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ORIGINAL ARTICLE

Investigation of Effect of Nifedipine on Surgicaly Repaired Rabbit Facial Nerve Regeneration

Adil Eryilmaz, Ayse Nur Cakar, Celil Gocer, Pergin Atilla Uz, Ayse Iriz, Sule Bilen, Melin Cayonu

Department of Otorhinolaryngology in Numune Education and Research Hospital Ankara, Turkey (AE, CG, AI, MC)
Department of Histology, Hacettepe University, Ankara, Turkey (ANC, PAU)
Department of Neurology in Numune Education and Research Hospital, Ankara, Turkey (SB)

Objective: Neuronal survival is an important factor in the achievement of functional restitution after peripheral nerve injuries. An experimental rabbit model was used to evaluate the effect of nifedipine on the outgrowth of regenerating axons of a transected peripheral facial nerve.

Materials and Methods: A complete transection injury was performed in 20 facial nerves of 16 rabbits. There were eight nerves in the first group; after the injuries, the anastomosis site was wrapped with a piece of gel foam soaked in phosphate buffered saline. In the second group there were 12 facial nerves; after the injuries, the anastomosis site was wrapped with a piece of gel foam soaked in nifedipine diluted. Functional studies were performed on 5th, 15th, 42nd days after the surgery. Electrophysiological studies were performed just before and 42 days after the surgery. At last, 42 days after the initial surgery, facial nerves were removed for the histological examination.

Results: After six weeks, the reduction in a mean compound muscle action potential (CMAP) and functional index, and the increase in latency index were determined by the use of nifedipine in the second group. The differences between the two groups were statistically significant. ($p<0.05$) On histological examination it was seen that nifedipine improved facial nerve regeneration by increasing the number of axons and myelin sheaths.

Conclusion: These results demonstrate that nifedipine may be an effective peripheral nerve protective agent when used locally at the surgical site after complete transection of the facial nerve.

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Introduction

Peripheral nerve injuries constitute one of the important health problems all around the world. Studies have shown that axonal regeneration rate is 1 mm / day [1-3]. Therefore, axonal regeneration takes months following a cut in the facial nerve. It is clear that everyone wishes to find an agent that fastens and increases axonal recovery and can be used in clinical practice. Various experimental studies in the literature reported that calcium channel blockers enhanced axonal regeneration and functional recovery in the central nervous system injuries [4].

This study is different from the other studies in the literature for using a calcium channel blocker (nifedipine) locally in the region of facial nerve injury. The effect of local nifedipine on facial nerve regeneration was investigated functionally, electrophysiologically and histopathologically.

Materials and Methods

This study was submitted to and approved by the Research Ethics Committee for procedures in experimental animals at the Turkish Scientific Institute (TUBITAK), protocol # 107S134.

In this study, we used 16 white male rabbits weighing on average 2,000g (ranging from 1,000 to 2,500). The animals were divided into two groups with 8 rabbits in each group to form a control (saline) and a study (drug application, nifedipine) group. A standard rabbit feeding and sheltering was performed for all animals.

Corresponding address:
Dr. Ayse Iriz,
Numune Hastanesi 3. Kulak Burun Bogaz Klinigi,
Samanpazari / Ankara - Turkey
Phone: 05058580370 • Fax: 03123121218
E-mail: ayseiriz@gmail.com

In the study group, the nifedipine solution was prepared; a 0.5 x 0.5 cm sized-gel foam was soaked in this solution and wrapped around the transected peripheral nerve after surgery. In the saline group, a 0.5 x 0.5 cm sized-gel foam was soaked in 100 microliters of phosphate buffered saline solution and wrapped around the cut peripheral nerve after surgery.

Preparation of nifedipine: Nifedipine is available in pharmacies in the form of capsules which contain the drug at a concentration of 2 %. In this study, nifedipine was diluted with phosphate buffered saline to a concentration of 0.006% before being used.

