



## Assessment of Neuroprotective Effects of Local Administration of 17-Beta- Estradiol on Peripheral Nerve Regeneration in Ovariectomized Female Rats

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### ► ABSTRACT

**Objective:** To assess the neuroprotective effects of local administration of 17- beta- estradiol on nerve regeneration.

**Methods:** Sixty female Wistar rats were ovariectomized and divided into four experimental groups (n=15), randomly: In autograft group a segment of sciatic nerve was transected and re-implanted reversely. In sham-surgery group sciatic nerve was exposed and manipulated. In transected group left sciatic nerve was transected and stumps were fixed in adjacent muscle. In treatment group defect was bridged using a silicon conduit filled with 10 µL (0.1 mg/mL) 17- beta- estradiol. Each group was subdivided into four subgroups of five animals each and nerve fibers were studied in a 12-week period.

**Results:** Behavioral, functional, biomechanical, electrophysiological and gastrocnemius muscle mass findings and morphometric indices confirmed faster recovery of regenerated axons in treatment group than in other groups ( $p < 0.05$ ). Immunohistochemical reactions to S-100 in treatment group were more positive than that in other groups.

**Conclusion:** Local administration of 17-beta-estradiol improved functional recovery and morphometric indices of sciatic nerve. It could have clinical implications for the surgical management of patients after facial nerve transection.

**Keywords:** Nerve regeneration; Sciatic nerve; 17-beta-estradiol; Local administration; Rat.

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### Introduction

The ideal surgical repair technique should accomplish good wound healing with minimal

scar formation and direct the nerve sprouts into their correct targets [1]. When primary repair cannot be performed without undue tension, nerve grafting is required. Autografts remain the standard for nerve

grafting material [2]. The results following nerve repairs are influenced by many parameters, such as the nature, location, and extent of the injury, the level and timing of the repair, the fascicular anatomy, and appropriateness of re-alignment of the injured nerve, and the surgical technique, as well as patient factors [3]. In addition to these factors in the regeneration of nerve repair, some pharmaceutical agents which are used locally at the site of nerve repair also have an effect [4].

Estrogen belongs to the unique superfamily of hormones which regulate expression of different genes involved in cell proliferation, growth, function and death [5]. In the nervous system, alpha and beta isoforms of estrogen receptors are not restricted to regions controlling reproductive function but are widely distributed in anatomically distinct regions [6]. Estrogen acts on neuronal cells through the classical genomic pathway by binding to steroid / thyroid super family nuclear receptors, which in turn activate expression of estrogen-regulated genes [5]. Another possible mechanism of action of estrogen in neurons is modulation of membrane-initiated cell signaling pathways, involving MAP kinases [7]. Effects of estrogen on the nervous system are quite diverse. The neuroprotective effect of estrogen on neuronal cells was observed in tissue and cell culture conditions [8,9]. It has been shown that estrogen enhances regeneration of neurons in hamster facial motor nucleus [10]. Estrogen–progesterone treatment was used to suppress the scar reaction after transection and suture of the sciatic nerve and to stimulate muscle re-innervation following crush injury of the sciatic nerve [11,12]. Both a and b isoforms of estrogen receptors are expressed in sensory neurons of dorsal root ganglions [13]. Recently, estrogen has been reported to be involved in proliferation of Schwann cells [14]. Schwann cells A major rate-limiting step in the induction of nerve regeneration across a gap is the proliferation, and migration of Schwann cells between the nerve stumps. Therefore, formation of a properly aligned extra cellular matrix scaffold is essential to enhance Schwann cell proliferation in a conduit, through which blood vessels and other cell types migrate and form primordial assembly for the formation of a new nerve structure [15]. We used silicon as a conduit to provide a scaffold to facilitate Schwann cells migration. Systemic treatment with estradiol is associated with several problems such as breast carcinogenesis [16]. Local administration of estradiol is selected in the present study as a therapy for injured nerves in order to avoid systemic side effects.

To the best of authors' knowledge the effects of local estradiol application at the site of small gap transected sciatic nerve has not been studied. Aimed to study local effects of 17- beta- estradiol on peripheral nerve regeneration, this study was designed to determine if its local administration could in fact reduce dysfunction after nerve injury

in the rat sciatic nerve transection model.

## Materials and Methods

### *Study Design and Animals*

Sixty ovariectomized female Wistar rats were included in this study. At baseline, body weight was determined and the animals were randomly stratified into four groups of 15 rats in each group: Sham operation group (SHAM), transected control (TC), silicon conduit (Silicon) and 17- beta- estradiol treated group (Con./Estradiol). Each group was further subdivided into three subgroups of five animals each and surveyed 4, 8 and 12 weeks after surgery. Two weeks before and during the experiments, the animals were housed in individual plastic cages with an ambient temperature of  $23\pm 3^{\circ}\text{C}$ , stable air humidity and a natural day/night cycle. The rats had free access to standard rodent laboratory food and tap water. All measurements were made by two blinded observers unaware of the analyzed groups.

