# Enhanced expression of neuronal nitric oxide synthase and phospholipase C-γ1 in regenerating murine neuronal cells by pulsed electromagnetic field

**Sung Sook Kim[1], Hye Jung Shin[2], Dae Woon Eom[1], Joo Ryung Huh[1], Yeongju Woo[1], Hyokyung Kim[3], Sung Ho Ryu[4], Pann Gill Suh[4], Moon Jung Kim[2], Jee Yun Kim[2], Tae Wan Koo[2], Yoon Hee Cho[2] and Sung Min Chung[2,5]**

[1] Department of Pathology, Ulsan University College of Medicine, 682-7-060, Ulsan, Korea
[2] Department of Otolaryngology, Ewha Womans University College of Medicine, Seoul 158-710, Korea
[3] Department of Neurology, Ulsan University College of Medicine, Ulsan 682-060, Korea
[4] Department of Life Science, Pohang College of Science and Technology, Pohang 790-784, Korea
[5] Corresponding author: Tel, +82-2-650-6163; Fax, +82-2-653-5135; E-mail, sungmin@mm.ewha.ac.kr

Accepted 20 February 2002

Abbreviations: nNOS, neuronal nitric oxide synthase; PEMF, pulsed electromagnetic field; PLC-γ1, phospholipase C-gamma 1

## Abstract

**Pulsed electromagnetic field (PEMF) has been shown to improve the rate of peripheral nerve regeneration. In the present study we investigated the expression of neuronal nitric oxide synthase (nNOS) and phospholipase C-γ1 (PLC-γ1) in regenerating rat laryngeal nerves during the exposure to PEMF after surgical transection and reanastomosis. Axons were found to regenerate into the distal stump nearly twice faster in PEMF-exposed animals than in the control. Consistently, motor function was better recovered in PEMF-treated rats. The expression of nNOS and PLC-γ1 was highly enhanced in the regenerated nerves.**

**Keywords:** PEMF, PLC-γ1, NOS, S-100

## Introduction

Over one decade, weak and asymmetric pulsed electromagnetic fields (PEMF) have been used to induce time-varying ionic currents in tissues, thereby modifying a number of cellular functions. Modifications include selective changes in cellular calcium, and cyclic AMP levels (Betty *et al*., 1993) and in the synthesis of collagen, proteoglycans, DNA, and RNA. In addition, enzymes and hormones involved in skeletal homeostasis are affected by PEMF. Moreover, PLC-γ2 was reported to be involved in PEMF-induced regeneration (Clejan *et al.,* 1996; Dibirdik *et al.,* 1998). Despite their wide use to treat ununited fractures with or without surgical repair, cellular mechanism of PEMF for enhancing repair process has not been elucidated yet.

Ideally, treatment for laryngeal paralysis should restore normal physiologic and dynamic movement of the vocal fold. Although numerous experiments to develop appropriate techniques to restore the function of the paralyzed larynx have been tried, none of these approaches have been consistently successful. The PEMF technique has been shown to enhance regeneration of injured nerves. However, PEMF treatment has not been applied to the injured recurrent laryngeal nerve (RLN). Therefore, in the present study we investigated the effect of PEMF on the regenerative capacity of the transected and reanastomosed RLN in rats physically and histologically and examined the alteration in the expression of nNOS and PLC-γ1 in PEMF-induced nerve regeneration.

## Materials and Methods

### Animal model

Thirty-six male Sprague-Dawley rats were used in this study. Thirty-two rats were underwent left recurrent laryngeal nerve transection and reanastomosis and ipsilateral superior laryngeal nerve also transected at the same time to minimize potential contamination of the cricothyroid muscle. Animals were anesthetized with intraperitoneal injection of Ketamine hydrochloride (Ketalar. 10 mg/kg). After fixation of the rat in supine position, the anterior neck was exposed and midline vertical incision was done at median portion. Trachea was exposed and left RLN coursing tracheoesophageal groove was found. Then, transection was done with microscissors to RLN at a level of 0.5 cm below the inferior edge of cricoid cartilage and the two cut end were reanastomosed carefully using operation microscope with 10-0 nylon without tension. All rats following neurorrhaphy of the RLN showed left vocal fold Immobility in paramedian fixed position during laryngoscopic examination. These

rats were randomly assigned two groups and the experimental group (n=16) received PEMF and the control group (n=16) were handled the same way as the experimental group without PEMF (Janet and Walker, 1994). The four remaining rats received sham operations including all procedures except for the actual nerve transection and two of these rats were placed into each groups.

