PROTECTIVE ROLE OF INTERFERON-B IN EXPERIMENTALLY INDUCED PERIPHERAL NERVE INJURY IN RATS

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ABSTRACT

Introduction: Interferon-beta (IFN-β) has been used for the treatment of multiple sclerosis, because of its immunomodulatory activity. IFN-β can also improve nerve regeneration after crush injuries. We aimed to investigate the potential of using IFN-β to treat peripheral nerve injury and the role of pro-inflammatory mediators and apoptosis in the effect of IFN-β.

Materials and methods: Fifty male rats, weighing 170–200 g, were randomized into five groups of 10 each: control (no injury), axonotmesis, axonotmesis+IFN-β, neurotmesis, and neurotmesis+IFN-β. The experimental trauma was performed on the sciatic nerve in anesthetized rats (intraperitoneal thiopental 25 mg/kg). IFN-β was applied intraperitoneally for 28 days (4×103 IU/kg per day). At 4 weeks after the surgical procedure, the right sciatic nerves were dissected out for histopathological, quantitative (density of myelinated axons), and molecular (mRNA expression of IL-1β and caspase-3) analysis.

Results: Many axons with severe damage and big gaps between axons were observed in sciatic nerve sections in the axonotmesis group. In the neurotmesis group, the axons were irregular in appearance and axon degeneration, from mild to severe, was observed. In both groups, the IFN-β treatment improved nerve healing significantly. IFN-β also decreased caspase 3 levels and IL-1β in traumatic nerves, suggesting anti-apoptotic and anti-inflammatory effects of IFN-β.

Conclusion: IFN-β may be a new therapeutic approach for the treatment of peripheral nerve injury. Further experimental studies are required to clarify the mechanisms of action of IFN-β, and clinical studies are required to determine optimum treatment doses and treatment durations for nerve injuries.

Key words: Interferon beta, axonotmesis, neurotmesis, peripheral nerve injury, rat.

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Introduction

Peripheral nerve injury is an important medical issue, and nerve regeneration after such injuries involves several highly specialized processes3. The peripheral nervous system displays more significant regenerative potential than the central nervous system after injury, but in many cases, the regeneration is far from perfect. In various types of peripheral nerve injury, especially those that cause large nerve gaps, nerve transection may have a disastrous effect on quality of life.

However, presently, the only treatment strategies for severe nerve lesions are microsurgical techniques using autologous nerve grafts and direct nerve suturing5. Previous animal experiments and clinical studies have confirmed that peripheral nerve regeneration can be improved by the application of neurotrophic factors, Schwann or stem cells, and nerve conduits3-5. However, the search continues for novel therapeutic factors that target regenerative mechanisms during peripheral nerve injury and which could be used in novel therapeutic approaches.
The overlap between immune and nervous system pathways has become a popular subject in recent years. The possibility that molecules originally seen as solely related to immune responses may also have particular effects on synaptic plasticity and nervous system development has opened new fields of investigation. Some cytokines are produced in peripheral nerve tissue during physiological conditions, while proinflammatory and anti-inflammatory cytokines are involved in pathological conditions. It is possible that glia may recognize the expression of certain levels of major histocompatibility complex class I (MHC-I) proteins in neurons as important signals of health or injury. If so, perhaps modulating MHC-I expression after a lesion could influence the outcome of the response to injury. In this context, interferon gamma (IFN-γ), IFN-α, IFN-β, and tumor necrosis factor alpha (TNF-α) potently induce MHC-I proteins.

Interestingly, IFN-β has been used for more than 10 years to treat multiple sclerosis (MS), due to its immunomodulatory activity, and it is considered a disease-modifying therapy. Despite current knowledge about the mechanism of action of IFN-β and its impact on the course of MS, little is known about the role of disease-modifying drugs in the nervous system itself. However, IFN-β treatment has been shown to improve nerve regeneration after a crush injury by inducing MHC-I expression in the spinal cord.

