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Anti-inflammatory Signalling Molecule Improved Peripheral Nerve Repair

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Abstract:

Introduction: Early inflammatory procedures may inhibit functional recovery after nerve injury. Tumor necrosis factor-α (TNF) plays a crucial role in the initiation of degenerative cascades after peripheral nerve injury.The aim of this study was to test the effects of TNF antagonist etanerceptonnerve regeneration in a rat model of sciatic nerve crush injury.

Experimental Procedures: 15 adult Wistar rats underwent a surgical procedure involving right sciatic nerve crush injury. Etanercept(6 mg/kg) was administered intra-peritoneally (IP)once immediately after nerve crush in the experimental group. Rats in the control group received saline IP post surgery. Genomic and immune-histochemical tests were done at 1, 3, 7, 21 and 42 days after injury. Functional and electromyography (EMG) assessments of nerves were performed at 7, 21 and 42 days after injury. Functional recovery was analyzed using hot plate test, a walking track assessment and quantified using the sciatic functional index (SFI).

Results: TNF mRNA expression was induced at 1 day and returned to baseline at the end of 1 week after injury in nerve. Etanerceptprevented increase of TNF in the crush site and enhanced the rate of axonal regeneration, as determined by increased number of characteristic clusters of regenerating nerve fibers distal to nerve crush segments, but it was not significant statistically with control group on 42 days. The statistical analyses of EMG studies showed that the latency and the amplitude in experimental group of 21 and 42 days were significantly different from the control group. SFI in the experimental group on 21 and 42 days was significantly higher than that of the control group.

Conclusion: This study established that immediate therapy with etanercept promotes the regeneration of peripheral nerve injuries in a rat model.

Key Words: Tumor necrosis factor-α; Nerve Regeneration

1. Introduction

Tissue engineering concepts integrate cells, scaffolds, signalling molecules. The main role for using signalling molecules is to activate a cell signalling cascade to enhance cell proliferation and cell migration from the defect into the newly regenerating tissue. Substantial number of signalling molecules are involved in peripheral nerve regeneration procedure. Peripheral nerve repair is a complicated process including different changes in cell processing such as Schwan cells proliferation and migration, 'bands of Büngner' formation, signalling molecules environment and axon sprouting. In the peripheral nerves extra cellular matrix, macromolecules are placed among cells. Peripheral nerve injury leads to a individual series of cellular and molecular events required for axon regeneration. Inflammatory reaction is one of the critical events, that very important for the orchestration of all the processes that occur during wallerian degeneration (GaoXet al 2013; Hall S. 2006).

Nerve fibers of the peripheral nervous system (PNS) are able to regenerate and reinnervate distal targets (Frisen 1997). These processes begin almost immediately after injury (KatoLiuKikuchiMyers and Shubayev 2010). Experimentally, a lot of medications were used in rat crush injury models such as steroids, nonsteroidalantiinflammatory drugs and vitamins (Le Prell Hughes and Miller 2007).

In the intact nervous system, tumor necrosis factor-α (TNF) is minimally expressed in the peripheral nerve. Early after nerve injury, TNF protein and mRNA at the injury site are upregulatedendoneurially primarily in nonneuronal cells, and after a brief temporal delay in dorsal root ganglion (DRG) neurons and the spinal cord (Scha¨ fersChristian GeisBrorsYaksh and Sommer 2002). The secretion of TNF and IL-1 proteins is detected within 5 to 6 hours after injury(Rotshenker 2011).The local increase in TNF activity initiates an inflammatory cascade that produces severe persistent neuropathic pain (KatoKikuchiShubayev and Myers 2009). TNF and IL-1 most probably play a critical role in setting the cytokine network in motion under normal conditions, as they do in other inflammatory conditions and disease states (Shamash Reichert and Rotshenker 2002).

Etanercept, a dimeric fusion protein containing a soluble TNFRII (Tracey Klares kog Sasso Salfeld and Tak 2008) and the Fc portion of human immunoglobulin G (IgG) (Zanella and others 2008), acts as a TNFantagonist with anti-inflammatory effects (Chio and others 2010), rendering TNF biologically inactive (Sehirli and others 2011).Etanercept has been shown to produce neuroprotective changes after brain and spinal cord injury (Campbell and others 2007). But potential beneficial effects of TNF on neurite outgrowth raise concern for use of anti-TNF agents for therapy of pain and neurodegenerative conditions (Kato Liu Kikuchi Myers and Shubayev 2010).

