

Effect of FK506 administration after obturator nerve injury: A functional and ultrastructural study

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The frequency of obturator nerve damage due to pelvic diseases, fractures or gynecologic procedures is uncertain. In the present study, we investigated the effect of FK506, a potent macrolide antibiotic and immunosuppressant, on obturator nerve recovery at morphological and functional levels. Forty female Wistar rats were randomly divided into four groups (control, sham, FK506-treated, vehicle-treated). In half of animals (FK506-treated and vehicle-treated) an obturator nerve crush (30 seconds clamp) was created. In FK506-treated group FK506 administration (1 mg/kg/day, subcutaneously) was performed on each postoperative day. All the rats were functionally evaluated by pinch and adduction tests preoperatively and postoperatively at one, two, three and four weeks after nerve injury. On the 28th postoperative day obturator nerve samples were collected and analyzed qualitatively by light and electron microscopy. FK506 treatment resulted in dramatic improvement in nerve function and in the ultrastructure of nerve fibers suggesting its therapeutic potential in traumatic obturator nerve injury.

Key words: FK506, obturator nerve, injury, functional recovery

INTRODUCTION

Gynecologic patients undergoing open lymphadenectomy or extensive retroperitoneal surgery along the pelvic wall are at increased risk of obturator nerve injury. Obturator nerve injury-related symptoms can be motor or sensory, including variable gait disturbance, constant pain or anesthesia along the nerve distribution (Cardosi et al. 2002, Sorenson et al. 2002). The causes and mechanisms of obturator neuropathy are multiple: iatrogenic (Spaliviero et al. 2004), traumatic (especially after traffic accidents), compression due to psoas muscle hematoma, inguinal hernias, arterial aneurysms, or

a synovial cyst (Bischoff and Schonle 1991, Sorenson et al. 2002, Stuplich et al. 2005, Holub 2006).

A novel immunosuppressant FK506, a macrolide antibiotic derived from the soil fungus *Streptomyces tsukubaensis*, is about 100 times more potent than Cyclosporin A (Horton 1991) and its toxicity in humans is reported to be less than that associated with Cyclosporin A (Wang et al. 1997, Wang and Gold 1999). FK506 is used clinically for the prevention of allograft rejection. Its immunosuppressive effect is understood to occur through modulation of calcineurin activity together with FK506 binding protein, FKBP-12. In addition to its ability to suppress immune system cells (T-cell activity), it has a number of non-immune effects (Heitman et al. 1993, Lyons et al. 1994, Gold et al. 1995, Drake et al. 1996). FK506 has been shown to accelerate the rate of nerve regeneration and to promote neurite

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outgrowth following a nerve crush or immediate nerve repair. Although an increased rate of axonal regeneration has been reported (Lyons et al. 1994, Gold et al. 1995, Sarikcioglu et al. 2006), neurotoxic complications from immunosuppressant therapy with FK506 have also been described in the central and peripheral nervous systems (Fansa et al. 1999). In the current study, our goal was to investigate the effect of FK506 on obturator nerve recovery by evaluation of changes in morphology and function.

METHODS

Animals

Forty female Wistar rats (200 to 250 g) were randomly divided into four groups (control, sham, FK506-treated, vehicle-treated). The animals were housed in groups of five or six per Macrolon cage on sawdust and received rat chow and water *ad libitum*. They were maintained on a 12-h light-dark cycle; light on from 07:00 AM to 07:00 PM. All procedures were reviewed and approved by animal care and usage committee of Akdeniz University, Antalya, Turkey.

Obturator nerve crush

Before surgical procedures, animals were anesthetized with an intramuscular injection of a mixture of a xylazine HCl (Rompun, Bayer HealthCare, Monheim, Germany) (15 mg/kg) and ketamine (Ketalar, Eczacibasi Drug Co., Istanbul, Turkey) (100 mg/kg). The surgical area was shaved and swabbed with an antiseptic solution. Following midline laparotomy and retraction of the intestines, the obturator nerve was approached by retraction of the psoas muscle laterally. Then the obturator nerve was clamped for 30 seconds as previously described (De Koning et al. 1986). A suture was tied to the adjacent muscle to mark the crush site. The procedure was carefully performed to avoid injury of the common iliac vessels. The wound was closed with a 2-0 Ethilon suture (Ethicon, Livingston, UK) and the rats were allowed to recover in the postoperative room. In the sham-operated group, the same surgical procedures were performed except that the obturator nerve which was left uncrushed.