### Pulsed electromagnetic field exposure

The PEMF was delivered *via* four custom-made cages equipped with Helm-holz coils 30 cm in diameter, placed 15 cm apart (Figure 1). These coils connected to a signal generator (LG function generator FG-8002, LG precision Co., Seoul, Korea) producing a magnetic field of 120 Hz sine wave and led to a stereo amplifier. The output of the amplifier was adjusted to create a 0.4 millitesla magnetic filed as measured with the gaussometer in parallel to each of the 4 cages. Experimental animals were exposed to this field and control animals were placed between coils not connected to the generator. The cages were placed in a low-light sound booth with ventilation. The experiment was carried out three hours per day and five days per week for 12 weeks from post-surgical day 2.

### Determination of functional recovery

Videoendoscopic examination was performed just after operation and at every week following neurorrhaphy to determine whether there had been a return of the vocal fold movements. After 12 weeks, laryngeal electromyography (EMG) during respiration was performed on both posterior cricoarytenoid muscles just before sacrifice. Functional recovery was determined only when reinnervation potential activities occurred to coincide with respiration.

Rats were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS) and nerve tissues were obtained and immediately fixed with 10% neutral buffered formalin, dehydrated in ethanol, embedded in paraffin wax. Fresh specimens of half tissue were immediately frozen in liquid nitrogen and stored at -80°C for Western blot analysis.

### Tissue preparation

Immunohistochemical and Western blot analysis was performed on the normal and operative sides. A segment of left RLN was removed proximally 4 mm from anastomotic area. Immunohistochemistry was performed on RLN of both PEMF exposed (n=14) and control rats (n=8), for S-100 protein, nNOS to confirm neuronal regeneration and PLC-γ1 to elucidate its role in PEMF-enhanced neuronal regeneration.

### Immunohistochemistry

Immunohistochemistry was done as previously described (Kim *et al*., 2001). Serial 5 μm sections were cut from paraformaldehyde-fixed and paraffin embedded tissue. After deparaffinization and hydration, sections were incubated for 20 min with 10% normal goat serum to block nonspecific serum protein bindings. A block for endogenous peroxidase activity using 3% hydrogen peroxide for 15 min followed this. The antibody was made as previously described (Suh *et al*., 1988), and diluted to 1 : 200 for anti PLC-γ1 antibody and 1 : 50 for anti S-100, nNOS antibody. After 2 h incubation, sections were washed three times in phosphate-buffered saline and incubated with streptavidin-biotin reagents from the Dako LSAB (labeled streptavidin biotin) Stain kit (Carpenteria, USA). In cases of negative control of PLC-γ1 peptide, substituting primary antibodies. All sections were reacted with 3, 3-diaminobenzidine (DAB, Dako), as the chromogen and photographed under Olympus BH2 light microscope. The staining for PLC-γ1 was performed after 12 weeks following neurorraphy and the staining for nNOS and S-100 protein was performed at 4th week and 12th week from nerve injury and ligation. The result of immunohistochemistry was evaluated as follows; +, positive; −, negative.

### Western blot analysis

The Western blotting was done to confirm PLC-γ1 immunoreactivity. The tissues were isolated and homogenized with buffer A (20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT with 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 500 *g* for 5 min. The supernatant was centrifuged at 10,000 *g* for 30 min. The equal amounts of supernatant of control and experimental tissue were subjected to SDS-PAGE, and transferred to nitrocellulose paper and incubated with PLC-γ1 monoclonal antibody for 4 h. Immunoblotting of β-actin was done as internal control. Immunoreactivity for PLC-γ1 was detected with horseradish peroxidase conjugated to anti mouse IgG and ECL system (Amersham. Arlington Heights, USA).