In this experimental study, we aimed to investigate the potential role of IFN-β treatment in peripheral nerve injury. Specifically, we compared its effects in two different injury models: axonotmesis and neurotmesis. We also evaluated the role of pro-inflammatory mediators and apoptosis in the effect of IFN-β by measuring mRNA expression of IL-1β and caspase 3 in the neural tissue.

Materials and methods

Study design and animals

This is an experimental study performed in rats in accordance with national guidelines for the use and care of laboratory animals. The experiments were approved by Ataturk University’s Animal Care Committee (approval number: 28.02.2011/15).

Rats were obtained from Ataturk University’s Experimental Animal Laboratory at the Medicinal and Experimental Application and Research Center (ATADEM). In total, 50 male rats (Sprague Dawley), weighing 170-200 g, were used. The rats were housed in standard plastic cages with sawdust bedding in an air-conditioned room at 22±1°C. Standard rat food and tap water were available ad libitum.

All animals were fasted for 6 h prior to surgical procedures and their neurological scores were determined. Rats were divided randomly into five groups of 10 each:

Group 1 (control): The sciatic nerves were exposed under general anesthesia (intraperitoneal [i.p.] thiopental sodium: 25 mg/kg [Ibrahim Ethem Ulagay A.S., Istanbul, Turkey]) with no further procedure. Rats received 1 mL 0.9% NaCl isotonic i.p., and the surgical site was sutured.

Group 2 (axonotmesis): An aneurysm clamp (12.5 cm) that provided a force of 0.6 N (Aesculap, Yaşargil standard aneurysm clamp, FE783, Tuttingen, Germany) was applied to the sciatic nerves for 1 min to create lesion (axonotmesis); then the clamp was removed. Rats received 1 mL 0.9% NaCl isotonic treatment.

Group 3 (axonotmesis+IFN-β): Following axonotmesis, rats received 4×103 IU/kg per day i.p. IFN-β (Avonex, Gene Pharmaceuticals and Healthcare Products Industry A.S., Ankara, Turkey) for 28 days.

Group 4 (neurotmesis): For neurotmesis model, sciatic nerves were cut with a bistoury (no. 10). Then an end-to-end anastomosis was performed using 8/0 nylon sutures. Rats received 1 mL 0.9% NaCl isotonic treatment.

Group 5 (neurotmesis+IFN-β): Following neurotmesis, rats received 4×103 IU/kg per day intraperitoneal IFN-β for 28 days.

Surgical procedure

In the surgical procedure, rats were anesthetized with i.p. 25 mg/kg thiopental, and their right thighs were shaved. Then the shaved area (4×2 cm2) was sterilized with povidone iodine. Under sterile conditions, a longitudinal incision was performed on the gluteal muscle to expose the sciatic nerve using a surgical microscope. After the experimental trauma procedure, the muscle layer and skin were closed with 4-0 nylon sutures (Ethicon Vicryl, Johnson & Johnson, USA). Wounds were treated with betadine for 10 days. Animals were placed in cages for recovery.

At 4 weeks after the surgical procedure, the
animals were euthanized with high-dose thiopental (50 mg/kg, i.p.). In all rats, the right sciatic nerves were dissected out and fixed in 2% paraformaldehyde+2% glutaraldehyde (150 mL) solution for histopathological analyses.

**Histopathological and quantitative analyses**

A 3 mm length of each sciatic nerve was dissected and fixed in a mixture of 2% paraformaldehyde+3% glutaraldehyde (150 mL) in 0.1 mol/L phosphate buffer, overnight at 4°C. Then the tissues were post-fixed in 1% phosphate-buffered osmium tetroxide for 1 h, dehydrated through a graded alcohol series, and immersed in propylene oxide and then in a propylene oxide+embedding material mixture (Araldite 501, Cambridge, UK). Then the specimens were embedded in embedding material, and polymerized in an incubator at increasing temperature, from 45°C to 65°C over 3 days. Epon blocks were cut transversally with an ultramicrotome (Nova, LKB, Bromma, Sweden).