### *Preparation of Ovariectomized Rats*

The rats were ovariectomized based on method described by others [17]. In brief, after peritoneal cavity was accessed, the adipose tissue was pulled away until the right uterine tube with the ovary surrounded by a variable amount of fat was identified. The ovary and associated fat were easily located and exteriorized by gentle retraction. The procedure was repeated for the left ovary through the same incision. After identifying the ovary and uterine horn, a Vicryl (Ethicon, Norderstedt) 4/0 suture was performed around the area of the distal uterine horns, that was sectioned thereafter, and the ovaries were removed. The uterine horn was returned to the peritoneal cavity after the removal of ovaries. The peritoneum and the muscle layers were sutured with 4/0 Vicryl (Ethicon, Norderstedt) and the skin was sutured with 3/0 nylon (Dafilon, B/Braun, Germany). Povidone iodine was applied on the area to disinfect the skin after suturing. The procedure was maintained aseptic throughout the operation. After surgery, the rats were housed individually in polyurethane boxes for a period of one week to allow recovery and then re-grouped in their home cages.

### *Surgical Procedure*

Ovariectomized animals were anesthetized by intraperitoneal administration of ketamine 5%, 90mg/kg (Ketaset 5%; Alfasan, Woerden, The Netherlands) and xylazine hydrochloride 2%, 5mg/kg (Rompun 2%, Bayer, Leverkusen, Germany). The procedures were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of pain [18]. The University Research Council approved all experiments. Through a muscle splitting approach,

the plane between gluteus maximus and biceps femoris was developed and the right sciatic nerve was clearly visible on the underlying hamstrings muscles. Sutures were passed through the nerve epineurium (one on each side), 3 mm apart at a level of 1 cm above the trifurcation of the nerve. Sutures had the same circumferential orientation on the nerve to restore spatial longitudinal nerve continuity. Before transection, both needles were driven through silicon conduit at each side 2 mm from the edge of the conduit. This facilitated proper and prompt insertion before endoneurial edema obscured the cut ends. Afterward, a complete transection between the sutures was undertaken and the cut ends of the nerve were driven carefully with the aid of the sutures inside the silicon conduit and held in place.

A second epineurial suture was placed, at each side and through the conduit. After placement, the chambers were filled with 10  $\mu$ L a neutral pH sterile solution of 17- beta- estradiol (0.1 mg/mL) (Sigma-Aldrich Chemie, Munich, Germany). In the sham-operation group (SHAM), the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with Vicryl (Ethicon, Norderstedt) 4/0 sutures, and the skin with 3/0 nylon (Dafilon, B/Braun, Germany). The rats were observed on a heating pad during recovery.

The animals of each group were anesthetized by intraperitoneal administration of ketamine-xylazine (see above) and were perfused via left cardiac ventricle with a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH 7.4) at 4, 8 and 12 weeks after surgery.

### Behavioral Testing

Functional recovery of the nerve was assessed using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale for rat hind limb motor function [19]. Although BBB is widely used to assess functional recovery in spinal cord injured animals, however, it has been demonstrated that it could be most useful in assessment of never repair processes in peripheral nerve injuries [20]. Scores of 0 and 21 were given when there were no spontaneous movement and normal movement, respectively. A score of 14 shows full weight support and complete limbs coordination. BBB recordings were performed by a trained observer who was blinded to the experimental design. The testing was performed in a serene environment. The animals were observed and assessed within a course of a 4-minute exposure to an open area of a mental circular enclosure. BBB scores were recorded once before surgery in order to establish a baseline control and again weekly thereafter to assess functional recovery during 12 weeks.

### Functional Assessment of Reinnervation Sciatic Functional Index (SFI)

Walking track analysis was performed 4, 8 and 12

weeks after surgery based on the method of others [21]. The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The sciatic function index (SFI) of each animal was calculated by the following formula:

$$\text{SFI} = -38.3 \times (\text{EPL} - \text{NPL}) / \text{NPL} + 109.5 \times (\text{ETS} - \text{NTS}) / \text{NTS} + 13.3 \times (\text{EIT} - \text{NIT}) / \text{NIT} - 8.8$$

In general, SFI oscillates around 0 for normal nerve function, whereas around -100 SFI represents total dysfunction. SFI was assessed in the NC group and the normal level was considered as 0. SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

### Static Sciatic Index (SSI)

SSI is a time-saving digitized static footprint analysis described by others [22]. A good correlation between the traditional SFI and the newly developed static sciatic index (SSI) and static toe spread factor (TSF), respectively, has been reported by others [22]. The SSI is a time-saving and easy technique for accurate functional assessment of peripheral nerve regeneration in rats and is calculated using the static factors, not considering the print length factor (PL), according to the equation:

$$\text{SSI} = [(108.44 \times \text{TSF}) + (31.85 \times \text{ITSF})] - 5.49$$

Where:

$$\text{TSF} = (\text{ETS} - \text{NTS}) / \text{NTS}$$

$$\text{ITSF} = (\text{EIT} - \text{NIT}) / \text{NIT}$$

Like SFI, an index score of 0 was considered normal and an index of -100 indicated total impairment. When no footprints were measurable, the index score of -100 was given.

### Electrophysiological Assessment

After 12 weeks, following the track test, all animals were subjected to electrophysiological studies using Nacro bio system 320-3760 A trace 80 (USA). Under general anesthesia the left sciatic nerve was re-exposed by incision of the skin at the previous surgical site. Single electrical pulses (at supra maximal intensity) were delivered via bipolar electrodes placed in turn at the proximal and distal trunk of the regenerated nerve and electromyography (EMG) was recorded by inserting an electrode into the belly of gastrocnemius muscle. The latency and the amplitude of EMG were obtained. Also, the difference in latency of EMG was measured, and the distance between the proximal and distal sites of stimulation was measured to calculate the conduction velocity across the regenerated nerve. On the contralateral, right intact side of each animal, similar measurements were made for the determination of conduction velocity. The conduction velocity of the bridged nerve was expressed as a percentage of that on the intact side of each animal to cancel off variations between animals (% CVR).

