed no significant difference between experimental groups, however, they showed significant difference compared to the control group. At the same time, experimental groups showed similar results of inflammatory cell infiltration, axonal edema, and count with significant differences compared to control group. At the end of the study, group I and III showed a significant difference in functional recovery between group II and control. After fourth week significant histopathological difference of axonal count was observed in group III. On day 28th, only IHC assessment in group III showed more glial fibrillary acidic protein (GFAP) expression compared to the same group on day 14th. This study revealed subcutaneous administration of combined estrogen and tacrolimus could be effective with acceptable results in nerve regeneration.

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Introduction

Peripheral nerve injuries are major, frequent problems which cause mostly by motor vehicle accident, penetrating trauma, gunshot, stretching, and crushing injuries. Sometimes fractures of adjacent bones are commonly associated with the peripheral nerve injuries such as humeral fractures which cause radial nerve neuropathy. In addition to injuries that cause sharp or blunt lesions, sometimes the nerves may be displaced, contused, stretched, or even partially divided leading to neuromas or lesion in its continuity. Six different classifications have been assigned for types of the injury including the first degree (mildest) of injury corresponding with neuropraxia to the sixth degree of the most severe injury with neurotmesis.¹⁻⁵

Various options are presented as nerve repairs; however, the treatment of the nerve injuries (invasive or

minimally invasive) remain challenging and none of them may consider particularly effective and still somehow may be irreversible or hardly appropriate and reasonable treatments. The results of the following nerve repairs are influenced by many parameters such as nature, location, extent of the injury, the level and timing of the repairs; and finally appropriateness of the realignment of injured nerves. Primary direct nerve repairs of injuries are carried out using an end-to-end anastomosis which may cause complications including scar tissue formation and aberrant axonal migration. Many biological or synthetic materials have been used as sleeve or cuff which are placed around the anastomosis. The ideal surgical repair techniques should accomplish acceptable healing with minimal scar formation and direct the nerve sprouts into their correct targets. Peripheral nerve allograft functions, as a temporary scaffold; allowing host axonal regeneration

*Correspondence:

Hamidreza Fattahian. DVM, DVSc
Department of Clinical Science, Faculty of Specialized Veterinary Science, Science and Research Branch, Islamic Azad University, Tehran, Iran
E-mail: hrfattahian@srbiau.ac.ir



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towards the scaffold, are another choice of treatment; however, temporary administration of immunosuppressive is also required as a pharmacological agent to avoid rejection and allows promotion of regeneration across nerve allograft. Application of nerve graft, nerve glue, and nerve growth factors are some advanced choices for peripheral nerve regeneration. In addition, some pharmacological agents could be effective as therapeutic options for peripheral nerve injuries.^{2,5-11}

Estrogen is the unique superfamily of steroid hormone that has the main role in the survival and growth of neurons in a fetus and during the early stage of life, regulates expression of genes, enhance neural stability, protect neurons and glial cells after injuries and promotes regenerative process. Estrogen has been demonstrated to have neuroprotective effect via anti oxidative effect, angiogenesis, and neurogenesis during the recovery of peripheral nerve damage.^{6,7,12-14}

Tacrolimus (FK506) is an immunosuppressive drug that was first utilized clinically in organ transplantation for prevention of allograft rejection for several decades, however, accidentally it was found that it has nerve regenerative properties once administered following injuries to peripheral nerves via distinct mechanisms from immunosuppressive effects.^{9,10,11-16}

The present study was conducted on the evaluation of functional recovery (walking track analysis), histopathological, and immunohistochemical (IHC) results of estrogen and tacrolimus administration (isolated and combined) after sciatic nerve crush injury of Syrian mice as an animal model.