Surgical procedure: Anesthesia of the animals was performed using 50 mg/kg ketamine hydrochloride (Ketalar vial, Pfizer) intramuscularly. One hundred microliters of prepared nifedipine solution was used locally at the site of the transected nerve. Surgical procedure was performed under the surgical microscope and under sterile conditions using a microsurgical approach. After cutting the main trunk of the facial nerve with microscissors, the nerve endings were sutured using a epineural suturing technique. Later, a 0.5 x 0.5 cm sized-gel foam was soaked in the nifedipine solution and wrapped around the cut region of the nerve in the nifedipine group. In the control (saline) group, a piece of gel foam with the same size was soaked in the phosphate buffered saline and wrapped around the cut region of the nerve.

Animals were kept in invidual cages and given standard ration and water ad libitum in adequate environmental condition.

Functional evaluation: Experimental animals had functional evaluations on postoperative 5th, 15th end 42nd days. The animals were taken out of their cages and put in a place where they can be easily observed. The spontaneous movements of upper lip and the eyelid were examined in all animals and were recorded with a digital camera. Then, the nose and the forehead of the animal were touched delicately at the midline to stimulate facial movements, and the resulting movements were also watched and recorded. Recorded upper lip and eyelid movements were scored between 0 and 3, as follows: 0 = no movement at all; 1 = minimal movement; 2 = moderate movement; 3 = normal movement. Since functional evaluation is a subjective method, the digital recordings were watched and scored by a jury of doctors who were experienced in the facial paralysis and did not have any information on the details of the study.

Electrophysiologic evaluation: Electroneurography (ENoG) was performed to both groups concurrently in the preoperative period. In this way, normal latency times, compound muscle action potentials (CMAP) and the stimulation intensity needed for a supramaximal stimulation were determined for each animal. These values were used for the evaluation of the data obtained at the end of the study as well as for the standardization of ENoG findings. On the 42nd postoperative day, ENoG was performed in both groups concurrently. Before ENoG, the animals were anesthetized with intramuscular 50 mg/kg ketamine hydrochloride (Ketalar vial, Pfizer). The facial nerve was stimulated at the region of stylomastoid foramen, proximal to anastomosis line, and electrophysiologic recordings were obtained from orbicularis oculi muscle using Kohden Neuro Pack equipment (stimulation intensity: 2.0-3.0 mA, 1 impulse/second, 0.2 msec duration). CMAP and latency times were used in the electrophysiological evaluation.

Histological evaluation: The experimental animals were sacrificed using deep ether anesthesia. Previously transected facial nerve was removed including 5 mm proximal and 5 mm distal parts from the anastomosis line. In this way, eight nerves were obtained in the control group and 12 nerves were obtained in the study group. The unoperated left facial nerve of the experimental animal number 18 was removed in order to have sufficient information about the normal facial nerve histology, and to compare the normal nerve with the operated facial nerves. The facial nerve samples were fixed in 2% glutaraldehyde. The nerves were put into 1% osmium tetroxide for 90 min for the second fixation. The tissues were washed with Sorenson's buffer and a routine tissue processing was performed for electron microscopy (dehydration, providing transparency with propylene oxide, embedding into araldite and polymerization). One micrometer – thick sections were obtained with a LKB 11800 pyramitome

and the sections were stained with 1 % methylene blue – Azure II mix in 1 % borax. The sections were examined under Leica DM 6000 microscope and recorded. The facial nerves were evaluated using the digital records.

Statistical analysis: 'Unpaired t test' was used to compare the scores obtained from the digital records of the functional study. The analysis of the data obtained from the electrophysiological tests were performed using SPSS 11.5 (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, United States) package program. Shapiro Wilk test was performed to analyze normal distribution of continuous variables. Descriptive statistics were presented as median and 25-75 percentiles. The statistical comparison of preoperative and postoperative latencies and compound muscle action potentials (CMAP) measurements within the same group was performed with Wilcoxon signed rank test. Intergroup statistical comparison of preoperative and postoperative latencies and evoked response amplitude measurements was performed using Mann Whitney U test. $p<0.05$ was regarded as statistically significant.

