The effect of short-term treatments of a gonadotropin-releasing hormone analog (buserelin) on sciatic nerve regeneration

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Summary

Gonadotropin-releasing hormone (GnRH) has neuromodulatory roles in central and peripheral nervous systems. The purpose of this study is to evaluate the effect of GnRH analog (buserelin) on peripheral nerve regeneration. Forty adult male rats were divided into buserelin-treated, normal saline, sham surgery, and castrated + buserelin groups. The left sciatic nerve was crushed by a fine forceps and all animals were evaluated by sciatic functional index (SFI), electrophysiology, histology and immunohistochemistry testing. On post operation days 21 and 28, the difference between buserelin and normal saline groups was statistically significant ($P<0.05$), but no significant difference was found between the buserelin and castrated + buserelin groups ($P>0.05$). At the 28th day after operation, the diameters (μm) of the regenerated myelinated fibers of the buserelin group were significantly greater than those of the normal saline group ($P<0.05$). Although nerve conduction velocity (NCV) of the buserelin group was faster than the normal saline group, the difference was not statistically significant. The present study suggests that buserelin treatment might accelerate peripheral nerve regeneration.

Key words: Gonadotropin-releasing hormone, Buserelin, Nerve regeneration, Sciatic nerve, Rat

Introduction

Gonadotropin-releasing hormone (GnRH), also called luteinizing hormone-releasing hormone (LHRH), plays a key role in controlling neurohormonal reproduction (Botte *et al*., 1998).

Previous studies showed that GnRH receptors mainly exist in the anterior pituitary (Sealfon *et al*., 1997), but during the development of the nervous system, they are also distributed in different areas including cerebral cortical neurons of embryos and adult rats (Quintanar *et al*., 2007), mouse cerebellum (Albertson *et al*., 2008), rat hippocampus, amygdale, arcuate nucleus (Jennes *et al*., 1997) and the myenteric plexus of gastrointestinal tract (Huang *et al*., 2001).

Gonadotropin-releasing hormone analogues play an important role in reproductive processes (Moghiseh *et al*., 2008). However, the function of GnRH is not limited to reproduction (Chu *et al*., 2010); it also plays additional roles (Quintanar *et al*., 2009) such as inhibiting the proliferation of uterine tumor cells (Mizutani *et al*., 1998) and suppressing the growth of ovarian cancer (Maruuchi *et al*., 1998).

Previous studies showed that GnRH has neurotransmitter/neuromodulatory roles in central and peripheral nervous systems (e.g. sympathetic ganglion, mid-brain) (Millar *et al*., 1987). GnRH has excitatory effects on rat cerebral cortical neurons (Phillips *et al*., 1980) and up-regulates the Ca^{2+} channel density of sympathetic neurons (Ford *et al*., 2003). It has been recently reported that GnRH has neurotrophic effects on neurite outgrowth and neurofilament protein expression in cultured cerebral cortical neurons of rat embryos (Quintanar *et al*., 2008).

Quintanar *et al*. (2009) described that the GnRH receptor and its mRNA exist in spinal cord neurons of embryos and adult rats (Quintanar *et al*., 2009). In cultured cerebral cortical neurons of rat embryos, GnRH has increased the number and length of neuritis as well as the cytoskeletal neurofilament expression (Quintanar *et al*., 2008). Studies have suggested that GnRH could operate as a neurotrophic factor that acts in the hippocampus where GnRH treatments induce changes in the spine's synapse density (Prange-Kiel *et al*., 2008).

Recent findings suggest that pretreatment with certain concentrations of GnRH analogue could attenuate the apoptosis of hippocampal neurons. GnRH analogue has protective effects on neurons (Chu *et al*., 2010). Sand *et al*. (2013) suggested that the enteric neurodegenerative effects of GnRH analog treatments in humans can be

mimicked in rats (Sand *et al*., 2013).

For over a century, peripheral nerve lesions have been a significant challenge, not only from a therapeutic point of view, but also to understand the degenerative-regenerative phenomena of the nervous system (Santos *et al*., 1995).