Materials and Methods

Experimental design and animal grouping. Twenty-four adult male Syrian mice (25.00 ± 2.00 g) were obtained from Pasteur institute (Tehran, Iran), which all were healthy and had a normal gait. They were housed in individual cages with an ambient temperature of 23.00 ± 3.00 °C and stable air humidity. The mice had free access to standard rodent food which were acquired from Pasteur institute and fresh tap water. The mice were divided into four groups ($n = 6$). Group I received estrogen (4.00 mg kg^{-1} , q24h; Aburaihan, Tehran, Iran);⁶ group II received tacrolimus (5.00 mg kg^{-1} , q24h; Astellas, Leiden, The Netherlands);¹⁶ group III received a combination of estrogen and tacrolimus with the mentioned amount, and group IV received saline 0.90% (Samen, Mashhad, Iran). All agents were administered at the back side of each mouse subcutaneously. The present study has been approved by the Animal Ethics Committee of the Iranian Laboratory Animal Ethic Frameworks under the reference code IAEC 6-32/12.

Surgical procedure. Anesthesia was induced by a combination of ketamine (40.00 mg kg^{-1} , Alfasan, Woerden, The Netherlands) and medetomidine (10.00 mg kg^{-1} , Syva,

Toledo, Spain) intramuscularly and also maintained with the mentioned anesthetic agents. Standard unilateral left side sciatic nerve crush was performed with 2.00 mm width mosquito hemostat forceps tip (Schreiber, Fridingen, Germany) and closed for 10 sec of the first ratchet.^{17,18} The location of the injury was 5.00 mm proximal to the sciatic nerve bifurcation. The site was signed with 5-0 nylon suture (Supa, Tehran, Iran) of biceps femoris fascia exactly in the vicinity of the lesion. All surgical procedures were performed with an aseptic technique. Subcutaneous enrofloxacin (0.10 mg kg^{-1} , q24h; Hipra Co., Gerona, Spain), as antibiotic, and also subcutaneous meloxicam (0.20 mg kg^{-1} for the first day and was tapered to 0.10 mg kg^{-1} for two days, q24h; Razak, Tehran, Iran) were administered to all animals for 3 days; post-operatively. All groups underwent functional analysis, histopathological and immunohistochemical assessment on days 14th and 28th of the study.

Functional recovery evaluation. On days 14th and 28th, functional nerve recovery following sciatic nerve injury was analyzed using walking track assessment which showed sciatic functional index (SFI). The assessment was done by dipping the pelvic paws of mice in ink and allowing them to walk along a corridor lined. The measurement of paws print length was then recorded as the (SFI), which was calculated by the following formula:

$$SFI = -38.8 [EPL-NPL/NPL] + 109.5 [ETS-NTS/NTS] + 13.3 [EIT-NIT/NIT] - 8.8$$

where, *EPL* indicated the operated experimental paw length, *NPL* was normal paw length, *ETS* was operated experimental toe spread (the distance between the first and fifth toes), *NTS* was the normal toe spread, *EIT* was operated experimental intermediary toe spread (distance between the second and fourth toes) and *NIT* was normal intermediary toe spread. Score zero was considered normal and index minus 100 indicated total impairment.^{7,17}

Histopathological and immunohistochemical evaluation. On day 14, three mice of groups were euthanized by placing in a ether container and the rest of them were euthanized in this manner on day 28th. The nerves were harvested for histopathological and IHC studies. The assessment of samples was performed by light microscopy (Olympus, Hamburg, Germany) after fixing the sciatic nerves specimen in 10.00% formalin and embedding in paraffin which was sectioned at a thickness of 6.00 μm for Hematoxylin and Eosin (H&E) staining and also for IHC study. Two blinded pathologists evaluated all samples histopathologically for perineurium formation, axonal edema, axonal count, and the degree of inflammatory cell infiltration. Glial fibrillary acidic protein (GFAP) expression was assessed as a marker for neurogenesis after injury.