The present study was designed to analyze the effect of immediate systemic (IP) Etanercept therapy on functional regeneration rate of sciatic nerve after crush injury.

2. Experimental Procedures

A total of 15 Adult male wistar rats (Pasteur Institute Research Centre, Tehran, IRI) weighing 200–250 g were used. Rats were housed with a 12 h light/dark cycle with free access to food and water. Prior to surgery the animals were randomly divided into 3 groups: 1) control, rats were subjected to sciatic nerve crush injury plus an intraperitoneal (IP) dose of normal saline (1ml/kg body weight) 2) Experimental, this group was the same as control group with the exception that etanercept at the dose of 6 mg/kg was administered IP, 3) (no sciatic nerve crush). All injections were performed once immediately after surgery.

Each group in any experiment consisted of 5 rats. The investigation was performed in accordance with the guide for the care and use of laboratory animals published by the national institutes of health (1996), and was approved by the animal ethics committee of Baqiyatallah University.

2.1 Surgical procedure

The animals were anesthetized with ketamine (Alfasan, Holland) (90 mg/kg, IP) and xylazine (10mg/kg, IP).Rats were placed prone under sterile conditions and a skin incision was made from the greater trochanter to the mid-thigh. About 10 mm of right sciatic nerve was exposed unilaterally at the mid-thigh level through a gluteal muscle-splitting incision. Nerve crush injury was performed by using Julry forceps once for 30 Sec at 5 mm lower than sciatic notch. The crush site was labelled using toluidine blue. The muscle and skin layers were closed with silk sutures. The animals were allowed to recover and killed at various times; the required tissues were quickly removed and snap frozen on dry ice.

2.2 TNF-α mRNA detection by RT-PCR.

Sciatic nerve tissue was removed from non-operated and operated rats at various time points after injury and rapidly frozen in liquid nitrogen. Total RNA wasisolated from the tissues using Tripure isolation reagent kit. Qualitative evaluation of RNA was done by electrophoresis on ethidium bromide-stained 1% agarose gel, visualized by transluminator device. Quantitative evaluation of RNA was done by Nano Drop-spectrophotometer.

300 ng of total RNA were used to synthesize cDNA according kit instructions (Cyclescript RT Premix Korea/Bioneer). After that, PCR was done for product amplification.

Primer sequences for the PCR reaction were as follows (Table 1):

TNF-α	Forward	CCAGGAGAAAGTCCTCCT
	Reverse	TCATACCAGGGCTTGAGCTCA
β-actin	Forward	TCATGAAGATCCTCACCGAG
	Reverse	TTGCAATGGTGATGACCTG

Table1. Primer sequences for the PCR reaction and the number of cycles was 33 for TNF-α

β-actin reaction was used as a positive control. PCR amplification products were separated by electrophoresis on ethidium bromide-stained 2% agarose gel, visualized by transluminator device. For densitometry analysis, gels were scanned and analyzed by Scion image.

2.3 Histology study

At the end of 1st, 3rd and 6th weeks, the rats were sacrificed by ether. A 5-mm-long segment of the sciatic nerve distal to the crush area was removed and fixed immediately with glutaraldehyde solution, overnight. The specimens were dehydrated in graded concentration of ethanol and finally embedded in resin. Semi-thin transverse sections, 0.5 μm (500 nm) thick, were cut using an ultramicrotome (Leica, Germany) Sections were stained with 1% Toluidine Blue and 1% sodium-borate (borax) solution for 2-3 minutes. Slides were washed thoroughly with water and then analyzed with a light microscope equipped with a (Nikon, Ds-Fil-L2, Japan) digital camera and Image-j software.