## Result

### Functional recovery

After the twelve weeks' experiment, 14 out of 18 rats survived in the group which received PEMF while 8 out of 18 rats stayed alive in the control group. Ten rats (71%) in the PEMF-exposed group and 3 rats (38%) in the control group showed recovery of vocal fold movement, but it did not reveal any significant difference statistically. However the time taken for functional recovery was 3.93 ± 0.27 weeks and 7.87 ± 0.85 weeks for the PEMF-exposed group and the control group, respectively ($p<0.05$) (Table 1).

**Table 1.** Comparison of effects between PEMF-exposed group and control group

| Weeks | PEMF Group (N=18) | | | Control Group (N=18) | | |
|---|---|---|---|---|---|---|
| | No. of examined rats | No. of recovered rats | No. of expired rats | No. of examined rats | No. of recovered rats | No. of expired rats |
| 1 | 18 | 0 | 1 | 18 | 0 | 2 |
| 2 | 17 | 1 | 1 | 16 | 0 | 0 |
| 3 | 15 | 4 | 0 | 16 | 0 | 3 |
| 4 | 11 | 4 | 1 | 13 | 1 | 0 |
| 5 | 6 | 1 | 1 | 12 | 1 | 2 |
| 6 | 4 | 0 | 0 | 9 | 0 | 1 |
| 7 | 4 | 0 | 0 | 8 | 0 | 0 |
| 8 | 4 | 0 | 0 | 8 | 0 | 1 |
| 9 | 4 | 0 | 0 | 7 | 1 | 1 |
| 10 | 4 | 0 | 0 | 5 | 0 | 0 |
| 11 | 4 | 0 | 0 | 5 | 0 | 0 |
| 12 | 4 | 0 | 0 | 5 | 0 | 0 |
| Total | 18 | 10 | 4 | 18 | 3 | 10 |

Notes: Recovery rates (functionally recovered rats/survived rats) are 71% in the PEMF exposed group and 38% in the control group. By chi square test, p value was more than 0.05 because of the difference in the number of expired rats. Functional recovery could be observed earlier in the experimental group than control group and the difference was statistically significant ($p<0.05$)

### PLC-γ1 overexpression

We performed immunohistochemistry for PLC-γ1 on RLN of both PEMF-exposed (n=16) and control rats (n=16). PLC-γ1 is expressed in cytoplasm of nerve cells including ganglion cells. The markedly damaged and non-functioning nerve tissue did not show any PLC-γ1 (Figure 1A, B). Although PLC-γ1 is constantly expressed in RLN of functional recovery group rats, the PLC-γ1 expression was remarkably increased in the PEMF-exposed rats as demonstrated by immunohistochemistry