Results

When functional results were considered, the mean score of the control group was 0.375 while this value was 1.92 in the study group on the postoperative 15th day. The difference between two groups was statistically significant (unpaired t test, $p = 0.0001$). The mean functional score of the control group was 1.38 and the mean functional score of the study group was 2.42 on the 42nd day. The difference between two groups was statistically significant (unpaired t test, $p = 0.0003$).

Intragroup comparisons were performed for latencies and evoked response amplitudes obtained with electrophysiological evaluation (Table 1). The mean preoperative latency was found as 1.37 msec in the nifedipine group. The mean latency was found as 1.67 msec on postoperative 42nd day. This difference means 22% elongation in the latency, and the difference is not statistically significant. The mean latencies in the preoperative period and on postoperative 42nd day were 1.15 msec and 1.80 msec, respectively in the control group. This difference means 57% elongation in the latency and is statistically significant. Preoperative mean evoked response amplitude was 4.60 mV in the nifedipine group. This value was 4.00 mV on postoperative 42nd day. This difference means 13% reduction in evoked response amplitude when compared to normal, and is statistically significant.

Another parameter evaluated statistically was intergroup distribution of the differences between normal (preoperative measurements) and postoperative latencies and evoked response amplitudes (Table 2). When latencies were considered, the difference between the difference appeared in the postoperative period in the saline group and the difference appeared in the postoperative period in the nifedipine group was statistically significant ($p = 0.039$). When evoked response amplitudes are taken into consideration, the difference appeared in the postoperative period in the saline group and the difference appeared in the postoperative period in the nifedipine group was not statistically significant.

Table 1. In the groups, Distribution of Evoked Response Amplitude Levels and Latency times, pre-operatively and post-operatively

Difference	Group	Pre-operative	Post-operative	p*
Latency	Saline	1.15 (1.07 -1.24)	1.80 (1.49 - 2.56)	0.012
	Nifedipine	1.37 (1.25 - 1.54)	1.67 (1.25 - 1.85)	0.054
Evoked Response Amplitude	Saline	3.70 (3.22 - 4.07)	1.95 (1.32 - 3.07)	0.012
	Nifedipine	4.60 (4.20 - 4.85)	4.00 (2.90 - 5.97)	0.327

Table 2. Distribution of the Amount of the Changes Between The Preoperative and Postoperative Levels of the Latency Times and Evoked Response Amplitude in the Groups.

Amounts of the Changes	Saline	Nifedipine	p
Latency	0.72 (0.23 - 1.40)	0.40 (-0.17 - 0.54)	0.039
Evoked Response Amplitude	-1.60 (-2.35 - -0.85)	-0.95 (-2.37 - 1.22)	0.427

Histological findings: Examination of normal facial nerve revealed healthy axons. The myelin sheath surrounding them had nearly same thickness everywhere. The axonal diameters were similar. Collagen fibrils and connective tissue cells could be seen in the endoneurium that surrounded axon bundles, in perineurium that surrounded fascicles, and in epineurium that surrounded the nerve.

The proximal segment of the facial nerve in the saline group: The degeneration the proximal part of the nerve appeared as proceeding rapidly in the saline group. There were myelin figures in some of the axons while some others had lipid deposits. There was atrophy in some axons. The endoneurium that surrounded the axon fascicles was edematous, and contained a number of macrophages (Fig 1).

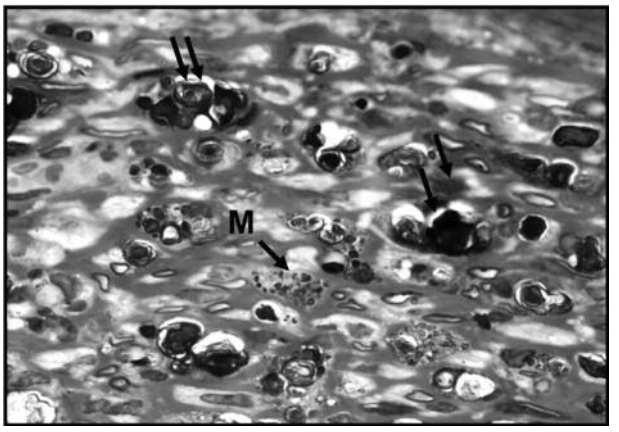

Figure 1. The proximal segment of the facial nerve in the salin group: There were degenerated axons (double arrow), tiny myelin figures and activiteas of macrophages (M) in large magnification. Methylene Bulue-Azur II X1000