Statistical analysis. Data were analyzed by ANOVA and Tukey post hoc, Kruskal-Wallis followed by Mann Whitney U test. All analysis was performed using SPSS Software (version 18.0; IBM Corp., Armonk, USA). The statistical significance was defined as $p < 0.05$.

Results

Functional recovery evaluation. Figure 1 showed paw tracks of all groups of the study on days 14th and 28th. As shown in Table 1, there was no significant difference between the experimental groups for walking track assessment on day 14th ($p > 0.05$), however, they had a significant difference with the control animals ($p < 0.05$). Clinically functional recovery improvement was observed in all animals from the second week till the end of the study. On day 28th, animals in groups I and III showed significant difference of functional recovery in comparison with group II and control group ($p < 0.05$), also group III had insignificant higher SFI value in comparison with the group I ($p > 0.05$).

Table 1. Sciatic functional index (SFI). Data were presented as mean \pm standard deviation.

Groups	SFI (Day 14 th)	SFI (Day 28 th)
I	-20.85 \pm 0.30 ^a	-8.05 \pm 0.08 ^a
II	-20.81 \pm 0.34 ^a	-10.34 \pm 0.20 ^b
III	-20.80 \pm 0.35 ^a	-7.99 \pm 0.14 ^a
IV	-30.60 \pm 0.49 ^b	-20.51 \pm 0.45 ^c

^{abc} Different letters indicate significant differences at $p < 0.05$.

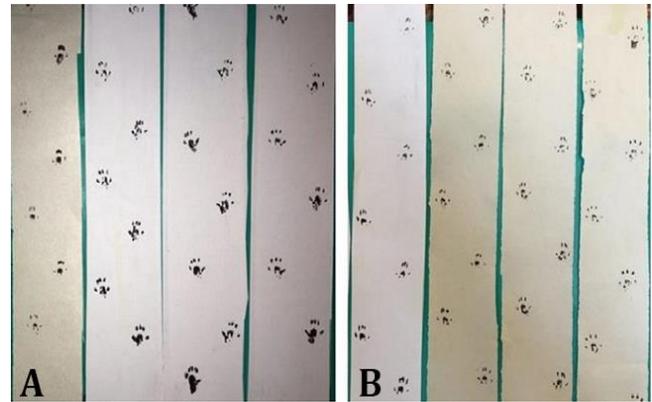


Fig. 1. Paw tracks. **A)** Day 14th; from right to left: group I, group II, group III, and group IV. **B)** Day 28th; from right to left: group I, group II, group III, and group IV.

Histopathological and immunohistochemistry evaluation results. Table 2 showed the score for histopathological analysis of all variables by evaluation of perineurium formation, infiltration of inflammatory cell, axonal count and edema. The longitudinal sections of the injured sciatic nerve were analyzed by H&E staining (Figs. 2 and 3).

Table 2. Evaluation of histopathological parameters and immunohistochemistry assessment.

Parameters	Score	Sample time (Day)	Groups				
			I	II	III	IV	
Perineurium formation	None	0					
	$\leq 25.00\%$	1	14	4 ^a	4 ^a	4 ^a	4 ^a
	25.00-50.00%	2					
	50.00-75.00%	3	28	4 ^a	4 ^a	4 ^a	4 ^a
	Complete	4					
Inflammatory cell infiltration	$\geq 75.00\%$	0					
	50.00-75.00%	1	14	3 ^a	3 ^a	3 ^a	1 ^b
	25.00-50.00%	2					
	$\leq 25.00\%$	3	28	3 ^a	3 ^a	3 ^a	3 ^a
	None	4					
Axonal edema	$\geq 75.00\%$	0					
	50.00-75.00%	1	14	2 ^a	2 ^a	2 ^a	1 ^b
	25.00-50.00%	2					
	$\leq 25.00\%$	3	28	2 ^a	3 ^b	3 ^b	2 ^a
	None	4					
Axonal count	$\leq 25.00\%$	0					
	25.00%	1	14	3 ^a	3 ^a	3 ^a	2 ^b
	50.00%	2					
	75.00%	3	28	3 ^a	3 ^a	4 ^b	3 ^a
	Normal	4					
GFAP expression (IHC)	None	0					
	Scatter	+1	14	+2 ^a	+2 ^a	+2 ^a	+3 ^b
	Mild ($\leq 25.00\%$)	+2					
	Moderate (25.00-50.00%)	+3	28	+1 ^a	+2 ^b	+3 ^c	+2 ^b
	High ($\geq 50.00\%$)	+4					
Histopathological total score	-	-	14	12	12	12	8
	-	-	28	12	13	14	13