FK506 administration

FK506 (Prograf, Eczacibasi, Istanbul) subcutaneous injections (1 mg/kg/day) were performed once a day, from the day of nerve crush to the day of animal sacrifice. The same volume of saline was administered to the vehicle-treated animals.

Evaluation tests

Assessment of function

The rats were functionally evaluated by both pinch and adduction tests preoperatively and postoperatively one, two, three and four weeks after nerve injury. The animals were handled gently, and the room where the animals were tested was kept quiet to minimize any stress that could interfere with their responses. The testing procedures were performed by two experienced researchers (L.S. and U.O.).

Sensory function (pinch test)

Functional sensory recovery was analyzed by pinch test. The medial side of the thigh was pinched with the same clamp used for crush injury. A gradual scale with four levels was used to assess functional recovery. The levels were no response (Grade 0); mild response (Grade 1); moderate response (Grade 2); and full response (Grade 3). Animals showing full withdrawal response to pinch (Grade 3) were recorded.

Adduction test

Both hindlimbs of the animals were abducted and released afterwards. Unoperated hindlimbs of the animals were adducted immediately. The position after release of the hindlimb and strength of the muscle of the operated hindlimb were evaluated by comparison with the unoperated hindlimb. The same gradual scale described above [no adduction (Grade 0); mild adduction (Grade 1); moderate adduction (Grade 2); and full adduction (Grade 3)] was used for this test.

Wet muscle weight

At the day of animal sacrifice, the wet muscle weight of all adductor muscles for each animal was measured.

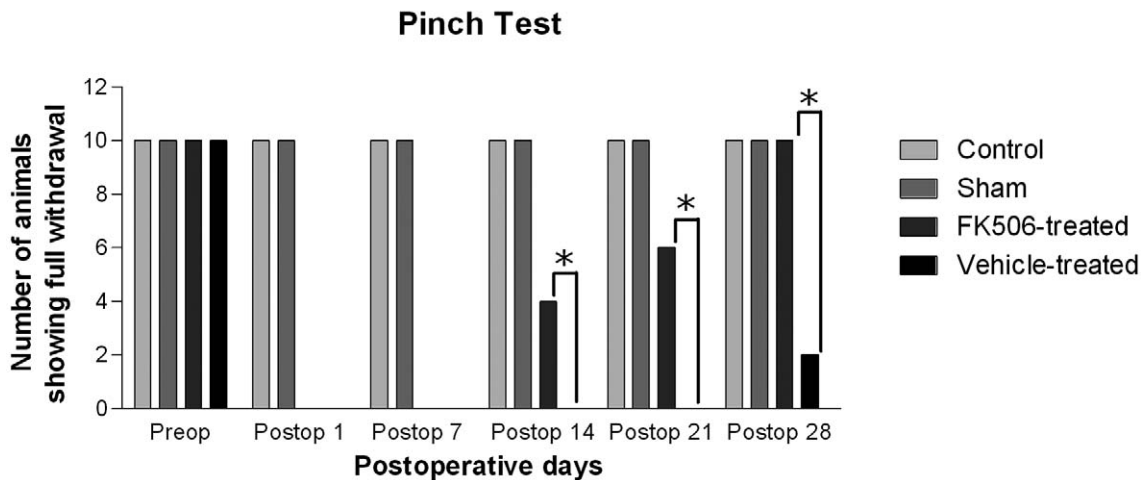


Fig. 1. Pinch test results on preoperative and postoperative days. Asterisk shows significant difference between groups.