The recovery index of EMG amplitude in all groups

was calculated based on Suzuki *et al.* using the following formula:

Recovery index=Peak amplitude of the operated side/Peak amplitude of the intact side [23].

### Biomechanical Testing

Following electrophysiological assessments the regenerated nerves were harvested and placed in a normal saline bath at room temperature. The samples were then fixed between frozen fixtures in a mechanical apparatus. The TA.XTPlus Texture Analyzer mechanical test device was used for the assessment (Stable Micro Systems, Surrey GU7 1YL, UK). After 5 minutes, the frozen fixtures were tightened to ensure that no slippage occurred during testing. The initial length was set to 10 mm. Each sample was stretched at a constant rate of 1 mm/min. The load and displacement were sampled 5 times per second. Each sample was stretched to complete tensile failure. Samples were kept wet moist during testing using a drop of normal saline solution to the nerve segments.

### Muscle Mass

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 weeks after surgery. Immediately after sacrificing of animals, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides and weighed while still wet, using an electronic balance.

### Histological Preparation and Morphometric Studies

Nerve mid-substance in silicon group, nerve mid-substance in Con./Estradiol treated group, midpoint of normal sciatic nerve (NC) and regenerated mid substance of TC group were harvested and fixed with glutaraldehyde 2.5%. They were post fixed in OsO<sub>4</sub> (2%, 2 h), dehydrated through an ethanol series and embedded in paraffin. The nerves were cut in 5  $\mu$ m in the middle, stained with toluidine blue and examined under light microscopy. Morphometric analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). Equal opportunity, systematic random sampling and two-dimensional dissector rules were followed in order to cope with sampling-related, fiber-location-related and fiber-size related biases [24].

### Immunohistochemical Analysis

In this study, anti-S-100 (1:200, DAKO, USA) was used as marker for myelin sheath. Specimens were post fixed with 4% paraformaldehyde for 2h and embedded in paraffin. Prior to immunohistochemistry nerve sections were dewaxed and rehydrated in PBS (pH 7.4). Then the nerve sections were incubated with 0.6% hydrogen peroxide for 30 minutes. To block non-specific immunoreactions the sections were incubated with normal swine serum (1:50,

DAKO, USA). Sections were then incubated in S-100 protein antibody solution for 1h at room temperature. They were washed three times with PBS and incubated in biotinylated anti-mouse rabbit IgG solution for 1h. Horseradish peroxidase-labelled secondary antibody was applied for 1 h. After that all sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride chromogene substrate solution (DAB, DAKO, USA) for 10 min. The results of immunohistochemistry were examined under a light microscope.

### Statistical Analysis

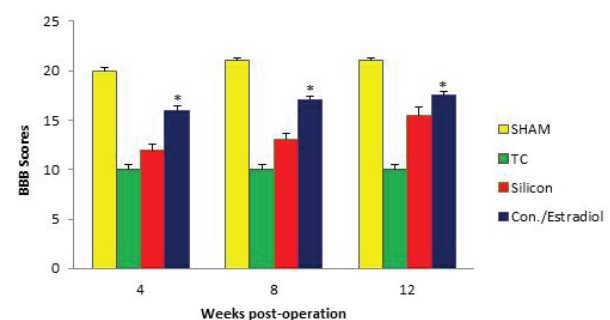
The results were expressed as means $\pm$ SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using a factorial ANOVA with two between-subjects factors. Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. The differences were set at  $p < 0.05$ .

## Results

### Behavioral Testing

#### BBB Recovery

In order to assess hind limb recovery the open field locomotor was used. Figure 1 shows BBB scores compared to the baseline. All experimental groups, except for SHAM, showed the greatest degree of functional deficit one week after surgery. The 17-beta-estradiol treated group showed significant improvement in locomotion of the operated limb compared to the silicon group during the study period ( $p=0.001$ ).

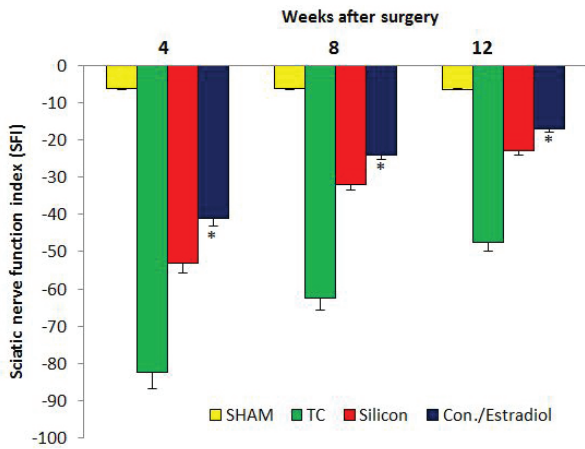


**Fig. 1.** Box-and-whisker plots of sciatic nerve BBB scores in each experimental group during the study period. Local administration of 17- beta- estradiol with silicon conduit gave better scores than in silicon group. Standard error at each data point is shown with bars.