^{abc} Different letters indicate significant differences at $p < 0.05$.

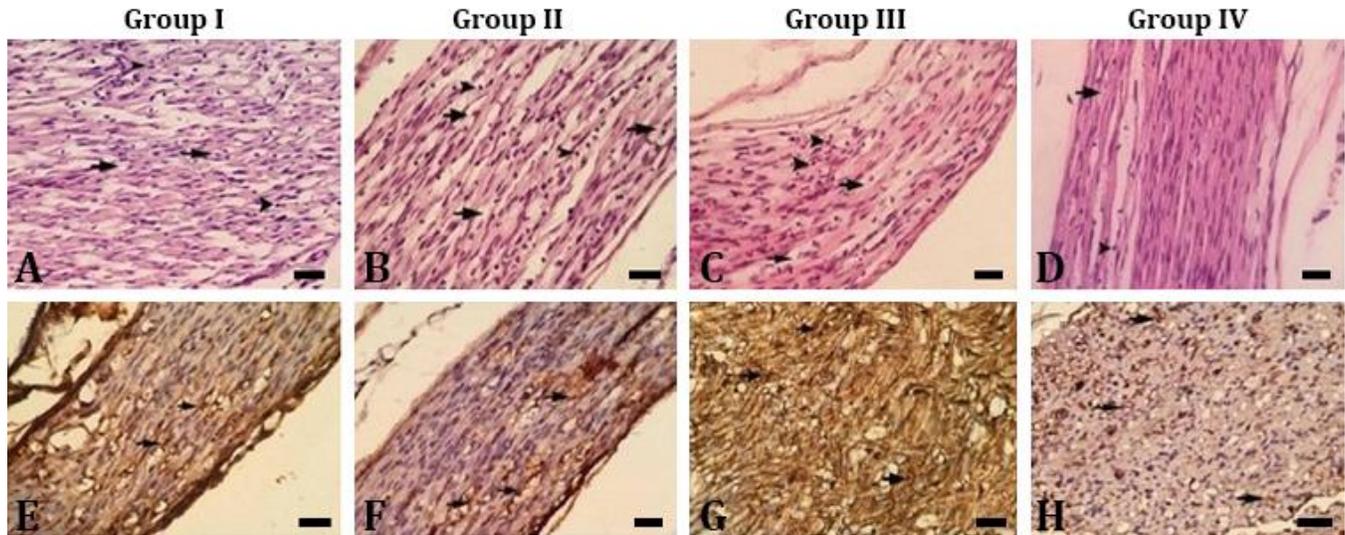


Fig. 2. A-D) Longitudinal section of sciatic nerve harvested on day 14th. Arrows show axonal edema and arrowheads show infiltration of inflammatory cell (H&E staining, scale bars = 30 μ m); **E-H)** Immunostaining for GFAP assessment. Arrows show expression of GFAP (Scale bars = 30 μ m).