The semi-thin sections obtained were stained with toluidine blue. They were evaluated under a light microscope (Leica DM 4000B) for histopathological and stereological examination. For stereological examination, the Stereo Investigator (Microbrightfield, USA) and a camera attachment were used. Sciatic nerve samples for each rat were examined at low magnification. A suitable grid size and unbiased counting frame size were estimated from a pilot study. The lined area was sampled systematically and randomly with an Optical Fractionator probe and myelinated axons were counted at high magnification. Finally, the mean numerical density per µm³ of myelinated axons was estimated as:

\[
N_v = \frac{\Sigma Q}{\Sigma S \times A}
\]

where \(N_v\) is the numerical density, \(\Sigma Q\) is the total markers counted, \(\Sigma S\) is the number of sampling slides, and \(A\) is the counting frame area.

**Molecular studies**

**Total RNA extraction and cDNA synthesis**

Total RNA extraction and cDNA synthesis were performed as described previously\(^{(14)}\). Briefly, tissues (20 mg) were stabilized in RNA Stabilization Reagent (RNAlater, Qiagen), and then disrupted using the TissueLyser II (Qiagen; 2×5 min). Total RNA was purified using the RNeasy Mini Kit according to the manufacturer’s protocol (Qiagen). The RNA samples were reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). Then, 10 µL total RNA was treated with 2 µL 10×RT buffer, 0.8 µL 25xNTPs mix, 2 µL 10×RT Random Primers, 1 µL MultiScribe reverse transcriptase, and 4.2 µL DEPC-H2O. Reverse transcription was carried out at 25°C for 10 min, followed by 120 min at 37°C, and finally 85°C for 5 min using a Veriti 96-Well Thermal Cycler (Applied Biosystem). The concentration and quality of cDNA were assessed and quantified using the Epoch Spectrophotometer System and Take3 Plate (Biotek).

**Relative quantification of gene expression**

Relative IL-1β and caspase-3 expression analyses were performed with the StepOne Plus Real Time PCR System (Applied Biosystem) using cDNA synthesized from rat sciatic nerve RNA. qPCR was run using TaqMan Probe mix (Applied Biosystem). Real-time PCR was performed using primers generated for rat IL-1β (Rn00580432_m1), rat caspase 3 (Rn00563902_m1), and rat β-actin (Rn00667869_m1). Results are expressed as relative-fold, compared to control animals. Expression data for β-actin in each tissue were used as an endogenous control. Primers and probes for β-actin were designed by Primer Design (Southampton, UK). For each tissue, triplicate determinations were performed in a 96-well optical plate for both targets using 9 µL cDNA (100 ng), 1 µL Primer Perfect Probe mix, and 10 µL QuantiTect Probe PCR Master mix (Qiagen, Hilden, Germany) in each 20 µL reaction. The plates were heated for 2 min at 50°C and 10 min at 95°C and subsequently 40 cycles of 15 s at 94°C and 60 s at 60°C. All data are expressed as fold-change in expression, compared to the expression in other animal groups, using the 2 ΔΔCt method\(^{(33)}\).

**Statistical analyses**

The SPSS software package (Statistical Package for Social Sciences, version 20.0, SPSS Inc., Chicago, Illinois, USA) was used for statistical calculations. Analysis of all parameters was conducted using one-way ANOVA with the Duncan test option for quantitative analyses of stereological findings and the Tukey test option for molecular analyses. Results were considered significant at \(p<0.05\). All results are expressed as means±standard deviation in each group.
Results

**Histopathological and Quantitative Results**

In the sciatic nerve sections of the control group, regular myelinated axons were conspicuous. Myelinated axon fibers filled the perineurium tightly (Figure 1A).