Light and electron microscopic evaluation

One month after obturator nerve crush, the animals were administered an overdose of chloral hydrate intraperitoneally. The obturator nerve was re-exposed and the distal part of the crushed site was sampled. Samples were fixed with 4% glutaraldehyde in 0.1 M Sorensen's phosphate buffer solution (pH 7.3) and post-fixed with 2% osmium tetroxide in the same buffered solution. After dehydration through a graded series of ethanol, they were embedded in epoxy resin (Araldite CY212, Agar Scientific Ltd, Stansted, UK). Semi-thin sections (1 μ m) were stained with toluidine blue and were examined and photographed with a light microscope (Olympus CX41). Afterwards, ultrathin sections (40–60 nm) were contrasted with uranyl acetate and lead citrate and were examined with Zeiss a LEO 906E transmission electron microscope. Six sections from each limb were obtained and processed for EM.

Data analysis

All data collection was carried out in a blinded fashion. Adduction test and pinch test data were analyzed by chi-square test. All statistical analyses were done using SPSS for PC version 10.0. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Immediately after acute compression injury, the crushed area of the obturator nerves became very thin,

but nerve continuity was not interrupted grossly. Two animals were excluded from the study since their iliac vessels were damaged during the approach to the obturator nerve or while marking the crush level. After surgery, 38 animals survived and no wound infections were detected.

Pinch test

In control and sham-operated groups, withdrawal responses to pinch were full (Grade 3) at each postoperative week. The onset day of full withdrawal response (Grade 3) to pinch stimulation was in the 2nd postoperative week in four animals of the FK506-treated group. In the rest of the FK506-treated group animals, four exhibited Grade 1 withdrawal and two showed no response (Grade 0). However, in the same postoperative week, only two animals in the vehicle-treated group were able to show Grade 2 withdrawal. The other eight showed no response (Grade 0). In animals showing full withdrawal, a significant difference ($P < 0.05$) was noted between the FK506-treated and vehicle-treated groups in the second postoperative week (Fig. 1).

The onset day of a full response (Grade 3) of withdrawal to pinch stimulation was in the fourth postoperative week in two animals of the vehicle-treated group. The rest of the vehicle-treated group animals exhibited Grade 1 withdrawal. However, in the same postoperative week, all of the FK506-treated group animals showed Grade 3 withdrawal. For the number of animals showing full withdrawal, a significant difference

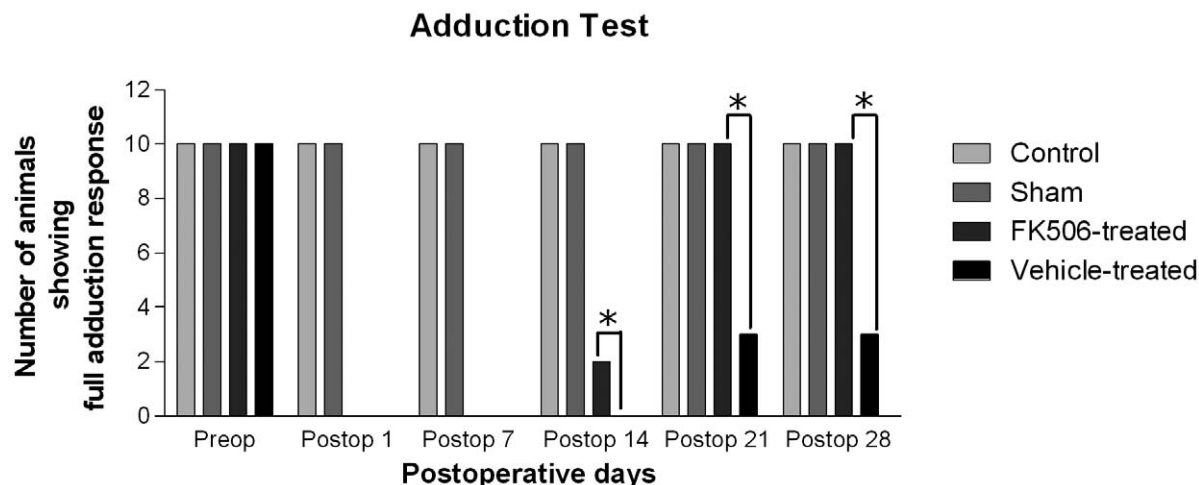


Fig. 2. Adduction test results on preoperative and postoperative days. Asterisk shows significant difference between groups.