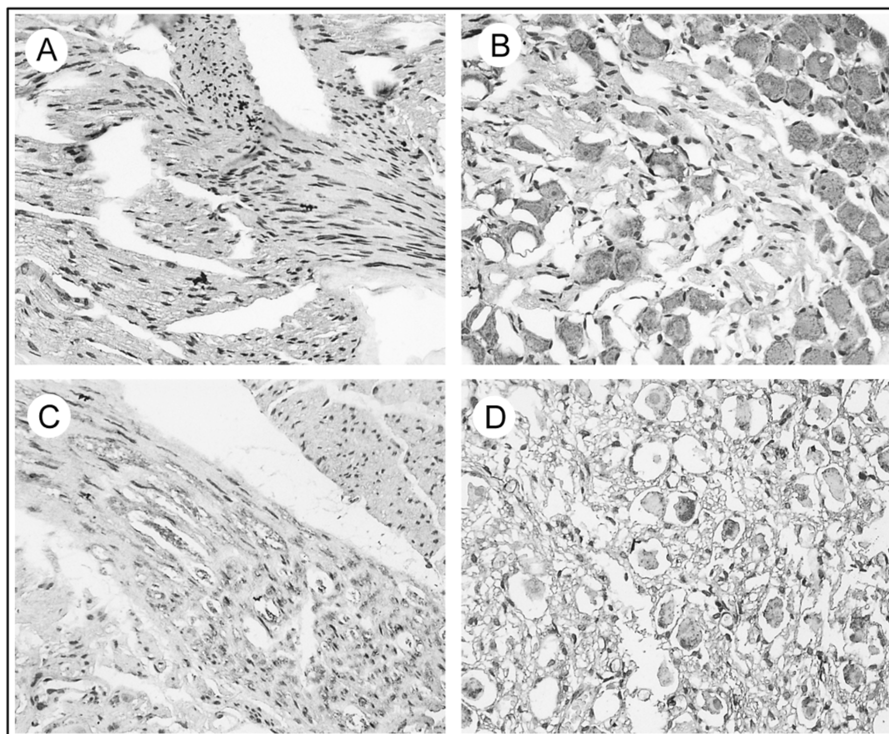


**Figure 1.** The expression of PLC-γ1(× 200). PLC-γ1 is not expressed in (A) and (B), compared that PLC-γ1 is diffusely expressed in (C) and (D). (A) nerve fiber without PEMF, not funtionally recovered until 12 weeks. (B) ganglion without PEMF, not funtionally recovered until 12 weeks. (C) nerve fiber with PEMF, functionally recovered at 4 weeks. (D) ganglion with PEMF, functionally recovered at 4 weeks.

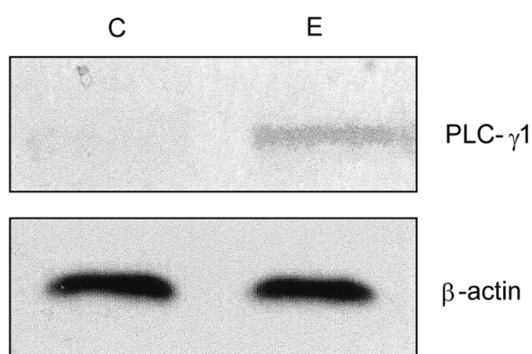


**Figure 2.** Nerve tissue of functionally recovered rat with PEMF at 4 weeks (experimental group: E) showed positive band for PLC-γ1 while no band was shown in nerve tissue without PEMF at 12 weeks (control group: C).

(Figure 1C, D), compared to control group. This over-expression was more remarkable in neuron, especially ganglion cells of nodose ganglions than in Schwann cells (Figure 1D). The results were confirmed by Western blotting (Figure 2, Table 2).

**Table 2.** Summary of PLC-γ1 expression. Although PLC-γ1 is constantly expressed in RLN of functional recovery group, the PLC-γ1 expression was markedly increased in the PEMF-exposed rats by immunohistochemisty and immunoblotting ($p<0.05$)

| Group | status of recovery (total No.) | PLC-γ1 overexpression | |
|---|---|---|---|
| | | – | + |
| PEMF-Exposed Group | FR (10) | 2 | 8 |
| | NFR (4) | 3 | 1 |
| Control Group | FR (3) | 3 | 0 |
| | NFR (5) | 5 | 0 |

### nNOS and S-100 expression

The expression of nNOS showed basically similar in that both protein are expressed in both neuron and Schwann cells of axon, although the expressions in neuron are very weak. The number of nNOS positive cells showed higher in functional recovery group (Figure 3A & B), but the difference between PEMF-exposed and control group was not found. Comparing to the expression of PLC-γ1, the nNOS is expressed mainly in Schwann cells, the PLC-γ1 is in ganglion cells (Figure 3B, D). Moreover, the intensity of expression was much stronger after 4 weeks following injury and returned to control level at 12th week ($p<0.05$). S-100 protein was