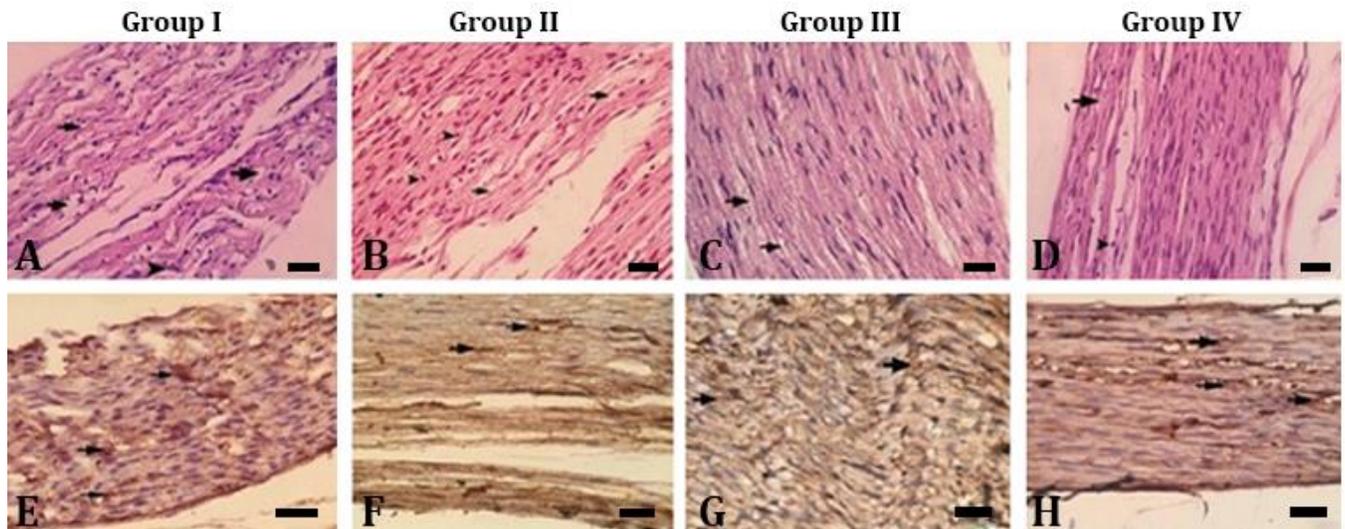


Fig. 3. A-D) Longitudinal section of sciatic nerve harvested on day 28th. Arrows show axonal edema and arrowheads show infiltration of inflammatory cell (H&E staining, scale bars = 30 μ m). **E-H)** Immunostaining for GFAP assessment. Arrows show expression of GFAP (Scale bars = 30 μ m).

Two weeks following crush injury, perineurium formation was tentatively identified in all groups with no significant difference between groups of study ($p > 0.05$). Experimental groups had a similar intensity of inflammatory cell infiltration, axonal edema accumulation, and axonal count with no significant differences ($p > 0.05$); however, significant differences were found in these parameters between them and the control ($p < 0.05$).

The histopathological results on the fourth week suggested no significant difference of perineurium formation between groups ($p > 0.05$). Histopathological inflammatory cell infiltration indicated no significant difference among groups at the end of the study ($p > 0.05$). Axonal edema was obvious in all groups; however, edema

was prominent in group II and III with significant differences from the group I ($p < 0.05$). The suppression of edema in the control on day 28th was obvious in comparison with the same group on day 14th. Axonal count analysis showed only a significant difference in group III compared to other groups ($p < 0.05$).

Immunohistochemistry evaluation of sections are shown in Figures 2 and 3. On day 14th, GFAP was mildly expressed in groups I, II, and III with no significant difference between them ($p > 0.05$). The control group showed moderate GFAP expression with a significant difference between the experimental groups ($p < 0.05$).

On day 28, GFAP expression was reduced in group I and the control group, also invariable expression was

observed in group II. Only group III showed increased GFAP expression with a significant difference compared to other groups of the study ($p < 0.05$).