The distal segment of the facial nerve in the saline group: The axons and the myelin sheaths were mostly degenerated in distal part of the facial nerve in the saline group. There was an increase in the collagen fibrils and edema in the endoneurium that surrounded axons. The thickened endoneurium replaced the places of the destructed neurons. There was an enormous increase of collagen in the perineurium that surrounded fascicles and in the epineurium that surrounded the nerve. There was proliferation of the Schwann cells, some axons were edematous, and there were occasional atrophic axons. There were axons with thin myelin in some parts of the fascicles (Fig 2).

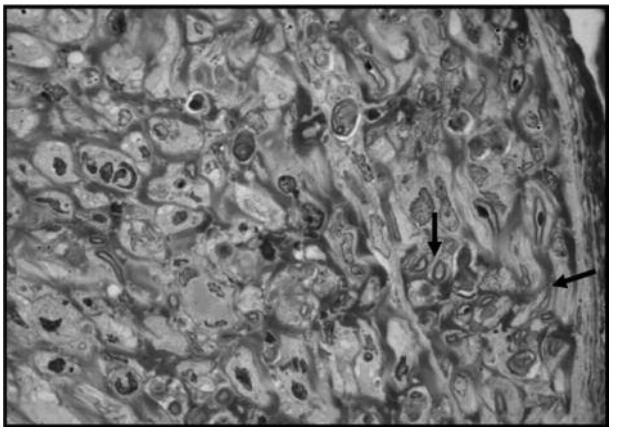

Figure 2. The distal segment of the facial nerve in the saline group; Schwann cells were proliferating in large magnification area of degeneration. There were edema and a few thin myelinated axons (arrow). Methylene Bulue- Azur II X 1000

The proximal segment of the facial nerve in the nifedipine group: Highly degenerated axons with myelin figure formation and lipid depositions seen in the proximal parts of the nerves in the saline group were not evident in the proximal nerve samples obtained in the nifedipine group. Schwann cells were found to be increased in number, and "Büngner bands" characterized by budding of the healthy axons were observed more extensively in some regions of the fascicles. There were some atrophic axons as well (Fig 3).

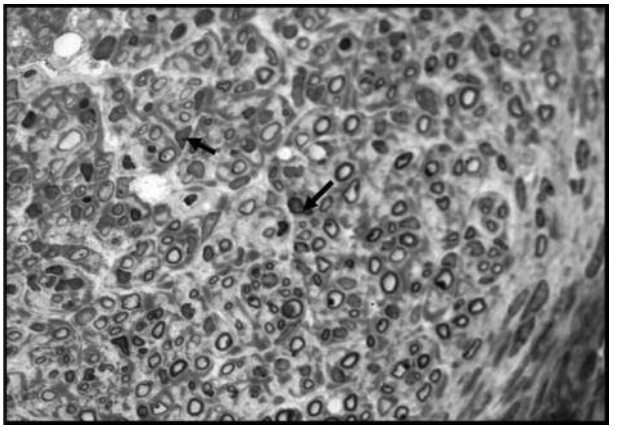

Figure 3. The proximal segment of the facial nerve in the nifedipine group; There were Büngner bands, myelinated axons which emerging new and Schwann cells (arrow) in large magnification. Methylene Bulue-Azur II X1000

The distal part of the facial nerve in the nifedipine group: Although there were myelin figures and lipid deposits as well as atrophy in some axons in the nifedipine group and these observations were similar to those seen in the saline group, new axon development was more frequent in this group. There was a significant increase in the number of the Schwann cells. Edema of the endoneurium that was seen in the saline group was not evident in the nifedipine group. Thickening of the connective tissues of endoneurium, perineurium and epineurium was less prominent when compared to saline group (Fig 4).