### Recovery of Sciatic Nerve Function

#### SFI Outcome

Figure 2 shows sciatic function index (SFI) values in all four experimental groups. Prior to surgery, SFI values in all groups were near zero. After the nerve transection, the mean SFI decreased to -100



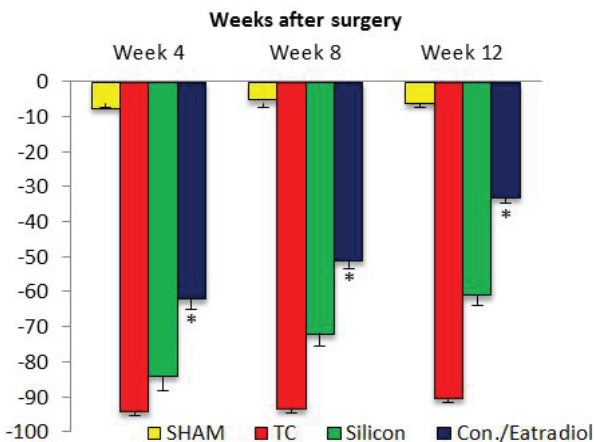
**Fig. 2.** Bar graph indicating sciatic nerve function index values in each experimental group during the study period. Local administration of 17- beta- estradiol with silicon conduit gave better results in functional recovery of the sciatic nerve than in silicon group. Data are presented as mean $\pm$ SE. \* $p$ <0.05 vs silicon group

due to the complete loss of sciatic nerve function in all animals. At the end of the study period, animals of Con. Estradiol group achieved a mean value for SFI of  $-33.5\pm 3.14$  whereas in group silicon a mean value of  $-51.5\pm 4.18$  was found. The statistical analyses revealed that the recovery of nerve function was significantly ( $p=0.001$ ) different between Con./Estradiol and silicon groups and application of the 17- beta- estradiol in silicon conduit significantly accelerated functional recovery in the course of time.

### SSI Outcome

Changes in SSI were similar to those observed in SFI, indicating significant deficit following the sciatic nerve transection (Figure 3). Changes in SSI were significant at weeks 4, 8 and 12 of recovery ( $p=0.001$ ). The contrasts indicate SSI values in group Con./Estradiol

### Static Sciatic Index (SSI)

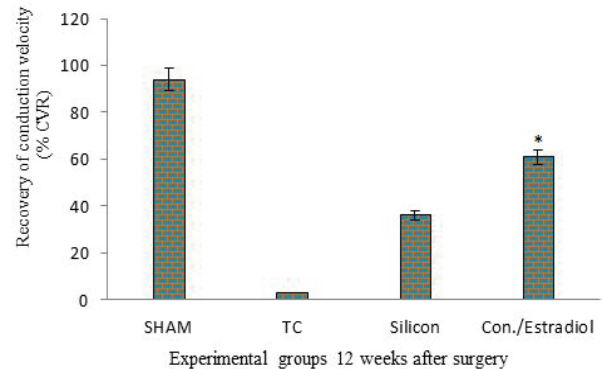


**Fig. 3.** Bar graph indicating static sciatic index (SSI) values in each experimental group during the study period. Topical administration of 17- beta- estradiol with silicon conduit gave better results in functional recovery of the sciatic nerve than in Silicon group. Data are presented as mean $\pm$ SE. \* $p$ <0.05 vs silicon group

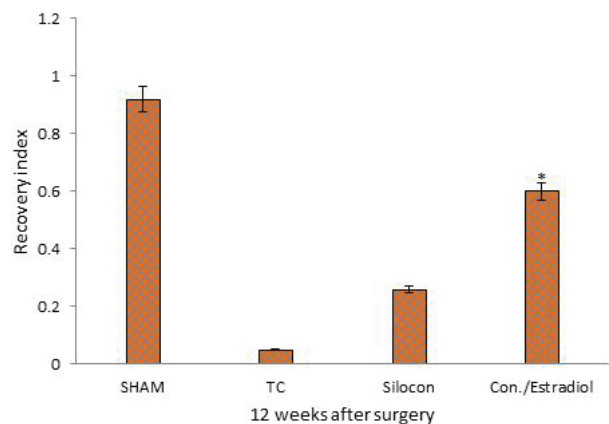
at week 12 to differ significantly from those obtained from silicon, a trend also noticed for SFI ( $p=0.001$ ).

### Electrophysiological Measurement

Figures 4 and 5 show nerve conduction velocity (NCV) along regenerated sciatic nerves in experimental groups. NCV in 17- beta- estradiol treated animals was significantly higher than that in silicon group ( $p=0.001$ ).



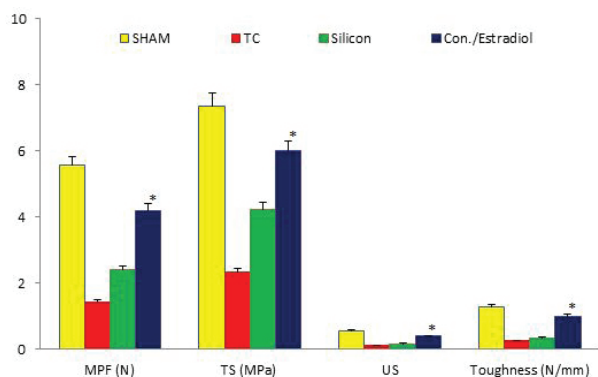
**Fig. 4.** Percentage recovery of conduction velocity in experimental groups. Data are presented as mean $\pm$ SE. \* $p$ <0.05 vs silicon group.