Discussion

Peripheral nerve lesion is a common clinical problem that may not be usually life-threatening, however, may cause permanent disability and sometimes long-term functional deficit.^{5,9} Usually crush injuries, fracture, laceration, compression, stretching or iatrogenic reasons, and so on are the main causes of nerve injuries and result in significant pathologic changes for the peripheral nervous system.^{5,9} Unsuccessful treatments of nerve injuries cause partial or total loss of nerve function.⁹ Despite widespread experimental studies, healing of peripheral nerve injuries are hardly reasonable.⁹ Nowadays, several studies have been carried out on experimental nerve lesions and different options were suggested of nerve repairs, such as direct nerve repair, nerve graft, nerve glue, stem cell transplantation, nerve growth factor stimulation and some pharmacological agents.^{11,17-25} With respect to acceptable microsurgical procedures; extent of lesions, realignment of nerve segments and scar formation around healed nerves are main challenges around anastomosis or lesion sites.¹¹ Numerous investigations have been performed to find appropriate pharmacological agents that could prevent scar formation and accelerate nerve regeneration. Corticosteroids, vitamin B12, hyaluronic acid, riluzole, melatonin, tacrolimus, cyclosporine A, free radical scavengers, calcium or potassium channel blocker, and anabolic steroid hormones, are tested for the aim of healing process.^{4,7-10,12,20,26,27} In this study estrogen and tacrolimus, as neuroregenerative agents, were used simultaneously to evaluate the effect of combined agents and to compare estrogen and tacrolimus effect.

The majority of researches agree with the neuroprotective role of estrogen with the mechanisms of enhancing synaptic transmission, axonal sprouting, neurogenesis and cell control of survival, proliferation, growth, and death.^{10,14} These effects relate to modulation of neurotransmitter receptor function as antioxidant activity and alteration, regulation; and activation of genes expression as an antiapoptotic process with a general trophic role.^{6,13,14,19,20} Estrogen can also regulate proliferation and reactive gliosis of peripheral nerve injuries.^{17,21-23}

Tacrolimus role was mentioned in the literature as neuroprotective and neurotrophic through enhancing neurite elongation and accelerating the rate of nerve regeneration that conjoin reducing of scar around the injured site and impede inflammatory reaction.⁹⁻¹¹ Tacrolimus displays its effects by binding to its receptors (FKBP12 and FKBP52). FKBP12 receptors are responsible for immunosuppressive effect whereas the FKBP52

receptors are related to neuroregenerative effect which is a distinct mechanism from immunosuppression properties.^{8,10,15,16}

Many investigations concentrate on facial, sciatic, and pudendal nerve regeneration following nerve injuries and estrogen administration of various animal models.^{6,12,14,20} Also regenerative effects of tacrolimus have been shown experimentally in multiple nerve injuries mostly facial and sciatic nerves, and in different animal models during the past decade. Many findings have supported the sub-immunosuppressive doses of this agent and the dose-dependency property of tacrolimus in nerve regeneration after crush nerve.^{8,9,11,15,16} Besides isolated administration of pharmacological agents, several studies have focused on how to act simultaneous application of neuroprotective agents together or with surgical techniques.^{8,9,11,16,23}

Walking track and histopathological analyses are the main aims of studies for nerve regeneration assessment.¹⁷ As previously mentioned, in the second week of the present study, walking track analysis showed a significant difference between the experimental groups and the control group. At the end of the study, animals in groups I and III demonstrated the most favorable functional recovery with a significant difference between group II and the control group. Improvement of functional recovery in animals that received estrogen in our study; agreed with Nobakhti-Afshar *et al.*⁷ and Islamov *et al.*^{14,21} studies that demonstrated the enhancement of the functional recovery followed estrogen administration after nerve injuries. Grand *et al.*⁸ and Shabeed *et al.*⁹ observed improvement of the level of functional recovery in tacrolimus treated animals after sciatic nerve crush; that agreed with our findings. Insignificant improvement of functional recovery in group III compared to group I at the end of fourth week might be due to the synergistic effect of concurrent estrogen and tacrolimus administration. The result might be related to the promising effect of isolated estrogen on nerve function in comparison with tacrolimus. Islamov *et al.*²¹ showed acceleration of functional recovery in the estrogen administrated animals and found SFI returned to pre-operative level by the day 21. We also found estrogen was efficacious for improvement of functional recovery at the end of our study; which agreed with Islamov *et al.* study.²¹