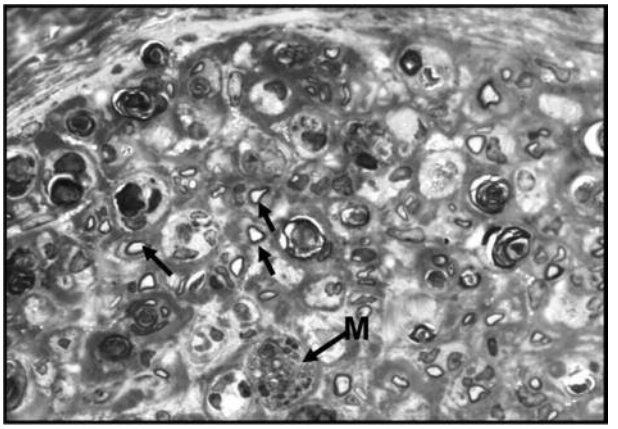

Figure 4. The distal part of the facial nerve in the nifedipine group; Axons which wrapped a thin myelin (arrow) with macrophages (M) are selected in large magnification. Methylene blue-Azur II X1000.

Discussion

Cranial nerves are quite susceptible to trauma. Facial nerve is especially important for otorhinolaryngologists since it may be damaged in chronic otitis media and otosclerosis surgery, surgery of the tumors of the temporal bone and parotid gland, and after blunt and penetrating trauma to the temporal bone and parotid region. Trauma is the second most common cause of facial paralysis, ranking after Bell's palsy. May et al. reported that trauma was responsible for facial paralysis in 23% of their patients [5]. Fourth and 5th degree neural injuries according to Sunderland classification require a surgical repair [6].

In this study, nifedipine, a calcium channel blocker, was locally applied to the surgical field. Nifedipine antagonizes L – type and voltage dependent calcium channels and inhibits calcium influx into the cell. Additionally, it binds calcium binding proteins such as calmodulin in the cell and inhibits the activation of the proteases. L–type calcium channels have been demonstrated in the facial nerve nucleus in the brain stem and in the peripheral axons of the facial nerve. Increased calcium influx from the plasma membrane and intracellular compartments has a critical role in basic neuronal functions. This influx has particular importance in the regulation of axonal growth, synaptogenesis, synaptic transmission and neuronal plasticity [7]. Tissue cell cultures with calcium channel blockers showed that these agents inhibited glutamate – mediated neurodegeneration mechanisms [8]. Another study showed that a reduction of calcium influx frequency into the cell resulted in an increased regeneration in the growth cone and increased axonal lengthening [9-11]. Animal studies showed that different calcium channel blockers might cause an improvement in the compound muscle action potentials when compared to the controls [12]. Similar experimental animal studies where intracranial damage was created reported that calcium channel blockers that can pass blood – brain barrier such as nimodipine, improved axonal healing. Theoretically, nifedipine used in this study might have reduced the frequency of calcium influx, and by this way might have increased axonal regeneration [4].

Measuring neural regeneration is one of the important problems of peripheral nerve investigations. Today, histology, morphometry, electron microscopy, nerve conduction rate measurement, muscle mass measurement and electromyography are used together and correlated to evaluate neural regeneration in experimental nerve repair models [1-3]. In this study, facial muscle movements of experimental animals were followed and video - recorded, facial nerve was stimulated around the stylomastoid foramen to obtain electromyographic recordings of the latencies and amplitudes from quadratus labii superior and orbicularis oculi muscles, and histological examination of the facial nerve including the anastomosis site was performed to evaluate neural

regeneration. By using aforementioned three methods together, we aimed to increase the reliability of the results, and to crosscheck the data obtained with each method.