Recent evidence suggests that GnRH could have neurotrophic effects on spinal cord neurons as well; hence, GnRH could be studied in neuronal regenerations (Quintanar *et al*., 2009). In males, for example, when a GnRH analog is administered at a low dose or in a short-term study, testosterone secretion is stimulated (Sharpe *et al*., 1982). Testosterone has been reported to accelerate functional recovery in peripheral nerve regeneration (Brown *et al*., 1999; Tanzer and Jones, 2004) through an androgen receptor-mediated mechanism (Tanzer and Jones, 1997). The castration of male rats has been shown to decrease hypothalamic GnRH content and serum testosterone (Rudenstein *et al*., 1979). There is no report that GnRH analog (buserelin) has acted independently of testosterone on peripheral nerve regeneration.

The aim of this study is, therefore, to evaluate the effect of GnRH analog (buserelin) on peripheral nerve regeneration using a model of sciatic nerve crushing in rats by measuring sciatic functional index (SFI), electrophysiology, histology and immunohistochemistry testing.

Materials and Methods

Animals

Forty adult male Sprague-Dawley rats (250-275 g) were divided into four groups: (I) buserelin-treated group, (II) normal saline group, (III) sham surgery group, and (IV) castrated + buserelin group. Experimental procedures were approved by the ethical committee of Urmia University of Medical Sciences.

Surgical procedure

Under general anesthesia with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg), the left sciatic nerve was exposed and isolated from the adjacent tissues through a gluteal muscle splitting incision. In the buserelin, castrated, and normal saline groups, the left sciatic nerve was crushed during 30 s by a fine forceps, approximately 1 cm distal to the sciatic notch.

To regulate the blood level of testosterone, rats were surgically castrated as described previously and were allowed to recover from surgery for 7 days before nerve crushing (Sanni *et al*., 2012).

In sham operations, left sciatic nerves were briefly exposed. The muscle was closed with 4-0 dexon sutures, and the skin with 3-0 nylon sutures. The surgery was performed under aseptic conditions using an operating microscope. After nerve crushing, buserelin treated and castrated + buserelin animals were given 20 µg (1 mg/ml) of the GnRH analog buserelin (Superfact, Sanofi-Aventis Bromma, Sweden) subcutaneously, once daily for 5 days. The dosage and administration of buserelin were based on a previous study (Trindade *et al*., 2008). Saline treated rats received sterile normal saline injections.

Blood plasma samples and testosterone levels

Testosterone level was measured using an estradiol coat-a-count radioimmunoassay kit (DPC) according to the manufacturer's instructions. Blood samples were collected 2 h before (as the intact) to 7, 28, and 56 days after nerve crushing and kept on ice for 2 h in bottles containing lithium heparin as the anticoagulant. Serum was separated by centrifugation at 3000 rpm and stored at - 70°C until the time of measurement.

Functional tests

Animals were tested before and at days 7, 14, 21, 28, 35, 49 and 56 after the injury. Indian ink was applied to the plantar surface of the hind feet to cover all anatomical regions. The footprints of both operated and unoperated limbs were used to calculate sciatic functional index (SFI) using the formula developed by Bain *et al.* (1989). An SFI value of zero was considered normal, whereas an SFI of -100 meant total impairment as would be expected from a complete transaction of the sciatic nerve (Bain *et al*., 1989).

Electrophysiological study

Animals in each group were subjected to electrophysiological studies using Narco bio-system (USA) 56 days after the operation and anesthetized by intraperitoneal injection urethane (1 g/kg). Their left sciatic nerve (operated side) was then re-exposed by incision of the previous surgical site at the mid-thigh level. On each side of the crush, stimulating electrodes were placed 20 mm apart and a recording electrode was inserted into the gastrocnemius muscle. Physiologic parameters (latency and peak amplitude of compound action potentials) were recorded. Nerve conduction velocity (NCV) was determined by stimulus latency and the distance between the two electrodes.