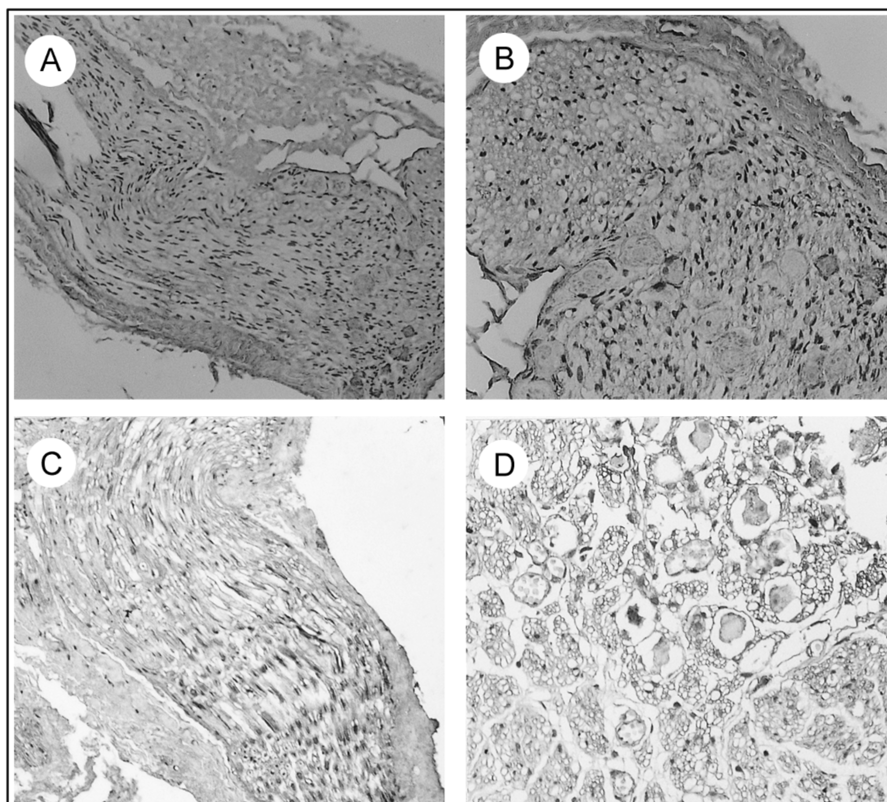


**Figure 3.** Comparison of the expression of nNOS (A and B) and PLC-γ1 (C and D) in nerve tissue and ganglion of functionally recovered rat with PEMF treatment for 4 weeks. (A and B) nNOS in nerve tissue with PEMF (A) showed stronger immunoreactivity than in ganglion cells (B). (C and D) PLC-γ1 showed stronger reactivity in ganglion (D) than nerve tissue (C).

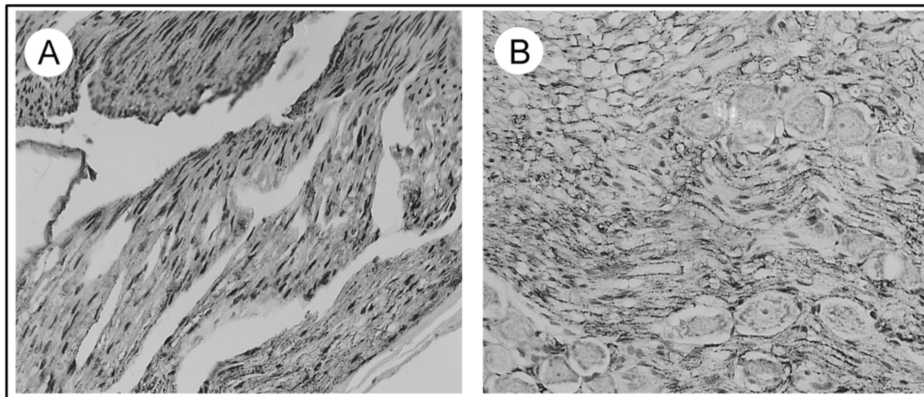


**Figure 4.** S-100 expression did not show any difference between control and experimental group. (A) nerve tissue without PEMF, not funtionally recovered until 12 weeks. (B) ganglion with PEMF, functionally recovered at 4 weeks.