In the axonotmesis group (Group 2), many axons with severe damage were observed. Also, unlike the control group’s sciatic nerve sections, there were big gaps between axons in the sciatic nerve sections (Figure 1B). In Group 3 (axonotmesis+IFN-β), regular axons were detected in many areas, resembling those in the control group. However, axons with irregular myelin sheaths and hyperchromatic stained axons in the middle of the myelin sheaths were also seen in this group, unlike the control group (Figure 1C). Also, some inter-axonal areas, especially between darkly stained axons, were enlarged (Figure 1C). In Group 4 (neurotmesis), the axons were irregular in appearance, unlike those of the control group. Some of the axons had degenerated completely while some had damage only on the myelin sheath. Some axons with small diameters and very close to each other were detected (Figure 1D). Some enlarged spaces between damaged axons were also seen, but less than in the axonotmesis group (Figure 1D). Group 5 (neurotmesis+IFN-β) had many axons with irregular shapes, but not obviously damaged, and their arrangement was congested. Although a few damaged neurons were seen in some areas, axon groups with small diameters suggested that axonal regeneration was occurring between damaged axons (Figure 1E).

The estimated mean density of myelinated axons in each group is shown in Figure 2.

The numerical density of axons decreased significantly in the axonotmesis and neurotmesis groups, compared to the control group (p<0.001). IFN-β administration increased the numerical density of myelinated axons significantly compared to both the axonotmesis and neurotmesis groups (p<0.05).

**Molecular results**

IL-1β and caspase 3 gene expression increased in both the axonotmesis and neurotmesis groups relative to the control group (p<0.01). IFN-β showed a significant downregulatory effect on IL-1β and caspase 3 expression (p<0.001) in both groups (Figures 3, 4).
Discussion

Nerve injuries can produce severe disability and greatly compromise patient quality of life. Injuries to peripheral nerves, occurring in ~2.8% of trauma patients, cause partial or total loss of motor, sensory, and/or autonomic function\(^{(16)}\). Peripheral nerve injuries lead to axon discontinuities and the degeneration of myelinated fibers, which may eventually result in the death of axotomized neurons\(^{(17,18)}\). After peripheral nerve injury, the severed axons have some capacity to regenerate and recover functional connections. However, the rate of axonal regeneration is far from satisfactory, especially after severe injuries\(^{(17)}\).

Nerve healing depends on many factors, such as the time period between trauma and surgery, severity of trauma, type of damaged nerve, the patient’s age, and the surgeon’s experience\(^{(19)}\). To control such factors, we performed all surgeries just after nerve damage occurred. Also, age- and weight-matched animals were used, and dissections were performed by the same surgeon according to a standardized method. Thus, a reasonably objective evaluation was made with regard to nerve healing (alone).

The regeneration process in nerve injuries includes breakdown of the blood-nerve barrier\(^{(20,21)}\), proliferation of Schwann cells\(^{(22,23)}\), recruitment of circulating macrophages\(^{(24,25)}\), a burst in cytokine production, reorganization of the endoneurial space\(^{(26)}\), and production of extracellular matrix components\(^{(27,28)}\). Also, many different mechanisms, such as an increase in oxidative stress after tissue damage and exacerbation of damage via inflammatory mediators, are involved in peripheral nerve injury and related organ damage.

Consistent with our study, overlap between immune and nervous system pathways has become an area of focus. The possibility that molecules originally seen as being solely related to immune responses may have particular effects in synaptic plasticity, and nervous system development has opened new fields of investigation\(^{(28)}\).