($P<0.0001$) was noted between the FK506-treated and vehicle-treated groups in the fourth postoperative week (Fig. 1).

Adduction test

In the control and sham-operated groups adduction responses were full (Grade 3) in all postoperative weeks. The onset day of full response for the adduction test (Grade 3) was in the second postoperative week in two animals in the FK506-treated group. In the rest of the FK506-treated animals, two exhibited Grade 1 and two showed Grade 2 adduction responses. However, in the same postoperative week, no Grade 3 adduction response was detected. Eight animals in the vehicle-treated group exhibited Grade 2 responses. The rest showed Grade 1 adduction responses. A significant difference ($P<0.05$) was noted between the FK506-treated and vehicle-treated groups (Fig. 2) in the number of animals showing a full response.

The first day of a full response (Grade 3) in the adduction test was in the third postoperative week in six animals in the vehicle-treated group. The rest of the vehicle-treated group animals showed Grade 1 adduction responses. However, in the same postoperative week, all of the FK506-treated group animals were able to show Grade 3 adduction responses. A significant difference between the number of animals showing a full response ($P<0.05$) was found between the FK506-treated and vehicle-treated groups. In the fourth postoperative week, the results of the FK506 and vehicle-treated groups did not change (Fig. 2).

Animal weight and wet muscle weight

A tendency to lower body weight found in animal group with FK506 administration was not statistically significant.

On the day of animal sacrifice, the wet muscle weight of all adductor muscles of the control, sham, FK506 treated, and vehicle treated groups were 2.376 ± 0.08 , 2.398 ± 0.03 , 2.495 ± 0.1 , 1.501 ± 0.05 grams, respectively. There was no significant difference between control, sham, and FK506-treated groups. A significant difference was observed between FK506 and vehicle-treated groups (Fig. 3).

Light and electron microscopic evaluation

Obturator nerves were found to contain both myelinated and unmyelinated nerve fibers (Figs 4, 5).

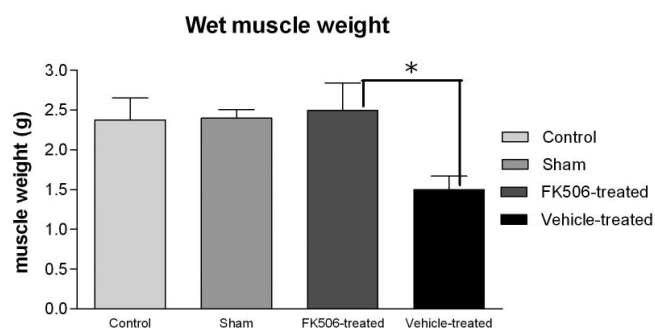


Fig. 3. Wet muscle weight of the adductor muscles on the sacrifice day. Asterisk shows significant difference between FK506 and vehicle-treated groups.

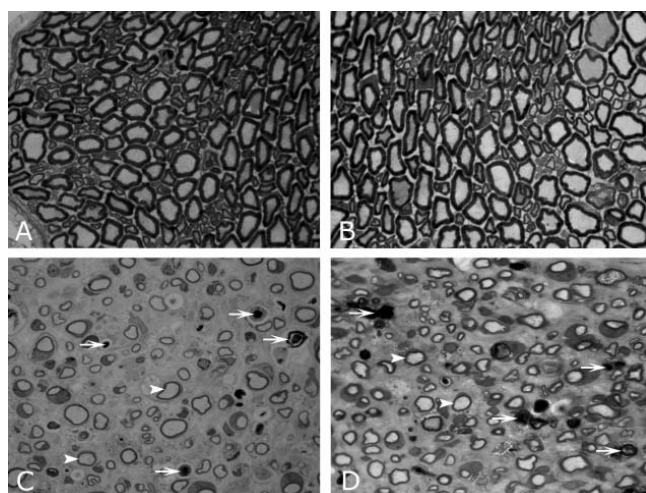


Fig. 4. Light micrographs showing obturator nerve appearance in control (A), sham-operated (B), FK506-treated (C) and vehicle-treated (D) groups. White arrows: myelin residues and deformed sites; arrowheads: newly remyelinated fibers.