2.4 Immunohistochemistry analysis

After total anesthesia at 1 day, 3 and 6 weeks post injury 1 mm from crushed, 1 mm from proximal and 3 mm from distal to crushed area of the sciatic nerve were removed and fixed in 4% paraformaldehyde in 0.01 M phosphate buffer. 15 μm thick sections of the nerve were cut using a cryomicrotome and placed on Silan coated slides. Slides were dried, immersed in 0.3% Triton solution, rinsed in phosphate buffered saline (PBS), immersed in Methanol/H_2O_2 solution, rinsed in PBS, treated with goat serum, incubated with Primary antibody overnight at 4 °C and Secondary antibody for 1h at room temperature in a humidified chamber, incubated for 60 min with Avidin-ABC Biotin Complex, treated with 3′-3-diaminobenzidine (DAB), rinsed in distilled water, rehydrated in graded ethanol, deparaffinised in xylene. The sections were analyzed using light microscope (Nikon 50i).

2.5 Electrophysiological evaluation

At the end of 3rd and 6th weeks after crush injury, the electrophysiological studies were performed under general anesthesia with a Neuromatic 2000 electromyography machine (Biomed 3250). The environment temperature was maintained at 30±1°C during all stages of study. The sciatic nerve was directly stimulated through needle electrode, proximal to the injury site at the level of the sciatic notch. For recording of muscle action potential, the two electrodes were inserted into the gastrocnemius muscle and 1 cm under tibia prominence. The distance between the electrodes was measured with a ruler.The gastrocnemius response was recorded by cap electrodes placed on the gastrocnemius muscle. The distance between the site of stimulation and the muscle was 2 cm which was kept constant in the repeated studies (Koudehi and others 2013). Immediately after each stimulation, latency and amplitude of muscle action potential were recorded.

2.6 Evaluation of sensory function of sciatic nerve

Hot-plate test, a pattern of behavioral tests, was performed to assess the nociceptive withdrawal reflex (WRL) at 3rd and 6th weeks after surgery. This reflex involves contractionof the flexors muscle of the hips, knee, and ankle. The rat was positioned to stand on a hotplate at 48ºC (Pars Azma CO, Esfahan, Iran). WRLis defined as the time elapsed from the onset of hotplate contact to withdrawal of thehind paw and measured with a stopwatch. The affected limbs were tested 3times, with an interval of 10 min between consecutive tests to prevent sensitization,and the three latencies were averaged to obtain a final result (Campbell 2001). If there was no paw withdrawal after 20 s, the heat stimulus was removedto prevent tissue damage, and the animal was assigned the maximal WRL of 20 s.

2.7 Evaluating motor function of sciatic nerve

The Sciatic Functional Index (SFI) is a quite useful tool for the evaluation of functional recovery of the sciatic nerve of rats in a number of experimental injuries.

Rats were tested at 3rd and 6th weeks after injury for SFI by walking tract analysis (WTA). Paw-prints were recorded by painting the hind paws with black ink and having them walk along a 130 × 25 × 25 cm corridor, lined with white paper. The paw-prints were collected. Paw length and toe spread were measured. SFI was calculated according to the following Bain formula (Boivin and others 2007a):

SFI = -38.5 (EPL-NPL/NPL) + 109.5 (ETS - NTS/NTS) + 13.3 (EIT - NIT/NIT) - 8.8

Where ETS is the experimental toe spread, NTS the normal toe spread, EPL the experimental paw length, NPL the normal paw length, and IT is the interval between 2nd and 4th toe (Fig. 1).

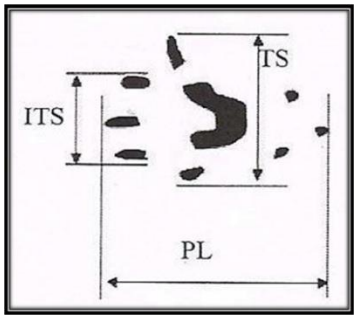

Fig.1. Parameters were measured in Sciatic Functional Index (SFI).

2.8 Statistical Analysis

The data were expressed as means ± SD. The data analysis wasperformed using the SPSSsoftware (version 16). Distributions of the data of the groups were considered normal. The repeated measures analysis of variance was conducted to test the treatment-by-time interactions and the effect of treatment over time on each score. One-way analysis of variance (Anova) was performed on the data to examine differences among groups. If a significant group effect was found, a Tukey HSD test was used to identify the location of differences between groups. $P_{value} < 0.01$ was considered significant.