Histological examination

For the morphologic analysis of regeneration, 4 mm sections of the sciatic nerves distal from the crush site were removed at days 28 and 56 of regeneration. The samples were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin blocks. Five-micron sections were cut across the transverse axis. The sections were stained with H&E and divided into four separate quarters of the same square. Average values corresponding to the total perimeter of the nerve sections were calculated via light microscopy at ×40 magnification. Total myelinated fiber counts and measurements of the myelinated fibers' diameters were performed in each nerve cross-section with the aid of a morphometric analysis system (OLYSIA Biorefort, Olympus, Japan).

Immunohistochemistry

In this study, anti S-100 (Dako, 1:200 dilution) was used as a marker for myelin sheath. Briefly, specimens

prior to immunohistochemistry were post-fixed in a solution containing 4% paraformaldehyde for 2 h. The tissue samples were embedded in paraffin and cut into 5 µm thick sections. According to the instructions of immunohistochemical staining kits, non-specific immunoreactions were blocked; sections were incubated in S-100 protein antibody solutions for 1 h at room temperature, washed three times with PBS, and incubated in biotinylated anti-mouse rabbit IgG solution for 1 h. Horseradish peroxidase (secondary antibody) solution was added to the sections using the diaminobenzidine method. Immunohistochemistry results were then examined under a light microscope.

Statistical analysis

Statistical analysis was carried out using a mixed-design (within and between group comparison) ANOVA computed with 95% confidence levels using SPSS (version 16.0 for windows) software. All data are presented as means ± SD (P<0.05 was considered as statistically significant).

Results

No clinical signs of infection, pain, or discomfort were observed over the regeneration period. SFI greatly decreased for buserelin, castrated + buserelin, and normal saline groups 7 days post operation. SFI improvements from first to last evaluations in the experimental groups at 21cd and 28th days post operation, and the difference between buserelin and normal saline groups were statistically significant (P<0.05), but no significant difference was found between the buserelin and castrated + buserelin groups (P>0.05). In addition, the buserelin, castrated + buserelin, and normal saline groups were all statistically different from the sham group (P<0.05). No statistically significant differences were found between buserelin and normal saline SFI values at day 56 post operation (P>0.05) (Fig. 1).

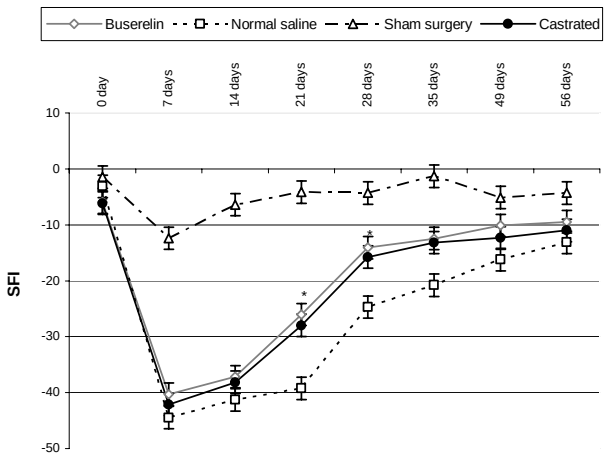


Fig. 1: SFI before and after crush injury in buserelin, castrated + buserelin, and normal saline groups. * Difference between ESM and normal saline groups (P<0.05, t-test)

For 5 days buserelin-treated rats released significantly more testosterone in their serum than normal saline and castrated + buserelin rats. Comparison of the serum testosterone level of the buserelin group with that of the normal saline group shows a significant difference (P<0.05) (Fig. 2).

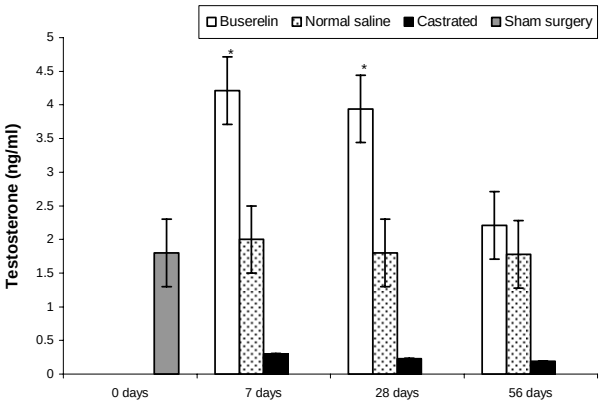


Fig. 2: Serum level of testosterone in buserelin-treated, castrated + buserelin, and normal saline groups at different time points of the sciatic nerve regeneration. * Difference between normal saline and buserelin-treated rat (P<0.05)