**Fig. 5.** Recovery index in experimental groups. Data are presented as mean $\pm$ SE. \* $p$ <0.05 vs silicon group

### Biomechanical Measurements

Maximum pull force ( $F_{max}$ ) of normal sciatic nerve was found to be  $5.42\pm 0.37$ .  $F_{max}$  of nerve samples in experimental groups are shown in Figure 6.  $F_{max}$  in Con./Estradiol group was significantly higher than that in silicon group ( $p=0.001$ ). Tensile strength, the amount of force per unit of initial cross-sectional area at tensile failure, was measured based on  $F_{max}$  and nerve cross sectional area. Assessment on week 12 revealed that tensile strength of regenerated nerves in Con./Estradiol group was higher than those in silicon group ( $p=0.001$ ). Ultimate strain, the amount of elongation divided by the initial specimen length achieved at the point of tensile failure, in Con./Estradiol group was significantly higher than that in silicon group ( $p=0.001$ ). Toughness, reflecting the properties of anti-deformation and anti-fracture of nerve, was determined by the nerve itself and



**Fig. 6.** Bar graph indicating biomechanical analyses of regenerated nerves for each of the experimental groups: Data are presented as mean±SE. \* $p < 0.05$  vs silicon group; MPF: Maximum Pull Force; TS: Tensile Strength; US: Ultimate Strain

could reflect “looseness” or “toughness” of nerve. Toughness in Con./Estradiol group was significantly higher than that in silicon group ( $p = 0.001$ ).

**Muscle Mass Measurement**

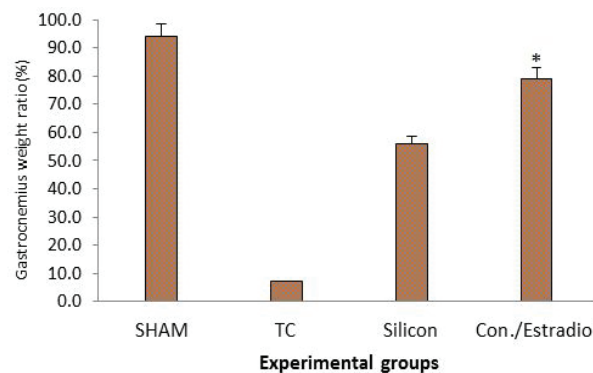
The mean ratios of gastrocnemius muscle weight were measured at the end of the study period. There was a statistically significant difference between the muscle weight ratios of the Con./Estradiol and silicon groups ( $p = 0.001$ ). The results showed that in Con./Estradiol the group, the muscle weight ratio was larger than in the silicon group, and weight loss in the gastrocnemius muscle was ameliorated by local administration of 17- beta- estradiol (Figure 7).

**Histological and Morphometric Findings**

The animals of Con./Estradiol group presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness during study period, compared to silicon animals ( $p = 0.001$ ). Normal control group presented significantly greater nerve fiber and axon diameter, and myelin sheath thickness compared to Con./Estradiol and silicon groups animals (Table 1). In case of myelin thickness there was no significant difference between Con./Estradiol and silicon groups, morphometrically ( $p = 0.0625$ ).

**Immunohistochemistry**

Immunoreactivity to S-100 protein was extensively observed in the cross sections of regenerated nerve segments. The expression of S-100 protein signal was located mainly in the myelin sheath. The axon also showed a weak expression indicating that Schwann cell-like phenotype existed around the myelinated



**Fig. 7.** Bar graph indicating percentage of gastrocnemius muscle weight ratio. The gastrocnemius muscles of both sides were excised and weighed in the experimental groups at 12 weeks after surgery. Data are presented as mean±SE. \* $p < 0.05$  vs. silicon group

axons (Figure 8). In both Con./Estradiol and silicon groups, the expression of S-100 and the findings resembled those of the histological evaluations.