As a host defense response, inflammation in the nervous system has been studied in the context of autoimmunity and infection. However, during nerve healing following axotomy, there is also a considerable inflammatory response, involving macrophages, Schwann cells, and inflammatory mediators in the distal stump, which set the stage for the success or failure of subsequent regeneration\(^{(24,27-29)}\). In this regard, previous studies have investigated the roles of interferons, key players in immune responses, in both central and peripheral nervous system damage and healing\(^{(3,7,30)}\). In this study, we evaluated the protective effects of IFN-β in two models of nerve injury, axonotmesis and neurontesis. Our results showed that in the control group, the sciatic nerves had a healthy peripheral nerve appearance on histological examination, while in the axonotmesis group, many axons with severe damage were observed. Also, unlike the control group sciatic nerve sections, in the axonotmesis group, there were big gaps between axons in the nerve sections. In the neurontesis group, the axons were irregular in appearance, and degenerated axons, from mild to severe, were observed. IFN-β application significantly improved nerve healing in both groups. Thus, the histopathological analyses and stereological counting demonstrated beneficial effects of IFN-β application.

We also found that axonotmesis- and neurontesis-induced sciatic nerve injury was associated with an increase in IL-1β levels in the sciatic nerve. Because cytokines appear early in the inflammatory cascade, they have been considered pro-inflammatory mediators\(^{(31,32)}\). The role of these cytokines has been documented in peripheral and central sensitization in neuropathic pain\(^{(33)}\). However, administration of IFN-β attenuated the trauma-induced rise in IL-1β levels. Thus, it may be that the beneficial effects of IFN-β in peripheral nerve injury are mediated, at least in part, through its attenuating effects on pro-inflammatory mediators. Similarly, a previous study demonstrated that IFN-β suppressed experimental autoimmune neuritis in Lewis rats by inhibiting the migration of inflammatory cells into peripheral nervous tissue\(^{(34)}\).

Peripheral nerve regeneration is closely related to apoptosis, a self-destructive programmed biochemical process. Neurons with high regeneration capacity are also at risk for programmed cell death\(^{(35,36)}\). The ability of resident cells to induce apoptosis in invading lymphocytes represents a major regulatory mechanism in the termination of inflammatory response within immune-privileged sites, including the nervous system\(^{(37,38)}\). By assessing the proportion of infiltrating T lymphocytes undergoing apoptosis in experimental autoimmune encephalomyelitis and neuritis, it has been shown that T cell clearance occurs in the central nervous system and, less efficiently, in the peripheral nervous system\(^{(39-41)}\).
These data suggest that both central nervous system and peripheral nervous system possess intrinsic mechanisms for resolving autoimmune attacks by inducing apoptosis in autoreactive lymphocytes\(^{[30]}\). In this regard, previous studies have suggested roles for interferons in nerve injury and the healing cascade\(^{[30,41-44]}\). Apoptosis is often mediated by caspase-related protein cleavage in which caspase 3 plays an important role\(^{[48]}\). Previous studies on nerve healing and nerve injury-related neuropathic pain revealed the potential role of apoptosis and caspase 3 activation in the injury process\(^{[46,47]}\). In the present study, increased mRNA expression of caspase 3 in both the axonotmesis and neurotmesis groups showed that apoptosis is induced during nerve injury. The decrease in caspase 3 expression with IFN-\(\beta\) shows that IFN-\(\beta\) prevented the loss of nerve cells by decreasing the apoptotic pathway. Thus, the protective effects of IFN-\(\beta\) may also be related to its anti-apoptotic affects.

In conclusion, we investigated the effects of long-term IFN-\(\beta\) treatment on nerve regeneration in two models of peripheral nerve injury: axonotmesis and neurotmesis. Our results suggest that IFN-\(\beta\) may be useful as a new therapeutic approach for peripheral nerve injuries. The protective effect(s) of IFN-\(\beta\) may be related to its anti-inflammatory and anti-apoptotic properties (amongst others). The regenerative potential of neurons may be improved by a better understanding of the mechanisms behind the response(s) to injury. Further experimental studies are required to clarify the mechanisms of action of IFN-\(\beta\), and clinical studies are required to determine optimum treatment doses and treatment durations for nerve injuries.

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