At day 56, mean nerve conduction velocities (NCV) for buserelin, castrated + buserelin and normal saline groups were 41.23 ± 4.62, 37.41 ± 2.66, 33.51 ± 3.62 m/s, respectively. Although NCV was faster for the buserelin group compared to the normal saline group, the difference was not statistically significant (P>0.05). Results of the comparison of mean amplitude (AMP) between the buserelin, castrated + buserelin, and normal saline groups were 7.55 ± 4.01, 6.99 ± 2.21, and 5.7 ± 1.82 mV, respectively. However, they were not statistically significant (P>0.05).

Sections of sciatic nerve were contained numerous Schwann cells, blood vessels, and myelinated axons within microfascicles. All myelinated axons present in each nerve cross-section were counted.

At days 28 and 56 post operation, the diameters (µm) of the regenerated myelinated fibers were 10.97 ± 5.12 and 15.04 ± 4.71 for the buserelin group, 9.31 ± 3.12 and 12.41 ± 2.99 for the castrated + buserelin group, and 7.11 ± 3.1 and 10.54 ± 6.24 for the normal saline group. Statistically significant differences were found between buserelin and normal saline groups on the 26th post operation day (P>0.05). At day 28, the number of myelinated fibers in the buserelin group was significantly greater than that of the normal saline group (P<0.01), but no significant difference was found at day 56 (P<0.05) (Fig. 3).

Immunoreactivity to S-100 was extensively observed in the cross sections from the midpoint of the buserelin group. For buserelin and normal saline groups, the expression of the S-100 protein was located mainly in the myelin sheath, and the Schwann cell was present around the myelinated axons. In the buserelin group, the structure of regenerated axons was more similar to those of normal nerves compared with the normal saline group

(Figs. 4a-d).

Discussion

To our knowledge, this is the first research on the effect of buserelin administration on peripheral nerve regeneration, specifically sciatic nerve regeneration, in adult rats. The results demonstrate that buserelin significantly enhances peripheral nerve regeneration *in vivo*.

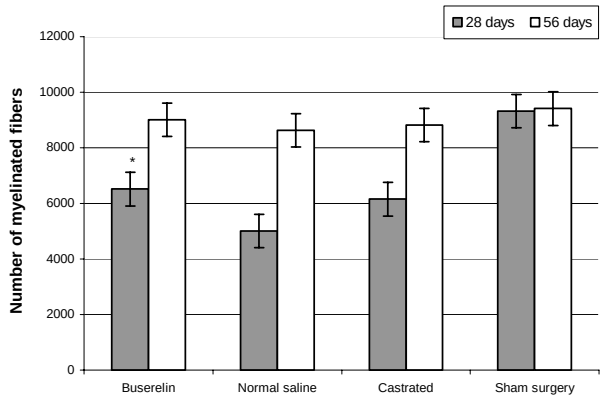
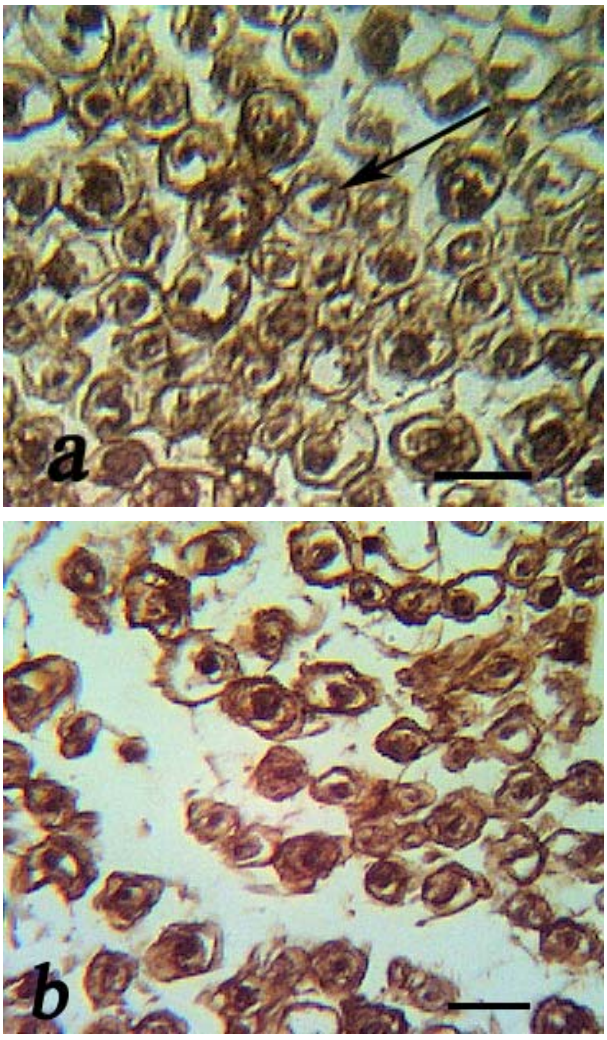