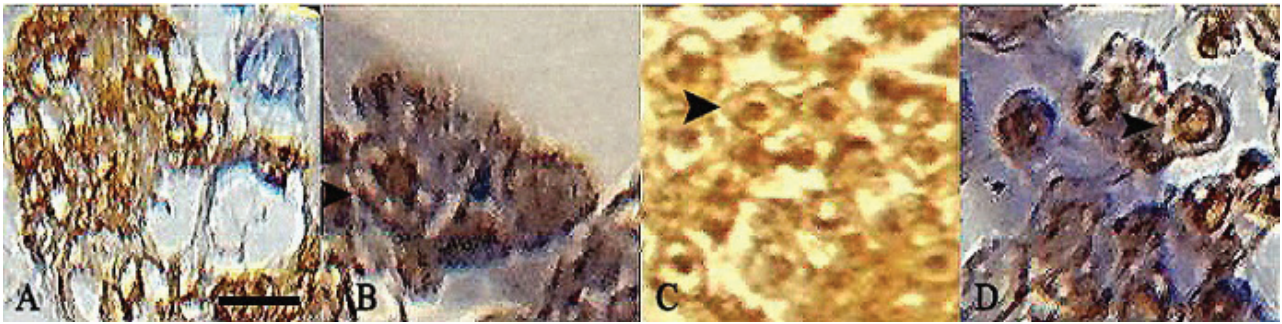
**Discussion**

Estrogen receptors are found in a wide range of cell types in the body, explaining the pleiotropic effects of this hormone [25]. 17- beta- estradiol has been demonstrated to play a role in neuroprotection [26]. 17- beta- estradiol protects cultured primary neurons against the neurotoxic effects of glutamate and oxidative stress and its neuroprotective effects are also observed in vivo. Animal studies have revealed that in the event of high 17- beta- estradiol levels during the premenopausal period, female rodents are reportedly less vulnerable to neurotrauma [27]. Neurospheres are free-floating heterogeneous aggregates that contain a minority of neural stem cells together with various progenitors and more differentiated cells. These cells, under appropriate culture conditions, can produce neurons, astrocytes, and oligodendrocytes [28,29]. Neurosphere-derived cells have been successfully used in models of brain injury, such as experimental autoimmune encephalomyelitis or spinal cord injury [29]. It has been indicated that neural stem cells can differentiate into both neurons and endothelial cells, and the ability of neural stem cells to differentiate into endothelial cells has been demonstrated in vitro and in vivo [30,31]. In cases of peripheral nerve injury, 17- beta- estradiol stimulate angiogenesis and neurogenesis during the process of recovery from nerve damage [16].

**Table 1.** Morphometric analyses of sciatic nerve in each of the experimental groups: Values are given as mean±SE

Groups	Axon counts fb/mm <sup>2</sup>	Axon diameter (µm)	Myelin sheath thickness(µm)
SHAM	29478±2312	11.36±0.18	2.63±0.02
TC	4096±2005	3.30±0.15	1.00±0.02
Silicon	20171±2100	6.14±0.10	1.19±0.02
Con./Estradiol	25104±2204 <sup>a</sup>	7.71±0.11 <sup>a</sup>	1.42±0.03

<sup>a</sup>The mean difference is significant at the 0.05 level vs. Silicon group



**Fig. 8.** Immunohistochemical analysis of the regenerated nerves 12 weeks after surgery from middle cable (A) SHAM, (B) TC, (C) Silicon and (D) Con./Estradiol. There is clearly more positive staining of the myelin sheath-associated protein S- 100 (arrowheads) within the periphery of nerve, indicating well organized structural nerve reconstruction in 17- beta- estradiol treated nerve compared to that of the silicon group. Scale bar:10  $\mu$ m

It is known from previous studies that regeneration process in rats would not have been completed by 12 weeks, a phenomenon which has been reported in a variety of experimental models [32]. Quantitatively, our results are consistent with these findings. However, a 12-week experimental period is sufficient for evaluation of regeneration process because in rats functional recovery after repair of a transected peripheral nerve occurs during this timeline [33].

The results of the present study showed that application of 17- beta- estradiol in a silicon conduit resulted in faster functional recovery of the sciatic nerve during the study period. Left gastrocnemius muscle weight was significantly greater in the Con./Estradiol group than in the silicon group, indicating indirect evidence of successful end organ reinnervation in the 17- beta- estradiol treated animals.

The conduction velocity depends on the diameter of axons and the thickness of myelin sheath [34]. The results of the present study showed significantly different conduction velocity between the 17- beta- estradiol treated animals and silicon bridged regenerated sciatic nerves, therefore, the silicon conduit in combination with 17- beta- estradiol could be assumed as a safe nerve guide with no nerve conduction interference. The strongest connective tissue layers in peripheral nerves are the perineurium and, to a lesser extent, the epineurium. Changes in the epineurium and perineurium extracellular matrix composition are likely to have significant effects on the biomechanical properties of acellular nerve [35]. The connective tissue from the epineurium forms a layer of fiber membrane at the 3rd day postoperatively and then forms collagen at the 8th day. The key point influencing functional recovery is the number of axons throughout the suture that enhances the anti-tension capacity of the nerve [36]. 17- beta- estradiol local administration in the present study resulted in the enhanced biomechanical indices that were in agreement with morphometric findings.

In immunohistochemistry the expression of myelin sheath special proteins was evident in both groups which indicate the normal histological structure. The location of reactions to S-100 in the 17- beta- estradiol treated group was clearly more marked than

in the silicon group implying that both regenerated axon and Schwann cell-like cells existed and were accompanied by the process of remyelination and the structural recovery of regenerated nerve fibers.

It has been demonstrated that morphometric indices are measures of regenerated nerve maturity and quality of regeneration [37]. Larger diameters of axons and thicker myelination give rise to improved nerve function compared to smaller and thinner myelinated fibers [38]. Loading of 17- beta- estradiol into silicon conduit at the nerve repair site increased fiber maturity.

At week 12 quantitative morphometrical indices of regenerated nerve fibers showed significant differences between the silicon and Con./Estradiol groups, indicating a beneficial effect of local application of 17- beta- estradiol on the nerve regeneration.

Although both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor [39].

Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling do not necessarily predict the reestablishment of motor and sensory functions [39]. Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery [39]. Therefore, research on peripheral nerve injury needs to combine both functional and morphological assessment. It has been suggested that arrival of sprouts from the proximal stump at the distal nerve stump does not necessarily imply recovery of nerve function [33]. Information taken from BBB scale may be invaluable in evaluation of peripheral nerve process. Correlation between BBB and SFI index has already been documented in other studies, therefore, BBB could also be a reliable method for assessment of peripheral nerve regeneration [40,41].