Histopathological analysis of perineurium formation did not show significant differences in all groups of study on days 14 and 28. Alvites *et al.*⁴ compared the degrees of peripheral nerve crushing lesion and the mechanism of recovery following injury that was in agreement with our results in pathophysiology of nerve lesion and also recovery after crush injury. With the attention of Alvites *et al.*⁴ study and to our knowledge, the insignificance presented in our study, demonstrated that nerve injuries such as crush nerve lesions may not have a negative effect on perineurium formation.

In the second week of the study, there was no significant difference between experimental groups in parameters of inflammatory cell infiltration, axonal edema, and axonal count, however, there were different significantly compared to the control group. Suppression of inflammation and increasing of axonal area following estrogen administration after nerve lesions were registered in Islamov *et al.* study,²¹ also reduction of inflammation and acceleration of axonal count in the tacrolimus treated groups were pointed out in Suchyta *et al.* and Yang *et al.* studies.^{14,15} Histologically suppression of inflammatory cell infiltration and enhancement of axonal area presented in our study, could be confirmed with these results. Our data showed that the synergistic impact of combined two agents could not synergistically act within two weeks, also could prove the probability of the anti-inflammatory role of isolated and combined agents in comparison with the control group.

At the end of the study the axonal edema was less in groups II and III that showed a significant difference between group I and the control group. This supported the efficacious role of tacrolimus in the elimination of edema and inflammation of axon in comparison with estrogen at the end of the study. Mekaj *et al.*¹⁰ and Suchyta *et al.*¹¹ studies confirmed our results. They explained that the suppression of edema with reduction of scar formation at the site of the nerve repair had acceptable nerve improvement. Acceleration of axonal count in the estrogen treated groups was shown in study of Nobakhti-Afshar *et al.*⁷ In transected peripheral nerves by 12 weeks, this finding was observed in Islamov *et al.*²¹ study in the period of 3 weeks after sciatic nerve crush injury. Shabeed *et al.*⁹ study defined that tacrolimus could increase axonal count and induce peripheral nerve regeneration with reduction of scar formation. These studies also showed the axonal accelerating role of estrogen and tacrolimus in comparison with the control groups.

In fourth week, the axonal count was significantly different in group III compared to the other groups, which could explain the synergistic effect of estrogen and tacrolimus to promote nerve regeneration in comparison with isolated administration of agents.

The expression of GFAP in the second week was the same with no significant difference between experimental groups. At the end of the fourth week, only animals of group III showed a significant difference in GFAP expression that supported the likely synergistic effect of the simultaneous administration of two agents. As there were no changes in GFAP expression in group II in comparison with group I at end of the study, the authors believed in implication of efficacious anti-inflammatory role and suppressive scar formation of tacrolimus in comparison with estrogen.

It seems estrogen may be responsible for higher SFI score, also we could declare tacrolimus might have better

anti-inflammatory and edema eliminated effects in comparison with estrogen at the end of the study. Walking track analysis and the histopathological study showed nerve regeneration, enhancement; and improvement of functional recovery due to the synergistic effect of two agents on day 28 in group III.

Our study supported the neuroprotective effect of isolated administration of estrogen and tacrolimus in groups I and II, also data determined a more acceptable result associated with the synergistic effect of combined two agents in group III. Thus, we recommended further investigations for confounding factors by increasing sample size, prolongation of study time; and examination of various etiology of nerve lesion to refine more data about the therapeutic effect of medical agents on nerve proliferation, regeneration, and its functional recovery.

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Conflict of interest

The authors declare no conflict of interest.

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