Fig. 3: Total number of regenerated myelinated nerve fibers after sciatic crush injury (n=5 on day 28 and n=5 on day 56 for each group). * Difference between buserelin-treated and normal saline groups on the 28 days post operation (P<0.05)



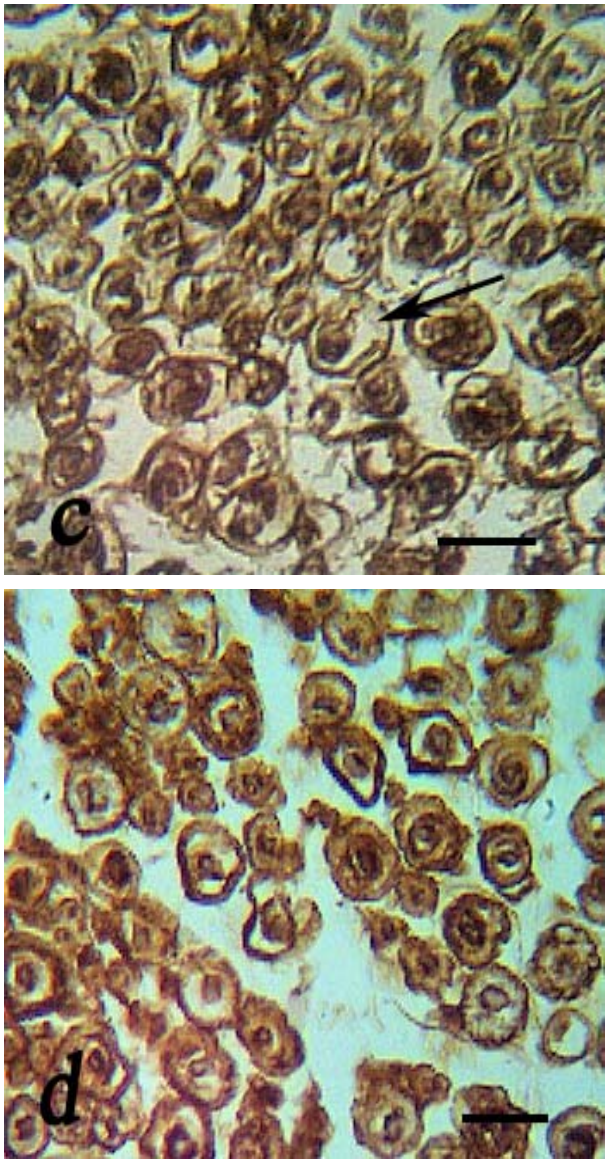


Fig. 4: Immunohistochemical analysis of cross sections to the main axis of the regenerated nerve 56 days after surgery distal from crush site of sham surgery (a), normal saline group (b), buserelin group (c), and castrated + buserelin group (d). There was positive staining of the myelin sheath-associated protein S-100 (arrow). Regenerated nerve fibers containing myelinated axons, Schwann cells and blood vessels were present throughout the tissue (scale bar 20 µm)

Extensive clinical applications of GnRH analogs enhance our understanding of the nervous system (Millar *et al*., 1987). The injection of a GnRH analog to intact rats is followed by a rapid and transient release of the pituitary content of gonadotropins, followed immediately by a transient stimulation of steroid secretion (Botte *et al*., 1999). *In vivo* studies in male or female adult hypophysectomized rats have shown that exogenous GnRH or GnRH analogs can both stimulate and inhibit gonadal functions (Hsueh and Jones, 1981).