3. Results

3.1 TNF mRNA expression

To determine the patterns of TNF mRNA expression in the course of rat sciatic nerve crush, PCR products were evaluated by electrophoresis. TNF mRNA expression was low in nerve homogenates from naive rats. After crush injury, local TNF increased rapidly with a five-fold increase on day 1(4.83 ± 0.86; P < 0.01) and remained elevated on day 3 (2.87 ± 0.28, P < 0.01) while this elevation was abolished in etanercept treated group. However, TNF mRNA expression was not significantly different at the end of 1, 3 and 6 weeks after the surgery between two groups (Fig.2).

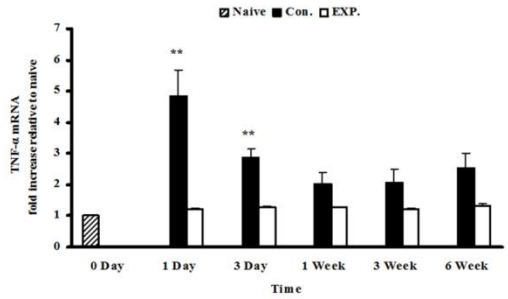

Fig.2. TNF-αmRNA expression in crushed sciatic nerveat each time point in control (Con) and experimental (Exp) groups.Data are expressed as the mean ± SD. Fold change relative to uninjured nerves in N = 5–7 samples. **P < 0.01 by one-way ANOVA followed by Tukey's post hoc test. Naïve (i.e. no crush injury).

3.2 Histology Results

The rate of axonal regeneration was evaluated by light microscope.Light micrographs of semi thin sections of regenerated sciatic nerves of the three groups are shown in Figures 3 a-f.

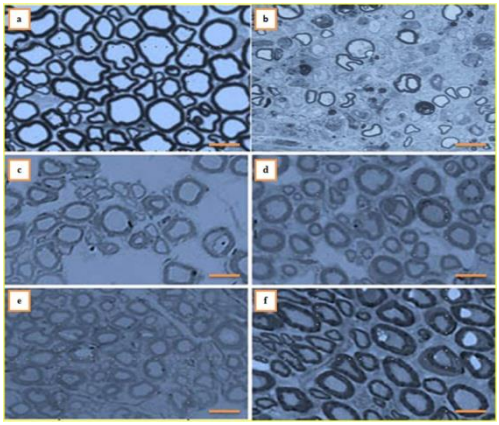

Fig.3. Light micrographs of Toluidine blue-stained sciatic nerve semi-thin sections at 1st, 3rd and 6th weeks after injury. a, b, c, d, e, f are normal,1 week control, 3 weeks control and experiment, 6 weeks control and experiment, respectively. The regenerated fibril number was higher in Exp. group at 3 weeks (d).

In the first week, no repair was observed. Axon regeneration occurred in both and control groups with a organized pattern however the number of nerve regeneration at 3 weeks was higher in the experimental group in comparison to control group at 6 weeks, no difference was seen in regeneration rates between two groups.

3.3 Immunohistochemistry for TNF in injured nerve

Tissue sections of sciatic nerve were stained with polyclonal TNF antibody at 1 day, 3 and 6 weeks after injury (Fig.4). Immunohistochemistry revealed prominent TNF immunereactivity on day 1 after crush injury.
Longitudinal sections of sciatic nerve in the control group were shown myelinated nerve fibers as regular and the wave- like degeneration.

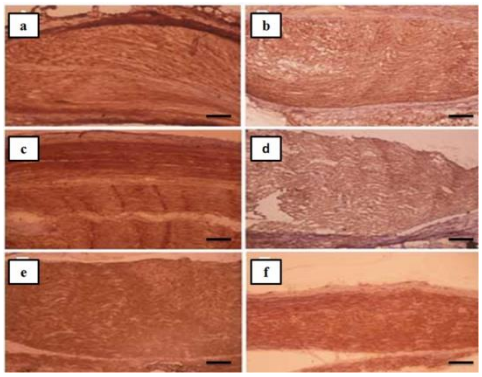

Fig.4. Immunohistochemistry for TNF in sciatic nerve tissue (crush site). a, c, e are 1 day, 3 and 6 weeks in control group. b, d and f are 1 day, 3 and 6 weeks in experimental group, respectively. TNF immunoreactivitywas significantly increased after injury; however it was seen lower following etanercept application in comparison to control group.