Control and sham-operated groups did not show myelin debris or damaged fibers when the sections were examined by light microscopy (Fig. 4). Numerous damaged myelin residues were observed in the vehicle-treated group. The amount of myelin debris was higher in the vehicle-treated group than in the FK506-treated group (Fig 4, 5).

Electron microscopic evaluation revealed that the control and sham-operated groups had evidence neither of edema nor an injured appearance. Myelinated and unmyelinated fibers revealed regular morphology (Fig. 5). In the vehicle-treated group, there were abundant phagocytic cells (macrophages or Schwann cells with phagocytic activity) in the endoneurium, which had plentiful esophaged myelin residues. Evaluation of EM sections indicated that the vehicle-treated group showed more phagocytic cells than the FK506-treated group (Fig. 5). Fibrous components of the connective tissue in the endoneurium had a normal appearance but there were some open areas among filaments of connective tissue. In the FK506-treated group, numerous thin myelin sheaths starting to form were observed (Fig. 5).

DISCUSSION

In the present study, we simulated traumatic injury of the obturator nerve as might be seen in fractures of the anterior pelvic ring, and demonstrated a beneficial effect of FK506 on obturator nerve regeneration. In

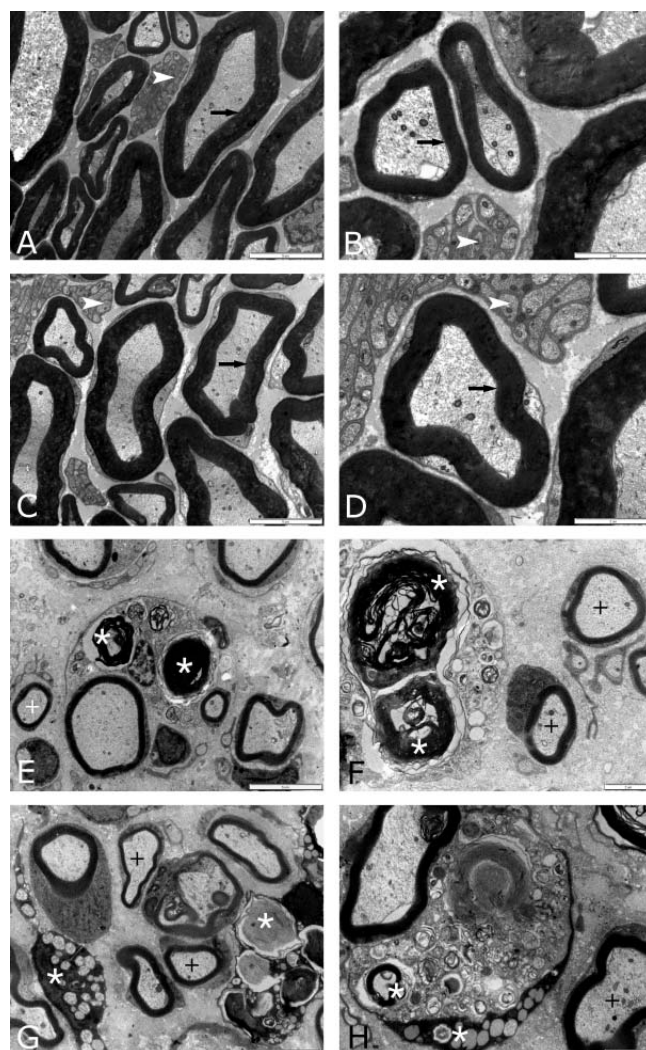


Fig. 5. Electron micrographs showing obturator nerve appearance in control (A, B), sham-operated (C, D), FK506 (E, F), and vehicle-treated (G, H) groups. The obturator nerve sections were taken 30 days following nerve crush. In the control and sham-operated groups obturator nerves show normal appearance. In the FK506-treated group, the obturator nerve showed well-shaped myelinated fibers. However, in the vehicle-treated group, myelin debris was observed. Black arrows: myelinated fibers. Arrowheads – unmyelinated fibers; (*) myelin residues; (+) newly remyelinated fibers.

other experimental studies, FK506 has been found to enhance the rate of axonal regeneration after injury of the rat sciatic nerve (Gold et al. 1995, Wang et al. 1997, Jost et al. 2000, Sarikcioglu et al. 2006). Evaluation of our data revealed that FK506 had a beneficial effect on obturator nerve regeneration as well.