Scoring facial paralysis by the movements of upper lip and eyelids was used in previous facial paralysis studies that employed rabbits as experimental animals [13, 14]. Although it is a subjective method, video recordings were carefully analyzed and were watched and scored by a jury of doctors who were experienced in the facial paralysis but did not have any information on the details of the study. When the scores obtained on 15th and 42nd days were analyzed, it was found that the difference between the saline and nifedipine groups was statistically significant.

Electroneuronography was performed as an electrophysiological study. ENoG is the most important quantitative test performed to determine the prognosis of facial paralysis in human studies [15-17]. Serially performed ENoGs are important for determining the candidates for surgery in patients with traumatic facial palsy, facial palsy due to Herpes Zoster oticus and Bell's palsy. A decrease in CMAP (compound muscle action potential) by 90 % in the paralyzed site when compared to the healthy facial nerve of the patient is regarded as an indication for surgery. A decrease in CMAP by 30 % in the paralyzed site when compared to the healthy site is interpreted as a positive result. However, the most important disadvantage of the test is the need for the technical equipment and experience [16, 19]. In addition, there are different opinions on the placement of the electrodes, and there is no standardization on this issue. In this study, as previously performed in the literature, ENoG was used to determine the differences between the groups for CMAP and latencies rather than to determine the prognosis.

In nifedipine group, there was an increase of 22% when preoperative and postoperative 42nd day latency times were compared, and the difference was not statistically significant. On the contrary, this increase was 57 % in the saline group and the difference between preoperative and postoperative 42nd day latency times was statistically significant.

Comparison of CMAP values revealed a reduction of 13 % in the postoperative period in the nifedipine group. This reduction was not statistically significant. On the other hand, postoperative CMAP was 47 % smaller than the preoperative value in the saline group and this result was statistically significant.

In this study, histological examination was another method used in order to show the differences between the groups. Important structural changes occur especially in the distal part of the nerve after the injury. Wallerian degeneration occurs in the distal segment of the nerve after injury. Increased macrophages cleanse the debris of Schwann cell tubes. At the same time, they produce "interleukin-1" (IL-1) to provide stimulus for the secretion of neurotrophic factors such as "nerve growth factor" (NGF) and "insulin- like growth factor" (ILGF). Proliferated Schwann cells are confined in the basement membrane and assemble in longitudinal columns to form “Büngner bands”. A biphasic neurotrophic factor, "nerve growth factor" (NGF) is synthesized after neural injury. The firth phase of the synthesis occurs in the proximal and the distal ends of the nerve during the first six hours after the injury. The second phase is seen 2 - 3 days after the injury and coincides with the increase in the number of proliferated macrophages and Schwann cells. Similar to NFG, other neurotrophic factors present in the cell are released in small amounts after the injury. These start a cycle and neurotrophic factors are released from a number of sources. These factors retrogradely move in the axon, reach the nerve cell and provide regeneration [9, 10, 12, 18].

On the histological sections, there was a significant difference in the nifedipine group for the presence of less degeneration and quicker beginning of regeneration. Nifedipine was observed to decrease intrafascicular myelin and axonal debris. These results are in conjunction with the data of the studies performed on the central nervous system and reported that calcium dependent mechanisms played a role in cell death, apoptosis, neurodegeneration and cell aging processes [8,12-14,20,21,23]. In addition, there is a correlation among the functional scores, neurophysiological results and histological results.

Conclusion

In this study, it was determined that use of local nifedipine inhibited calcium - dependent degeneration mechanism in a surgical field in which an end - to - end anastomosis was performed with epineural suturing after complete transection of the facial nerve, and caused faster appearance of regeneration findings in the distal and especially proximal parts of the injured nerve. In addition, there were no degeneration findings in the proximal part of the nerve in the nifedipine group. On the other hand, when distal parts of the nerves in the nifedipine group were compared with the distal parts of the nerves in the saline group, there was a very limited degeneration. Therefore it was concluded that use of nifedipine might be helpful in the treatment of peripheral nerve injuries, especially in the acute period.

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