3.4 Electrophysiological results

3.4.1 Amplitude of action potential

The results of amplitude values show that there was a significant difference between the groups at 3rd and 6th weeks after intervention, but not on the 1st week (P= 0.33). Amplitude values were significantly higher in experimental group. Statistical analysis showed amplitude changes were increasing during time course, even though increasing gradient was not different between both groups (Table 2).

	Experimental group	Control group	P
1 week	4.96 ± 2.85	4.35 ± 0.83	0.33
3 weeks	33.08 ± 4.86	22.06 ± 7.66	0.03
6 weeks	33.17 ± 2.93	24.71 ± 5.72	< 0.01

Table2. Amplitude values (mV) in control and experimental groups at 1st, 3rd and 6th weeks after injury. Results are presented as mean ± SD

3.4.2 Latency degree

Latency degree for 1st, 3rd and 6th weeks are given in Table 3. The latency at each time point of experimental group was significantly lower than control group. Statistical analysis showed Latency changes were decreasing during time course, even though decreasing gradient was not different between both groups (Table 3).

	Experimental group	Control group	P
1 week	1.6 ± 0.33	2.14 ± 0.20	< 0.01
3 week	1.48 ± 0.22	1.77 ± 0.12	0.04
6 week	1.36 ± 0.12	1.7 ± 0.11	< 0.01

Table 3.Latency degree in control and experimental groups at 1st, 3rd and 6th weeks after intervention. Results are presented as mean ± SD

3.5 Nociception function (WRL)

Hot plate test results showed that withdrawal response recovered progressively in the course of the 6 weeks but at different rates, this difference between control and experimental groups are significant. More analysis showed that change rate was different between both groups during time course. Sensory deficit progressively decreased along postoperative (Fig.5).

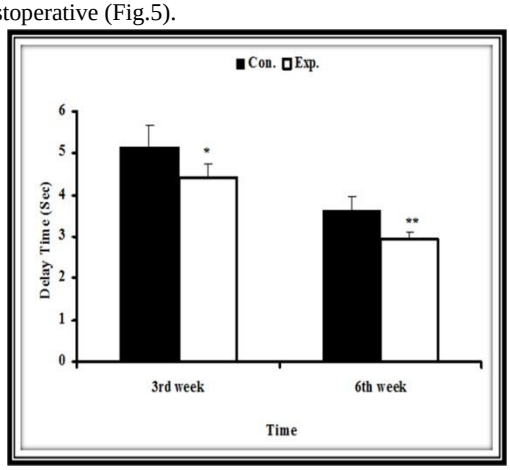

Fig.5. Values in seconds (Sec) were obtained performing Withdrawal Reflex Latency (WRL) test to evaluate the nociceptive function. This test has been performed at 3rd and 6th weeks after the surgical procedure, * P < 0.05 and ** P < 0.01 in comparison to control group. Data presented as Mean ±SD

3.6 Results from SFI:

The SFI was greatly decreased for both control and experimental groups 3 weeks post-injury, and began showing signs of recovery on 6 weeks. The SFI values of experimental group significantly were more than control group on 3 and 6 weeks (Fig.6). Statistic analysis showed

that SFI changes were increasing during time course in both groups. These change gradient were not different between groups (P = 0.06).

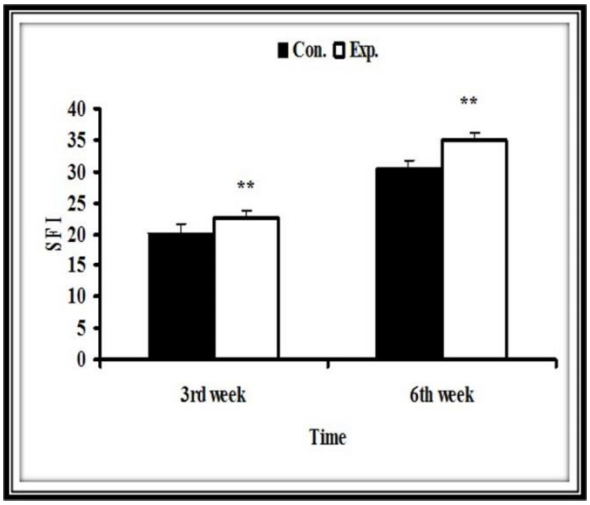

Fig.6. Comparison of SFI values between control (Con) and experimental (Exp) groups on 3rd and 6th weeks. ** P < 0.01 in comparison with control group. Data presented as Mean ±SD.