Results of the present study showed that the 17- beta- estradiol treated animals had been improved in locomotion of the operated limb compared to the silicon group during the study period. In the present

study, first of all it was important to know whether local exogenous administration of 17- beta- estradiol in silicon tubes was able to stimulate the regeneration of the transected rat sciatic nerve. With this aim, we compared the regeneration of the transected sciatic nerve within silicon nerve guides. Our functional results revealed that the silicon guides allow 17- beta- estradiol to exert the stimulation of nerve regeneration. In addition, gasterocnemius muscle mass, obtained from muscles of operated and unoperated limbs indicated that motor functional recovery in transected sciatic nerve bridged by silicon conduits achieved a faster rate. The functional, morphometric and immunohistochemical results indicated that silicon nerve guide did not prevent the stimulating action of 17- beta- estradiol. Even though our study shows the regenerative action of local 17- beta- estradiol in peripheral nerve injuries, data regarding the molecular mechanisms leading to the action remain to be investigated in depth. We have not given the histological and molecular evidence for regenerative action of 17- beta- estradiol. This may be considered as a limitation to our study. Therefore, the authors stress that the aim of the

current investigation was to evaluate a single local dose and clinical treatment potential of 17- beta- estradiol on nerve regeneration.

The results of the present study indicated that a single local administration of 17- beta- estradiol at the site of transected nerve could be of benefit after silicon tubulization. Detailed mechanism of regenerative action remains to be investigated. In Conclusion results of the present study demonstrated that a single local application of 17- beta- estradiol could accelerate functional recovery after transection of sciatic nerve and could have clinical implications for the surgical management of patients after facial nerve transection.

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**Conflict of Interest:** None declared.

### References

- Ferguson MW, O'Kane S. Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. *Philos Trans R Soc Lond B Biol Sci.* 2004;**359**(1445):839-50.
- Yousef MA, Dionigi P, Marconi S, Calligaro A, Cornaglia AI, Alfonsi E, et al. Successful Reconstruction of Nerve Defects Using Distraction Neurogenesis with a New Experimental Device. *Basic Clin Neurosci.* 2015;**6**(4):253-64.
- Wlasczuk A, Pietrucha-Dutczak M, Marcol W, Jedrzejska-Szypulka H, Lewin-Kowalik J. The use of chitosan to facilitate nerve regeneration. *Wiad Lek.* 2011;**64**(3):208-16.
- Mekaj AY, Morina AA, Bytyqi CI, Mekaj YH, Duci SB. Application of topical pharmacological agents at the site of peripheral nerve injury and methods used for evaluating the success of the regenerative process. *J Orthop Surg Res.* 2014;**9**:94.
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. *Cell.* 1995;**83**(6):835-9.
- Shughrue PJ, Merchenthaler I. Distribution of estrogen receptor beta immunoreactivity in the rat central nervous system. *J Comp Neurol.* 2001;**436**(1):64-81.
- Boonyaratankornkit V, Edwards DP. Receptor mechanisms mediating non-genomic actions of sex steroids. *Semin Reprod Med.* 2007;**25**(3):139-53.
- Toran-Allerand CD. Estrogen and the brain: beyond ER-alpha, ER-beta, and 17beta-estradiol. *Ann N Y Acad Sci.* 2005;**1052**:136-44.
- Caceres LG, Uran SL, Zorrilla Zubilete MA, Romero JI, Capani F, Guelman LR. An early treatment with 17-beta-estradiol is neuroprotective against the long-term effects of neonatal ionizing radiation exposure. *J Neurochem.* 2011;**118**(4):626-35.
- Hrabovszky E, Kallo I, Steinhauser A, Merchenthaler I, Coen CW, Petersen SL, et al. Estrogen receptor-beta in oxytocin and vasopressin neurons of the rat and human hypothalamus: Immunocytochemical and in situ hybridization studies. *J Comp Neurol.* 2004;**473**(3):315-33.
- Nachemson AK, Lundborg G, Myrhage R, Rank F. Nerve regeneration and pharmacological suppression of the scar reaction at the suture site. An experimental study on the effect of estrogen-progesterone, methylprednisolone-acetate and cis-hydroxyproline in rat sciatic nerve. *Scand J Plast Reconstr Surg.* 1985;**19**(3):255-60.
- Smith PG, George M, Bradshaw S. Estrogen promotes sympathetic nerve regeneration in rat proximal urethra. *Urology.* 2009;**73**(6):1392-6.
- Wilson ME, Westberry JM. Regulation of oestrogen receptor gene expression: new insights and novel mechanisms. *J Neuroendocrinol.* 2009;**21**(4):238-42.
- Magnaghi V, Cavarretta I, Galbiati M, Martini L, Melcangi RC. Neuroactive steroids and peripheral myelin proteins. *Brain Res Brain Res Rev.* 2001;**37**(1-3):360-71.
- Horie H, Kadoya T, Sango K, Hasegawa M. Oxidized galectin-I is an essential factor for peripheral nerve regeneration. *Curr Drug Targets.* 2005;**6**(4):385-94.
- Sekiguchi H, Ii M, Jujo K, Thorne T, Ito A, Klyachko E, et al. Estradiol promotes neural stem cell differentiation into endothelial lineage and angiogenesis in injured peripheral nerve. *Angiogenesis.* 2013;**16**(1):45-58.
- Khajuria DK, Razdan R, Mahapatra DR. Description of a new method of ovariectomy in female rats. *Rev Bras Reumatol.* 2012;**52**(3):462-70.
- Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain.* 1983;**16**(2):109-10.
- Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. *J Neurotrauma.* 2006;**23**(5):635-59.