**Table 3.** Staining intensity in immunocytochemistry of nNOS and S-100 protein. The expression of nNOS revealed higher in functional recovery group, but the difference between PEMF-exposed and control group was not found. Moreover, the intensity of expression was much stronger after 4 weeks following injury but staining intensity was dim at 12th week ($p<0.05$)

| Group | status of recovery | weeks | nNOS | S-100 |
|---|---|---|---|---|
| PEMF-Exposed Group | FR | 4 | + | + |
| | | 12 | + | + |
| | NFR | 4 | – | + |
| | | 12 | – | + |
| Control Group | FR | 4 | + | + |
| | | 12 | – | + |
| | NFR | 4 | + | + |
| | | 12 | – | + |

observed in the cytoplasm of Schwann cell, and was positive in both PEMF-exposed and control groups (Figure 4A, B). The degree of staining showed no difference between the two groups (Table 3).

## Discussion

In the treatment for unilateral or bilateral laryngeal paralysis from recurrent laryngeal nerve injury, it has been difficult to achieve optimal result with expedient, movement-specific reinnervation. Current treatment for bilateral laryngeal paralysis with significant airway obstruction involves either bypass of the obstruction with a tracheostomy or destructive procedure involving the vocal fold or arytenoid. The completely satisfactory treatment for unilateral vocal fold paralysis has not been reported yet. To date, vocal fold medialization techniques including thyroplasty, arytenoid adduction, and injection are most commonly used. While these methods provide good results for most patients, medialization method still have some limitation due to their static nature.

Ideally, treatment for vocal fold paralysis should restore normal physiologic and dynamic movement of the vocal fold. This is most likely to require some form of laryngeal innervation. Physiologic restoration of function would allow the patient with one or two paralyzed vocal folds to return to normal activity with a normal voice.

Recently, numerous experiments have been performed to attempt reinnervation of the paralyzed larynx. None have been consistently successful probably due to synkinesis and an inadequate number of regenerated axons. Synkinesis may be defined as the synchronous contraction of muscles that do not contract together. Within the larynx this refer to the simultaneous contraction of antagonistic muscle group, with the end result being an immobile vocal cord fixed by opposing forces (Paul *et al*, 1991). The number of regenerated axons may be inadequate by failure of the sprouting axon to reach the muscle fiber, increase of the connective tissue between nerve fibers, and/or obstruction of the contractile mechanism on the muscle fibers.

Stimulation by electromagnetic field exposure can modify cellular function in bone and nervous tissue. Ample evidence has been accumulated that the regenerative capacity of the tissue may be affected. Thus, pulsed electromagnetic stimulation was reported to enhance the regenerative process of injured nerves. Pulsed electromagnetic fields have encouraged healing of fractured bones (John *et al*., 1998) and benefited reanastomosis of peripheral nerves after transection. However there is no direct evidence of this process to be beneficial for the transected recurrent laryngeal nerve.

The specific mechanism of PEMF-enhanced regeneration has not been proven yet, but numerous studies reported that the increased synthesis of collagen and proteoglycans, DNA and RNA have been demonstrated in osseous, nervous, and mesenchymal tissues (Tompson 1995). In addition, enzymes and hormones involved in skeletal homeostasis are affected by specific PEMF. Moreover it has been reported that PLC-γ1 was involved

in PEMF-induced regeneration (Clejan *et al.,* 1996; Dibirdik *et al.,* 1998). The proper and minimum intensity of the electromagnetic field to produce nerve regeneration has not determined yet. The pulsation of electromagnetic field, not its intensity, appears to be most important for the nerve regeneration. Theoretically, the changing polarity of the current alters nerve electrochemical environment favoring better axoplasmic flow, more effective enzymatic activity for protein synthesis, and inhibition of fibrosis at the injury site. The phagocytic activity of the Schwann cells was also enhanced, and these cells have some control over nerve growth factor release (Rusovan and Kanje, 1991). The pulsed electromagnetic stimulation also lessened the degree of nerve cell body injury during the degeneration phase of injury, along with increasing the protein synthesis during regeneration. In our study, animals were exposed to pulsed electromagnetic field (0.4 millitesla at 120 Hz) for 4 h/day, 5 days/week, during 12 weeks. other researcher demonstrated that rats treated by PEMF (0.3 millitesla at 2 Hz) for 4 h/day during 5 days showed enhancement of functional recovery following sciatic nerve crushing injury (Janet, 1994). Stimulation at 0.2 mT did not affect regeneration (Rusovan and Kanje, 1991), while stimulation at 0.4 mT increased regeneration distances (Tompson, 1995).