4. Discussion

In the present study, the rat sciatic nerve model was used for investigating the effect of etanercept after crush injury. For evaluation of successful nerve regeneration, electrophysiological and histological studies together with sensory and motor functional recovery tests were used.

Spontaneous regeneration through the distal nerve stump with good functional return can be expected after crush or tension injury(Sunderland 1990). This type of nerve injuries are treated pharmacological agents instead of surgery.

It is shown that TNF production typically starts to reach maximal levels within 24 h, which are maintained for over 2 weeks and then usually drop. Measurements performed at 3 and 4 weeks reveal weak signaling of the inflammatory process (OtoshiKikuchiKonno and Sekiguchi 2010; Sekiguchi and others 2009).The results showed that TNF mRNA expression in nerve was peaked at 1 day and returned to baseline 1 week after nerve crush. Immediate and single etanercept administration prevented increase of TNF in the crush site as shown by immunehistochemical investigation and RT-PCR. Di Paola et al demonstrated that treatment with etanercept attenuates TNFactivity, the infiltration of neutrophils, cell apoptosis and nitrotyrosine formation. According to the findings, Di Paola et al suggest that interventions which may reduce the generation of TNF may be useful in conditions associated with local or systemic inflammation (Di Paola and others 2007).Antagonism of TNF with etanercept has proved to be effective in the treatment of acute spinal cord injury (Genovese and others 2006).

Kato et al established that immediate therapy with TNF antagonist supports axonal regeneration after peripheral nerve injury (Kato Liu Kikuchi Myers and Shubayev 2010). The present results further showed that systemic delivery of etanercept significantly improved outcomes of sciatic nerve crush injury in rats. Under light microscope, an increased number of axons were seen in experimental group in compared to control group at 3rd weeks. So, etanerceptaccelerates nerve crush injury healing in rats. Several interesting observations on the effects of etanercept therapy on the rate of regeneration were made. For example, systemic (5 mg/kg) etanercept administration 1 hr before and 6 hr after spinal cord injury significantly ameliorated the recovery of limb function in mice (Genovese and others 2006) and endoneurial administration of etanercept once immediately after injury at 6 mg/kg produced sustained attenuation of mechanical allodynia after sciatic nerve crush (Kato Kikuchi Shubayev and Myers 2009).

Treatment with etanercept did not significantly influence the neuropathology of Wallerian degeneration. Thus, it is suggested that immediate etanercept therapy promotes axonal regrowth without delay in myelin debris clearance and may present an alternative to acute treatment with glucocorticoids that cause significant delays in myelin debris removal and functional recovery after sciatic nerve injury (Boivin and others 2007b; Kato Liu Kikuchi Myers and Shubayev 2010).

Electrophysiological examinations showed a significant increase in amplitude and a significant decrease in latency at 3rd and 6th weeks in experimental group compared with control group.

Delay time in hot plate test of experimental group was decreased at 3rd and 6th weeks in compared to control group. The SFI in experimental group was significantly higher than that in control group at 3rd and 6th weeks.SFI changes were increasing during time course in both groups. Medinacelli at al. reported walking gait analysis for rat sciatic nerve. Later this method is modified and named as sciatic functional index (Arslan and others 2003). Normal walking patterns was reported only after the first month of post crush in some studies (Gudemez and others 2002). In contrast to these experiments, some authors reported a full recovery at the third and fourth weeks. The difference in the rate of motor functional recovery may relate to the pathophysiologic response of peripheral nerves to the magnitude of different crushing loads (Kalender and others 2009).

Thus, based on the results of the present study and a review of all of the literature, we propose that this medication accelerates nerve crush injury healing in rats.

From a morphological point of view, the results showed that nerve regeneration
occurred in both groups and that, at time of withdrawal, Wallerian degeneration was almost completed and substituted by re-growing axons and the accompanying Schwann cells.

5. Conclusion

As a result, etanercept has been found effective in promoting nerve regeneration in sciatic nerve crush injury rat model. This drug can be used also for the human injured nerve but additional work is needed.

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