It would not be expected that an immunosuppressant drug (FK506) would alter axonal regeneration since

Wallerian degeneration is not an immune-mediated event (Griffin et al. 1993). Furthermore, even if a reduction in macrophage infiltration occurs following FK506 administration (Bruck and Friede 1990), such an alteration would be expected to impair nerve regeneration by delaying the removal of products of Wallerian degeneration from the distal stump (Chen and Bisby 1993, Ludwin and Bisby 1992). Thus, what can be the mechanism of FK506 "reparative" effect on regeneration?

The immunosuppressant drug FK506 acts *via* binding to receptor proteins, FKBP-12, which in turn can bind to and regulate a calcium-dependent phosphatase, calcineurin, and a calcium release channel, the ryanodine receptor. Axotomy increases FKBP-12 mRNA expression in both motor and sensory neurons. Levels of FKBP-12 mRNA expression during neural regeneration are parallel to those of growth-associated protein (GAP-43), a calcineurin substrate that regulates neurite extension. Thus FK506 could increase nerve regeneration by increasing the phosphorylation of GAP-43 *via* its known ability to inhibit the activity of calcineurin, and could enhance neurite outgrowth in motor and sensory neurons by increasing sensitivity to nerve growth factor (Heitman et al. 1993, Lyons et al. 1994, Gold et al. 1995, Drake et al. 1996). It is also possible that FK506 treatment suppresses the loss of myelin and therefore plays a protective or sparing role after injury. Further studies with specific nerve tracers should be performed to verify this possibility.

The obturator nerve can be compressed against the lateral pelvic wall during its course in the obturator canal. Obturator nerve injury can occur in association with pelvic lymphadenectomy, obturator hernia, endometriosis, forceps delivery, or prolonged surgery with exaggerated hip flexion, stretching (neurapraxia), electrofulguration injury, dissection injury, or traumatic injury after fractures of the anterior pelvic ring (Lam 1936). It results in decreased sensation over the medial thigh, weakness of the hip adductors and decreased ability to internally rotate. While the complaints and symptoms are not serious, in terms of life and death or ultimate function, the neurologic complication became the chief complaint throughout the period of hospitalization of the patients (Lam 1936). Therefore, acceleration of the recovery time should be main goal of physicians.

Recovery from peripheral nerve injury can be described in terms of quality and speed. In order to measure the effects of drugs on these parameters, numerous methods have been described (Gold et al.

1995, Wang et al. 1997, Jost et al. 2000, Sarikcioglu et al. 2006). To our knowledge, no precise procedure for testing motor recovery of the obturator nerve of the rat has been published. We used the adduction test in our study to overcome this limitation and we found that it was a simple and reliable test to measure functional recovery.

Spaliviero and coauthors (2004) reported a case of iatrogenic obturator nerve transection during laparoscopic pelvic lymph node dissection and radical prostatectomy. The nerve stumps were oriented and laparoscopically repaired by end-to-end coaptation. At a follow-up of 21 months, the patient had regained full adduction strength of the left lower extremity with only occasional, transient numbness or tingling of the left foot. After such inadvertent injury FK506 might have been used. As the aforementioned recovery time is considerably longer than those found in the rat (Wang et al. 1997, Kvist et al. 2003), experimental data should be carefully extrapolated to clinical data or usage.

CONCLUSION

Previously we have shown beneficial effects of FK506 on sciatic (Sarikcioglu et al. 2006) and optic nerve recovery (unpublished data) after crush injury. In the present study, we simulated the traumatic injury of the obturator nerve as might be seen in the fractures of the anterior pelvic ring, and demonstrated the beneficial effects of FK506 on obturator nerve regeneration both at the ultrastructural and functional level. Thus usage of FK506 in such injuries may result in acceleration of the recovery time and improvement of the quality of the functional recovery.

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