In this study, histological and functional results

revealed that regeneration in buserelin and castrated + buserelin groups was superior to the normal saline group. Clinical and experimental nerve repair deemed successful in terms of electrophysiologic and morphometric assessments has not always yielded a correspondingly favorable functional recovery (Dellon and Mackinnon, 1989). Nerve conduction velocity measures the fastest conducting nerve fibers, a measure that has been shown to be dependent on axon diameters, myelination, and intermodal distance (Brown *et al*., 1991). A nerve may have a few fibers that conduct very well despite the damage of a large number of remaining fibers. For this reason, nerve conduction velocity may not evaluate total nerve function, but the fastest and perhaps healthiest fibers (Kanaya *et al*., 1996). Although these morphometric parameters may measure nerve maturity, they may not measure its function because of improper reinnervation or inappropriate central nervous system integration (Kanaya *et al*., 1996).

The administration of buserelin aimed to stimulate increasing testosterone release from Leydig cells (Brown-Douglas *et al*., 2004). The significant increase in plasma testosterone levels in the buserelin group of this study is in agreement with results reported earlier by Kawakami *et al*. (2009). Testosterone accelerates the functional recovery from hind limb paralysis resulting from sciatic nerve injuries in rats (Brown *et al*., 1999) and facial nerve regeneration in hamsters (Tanzer and Jones, 2004) through an androgen receptor-mediated mechanism. Supraphysiological levels of testosterone have been shown to effect peripheral nerve regeneration most (Tanzer and Jones, 1997).

The major components of the sciatic nerve are the Schwann cell, processes of motor neurons and processes of sensory neurons of the dorsal root ganglion (Brown-Douglas *et al*., 2004). To study the specific effects of GnRH and avoid these variations, one way is to use testectomized rats (Caldetron-Vallejo and Quintanar, 2012). In the present study, castration caused a significant decrease in plasma testosterone levels (Sanni *et al*., 2012).

The surprising finding of this study was the accelerative effect of buserelin administration on axonal regeneration in castrated rats. It has been reported previously that the administration of GnRH analogs acts as a neurotrophic factor and may be used as a potential therapeutic agent for the treatment of spinal cord injuries (Caldetron-Vallejo and Quintanar, 2012). GnRH increases both growth and number of neurites, as well as the expression of neurofilaments (Quintanar and Salinas, 2008). Neurofilaments are neuron-specific intermediate filaments, and together with other axonal components, they maintain which will regulate neuronal cytoskeletal plasticity, thus affecting the axonal caliber, axonal transport and neurite outgrowth (Kesavapany *et al*., 2003).

There are mechanisms by which buserelin could affect peripheral nerve regeneration. One mechanism is the expression of GnRH receptors in motoneurons of rats' spinal cords (Quintanar *et al*., 2009) which might be due to the finding that sciatic nerve regeneration in animals treated with buserelin may be mediated by the activation of these receptors (Caldetron-Vallejo and Quintanar, 2012). This activation could induce axonal regeneration. Another mechanism is its effect on Leydig cells causing increasing testosterone release (Brown-Douglas *et al*., 2004).

A major advantage is that treatment with GnRH is non-invasive. In addition, this neuropeptide and its analogs are capable of crossing the blood-spinal cord barrier (Barrera *et al*., 1991) and reaching the injured area (Caldetron-Vallejo and Quintanar, 2012).

In conclusion, the present study shows that buserelin treatment increases nerve regeneration significantly more than normal saline. However, the mechanism of its action remains unclear. Further experiments are necessary to explore these issues.

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