20. Dinh P, Hazel A, Palispis W, Suryadevara S, Gupta R. Functional assessment after sciatic nerve injury in a rat model. *Microsurgery*. 2009;**29**(8):644-9.
21. Varejao AS, Melo-Pinto P, Meek MF, Filipe VM, Bulas-Cruz J. Methods for the experimental functional assessment of rat sciatic nerve regeneration. *Neurol Res*. 2004;**26**(2):186-94.
22. Bervar M. Video analysis of standing-an alternative footprint analysis to assess functional loss following injury to the rat sciatic nerve. *J Neurosci Methods*. 2000;**102**(2):109-16.
23. Sabongi RG, Fernandes M, Dos Santos JB. Peripheral nerve regeneration with conduits: use of vein tubes. *Neural Regen Res*. 2015;**10**(4):529-33.
24. Geuna S, Gigo-Benato D, Rodrigues Ade C. On sampling and sampling errors in histomorphometry of peripheral nerve fibers. *Microsurgery*. 2004;**24**(1):72-6.
25. Cavailles V. Estrogens and receptors: an evolving concept. *Climacteric*. 2002;**5** Suppl 2:20-6.
26. Islamov RR, Hendricks WA, Jones RJ, Lyall GJ, Spanier NS, Murashov AK. 17Beta-estradiol stimulates regeneration of sciatic nerve in female mice. *Brain Res*. 2002;**943**(2):283-6.
27. Roof RL, Hall ED. Gender differences in acute CNS trauma and stroke: neuroprotective effects of estrogen and progesterone. *J Neurotrauma*. 2000;**17**(5):367-88.
28. Gage FH. Mammalian neural stem cells. *Science*. 2000;**287**(5457):1433-8.
29. Pallini R, Vitiani LR, Bez A, Casalbore P, Facchiano F, Di Giorgi Gerevini V, et al. Homologous transplantation of neural stem cells to the injured spinal cord of mice. *Neurosurgery*. 2005;**57**(5):1014-25; discussion -25.
30. Wurmser AE, Nakashima K, Summers RG, Toni N, D'Amour KA, Lie DC, et al. Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. *Nature*. 2004;**430**(6997):350-6.
31. Ii M, Nishimura H, Sekiguchi H, Kamei N, Yokoyama A, Horii M, et al. Concurrent vasculogenesis and neurogenesis from adult neural stem cells. *Circ Res*. 2009;**105**(9):860-8.
32. Siemionow M, Sonmez E. Nerve allograft transplantation: a review. *J Reconstr Microsurg*. 2007;**23**(8):511-20.
33. Castaneda F, Kinne RK. Omental graft improves functional recovery of transected peripheral nerve. *Muscle Nerve*. 2002;**26**(4):527-32.
34. Chen CJ, Ou YC, Liao SL, Chen WY, Chen SY, Wu CW, et al. Transplantation of bone marrow stromal cells for peripheral nerve repair. *Exp Neurol*. 2007;**204**(1):443-53.
35. Ma X-L, Sun X-L, Yang Z, Li X-L, Ma J-X, Zhang Y, Yuan Z-Z. Biomechanical properties of acellular sciatic nerves treated with a modified chemical method. *Neural Regen Res*. 2011;**6**:252-256.
36. Jiang B, Zhang P, Yan J, Zhang H. Dynamic observation of biomechanical properties of sciatic nerve at the suture site in rats following repairing. *Artif Cells Blood Substit Immobil Biotechnol*. 2008;**36**(1):45-50.
37. Hamilton SK, Hinkle ML, Nicolini J, Rambo LN, Rexwinkle AM, Rose SJ, et al. Misdirection of regenerating axons and functional recovery following sciatic nerve injury in rats. *J Comp Neurol*. 2011;**519**(1):21-33.
38. Quintes S, Goebbels S, Saher G, Schwab MH, Nave KA. Neuron-glia signaling and the protection of axon function by Schwann cells. *J Peripher Nerv Syst*. 2010;**15**(1):10-6.
39. Sarikcioglu L, Demirel BM, Utuk A. Walking track analysis: an assessment method for functional recovery after sciatic nerve injury in the rat. *Folia Morphol (Warsz)*. 2009;**68**(1):1-7.
40. Mehrshad A, Seddighnia A, Shadabi M, Najafpour A, Mohammadi R. Local Effect of Heparin Binding Neurotrophic Factor Combined With Chitosan Entubulization on Sciatic Nerve Repair in Rats. *Bull Emerg Trauma* 2016;**4**(2):80-87.
41. Abbasipour-Dalivand S, Mohammadi R, Mohammadi V. Effects of Local Administration of Platelet Rich Plasma on Functional Recovery after Bridging Sciatic Nerve Defect Using Silicone Rubber Chamber; An Experimental Study. *Bull Emerg Trauma*. 2015;**3**(1):1-7.