Because nerve transection itself promotes intracellular process of many cells acting at early stage of regeneration, it seems that PEMF functions at 1-2 days after injury when DNA and RNA synthesis was prepared. Therefore, it is more effective when PEMF exposure begins as soon as possible after injury. The rats with functional recovery in viable animals were 71% (10 among 14 animals) in PEMF-exposed group and 38% (3 among 8 animals) in the control group. However, during the experiments, 4 of the PEMF-exposed animals and 10 of the control animals were expired. Therefore, there were no statistically significant differences between the percentage of functional recovery including expired animals of the treated group and the control group.

In our study, the time for functional recovery was 3.93 ± 0.27 weeks and 7.87 ± 0.85 weeks for the PEMF-exposed group and the control group, respectively. Thus, the functional recovery was developed earlier in the experimental group than control group and the difference was statistically significant ($p<0.05$). This result agrees with previous reports (Rusovan and Kanje, 1991; Tompson *et al*., 1995).

Other researchers made a report of the degree of nNOS expression according to nerve reinnervation and functional recovery after vagus and hypoglossal nerve injury with time sequence (Tompson *et al*., 1995). In our hand, the number of nNOS positive cells was increased in functionally recovered rats. nNOS staining intensity was reduced until 12 weeks after injury. The difference between PEMF group and control group was not found. This result may suggest that nNOS have a beneficial effect on nerve regeneration and it may function mainly in the early stage of recovery phase.

It is known that S-100 protein exists in cytoplasm of Schwann cell and enhances the growth of motor nerve. In this study, there was no difference between functional recovery group and nonrecovery group in the recurrent laryngeal nerve.

PLC-γ1 acts as an intracellular effector in signal transduction of growth factors and mitogens, hydrolyzing phosphatidylinositol 4,5-bisphosphate to two important second messengers, inositol 1,4,5-triphophate and diacylglycerol (Sanda *et al*., 1996). Although PLC-γ1 has been mostly studied in carcinogenesis, this molecule also plays a role in tissue regeneration (Saika, 1994; Lee, 2000). Moreover, there have been reported that PLC- γ2 was involved in PEMF-induced regeneration (Clejan *et al.,* 1996; Dibirdik *et al.,* 1998). Present study clearly demostrated that the expression of PLC-γ1 is also highly enhanced in PEMF treated neuronal regeneration after axonal injury, implying a possible role of PLC-γ1 in neuronal regeneration. However, the degree of expression is not well correlated with neuronal functional recovery.

Taken together, the results of these studies suggest that pulsed electromagnetic field can enhance the reparative process of injured nerves and achieve physiologic recovery of paralyzed nerve accompanying with the enhanced expression of nNOS and PLC-γ1. Further studies are required to delineate the exact role of nNOS in early stage of nerve regeneration and PLC-γ1 in PEMF-induced nerve regeneration.

## References

Clejan S, Ide C, Walker C, Wolf E, Corb M, Beckman B. Electromagnetic field induced changes in lipid second messengers. J Lipid Mediat Cell Signal 1996;13:301-24

Cordeiro PG, Seckel BR, Miller CD, Gross PT, Wise RE. Effect of a high intensity static magnetic field on sciatic nerve regeneration in the rat. Plast Reconstr Surg 1989;83:301-8

Dibirdik I, Kristupatis D, Kurosaki I, Tuel AL, Chu A, Pond D, Tuong D, Luben R, Uckun F. Stimulation of Src family protein tyrosine kinase as a proximal and mandatory step for SYK kinase dependent phospholipase C gamma3 activation in lymphoma B cells exposed to low energy electomagenetic fields. J Biol Chem 1988;13:4035-39

Firth JD, Putnins EE, Larjava H, Uitto VJ. Exogenous phospholipase C stimulates epithelial cell migration and integrin expresson *in vitro*. Wound Repair 2001;9:86-94

Gonsalez HT, Rustioi A. Expression of three forms of nitric oxide synthase in peripheral nerve regeneration. J Neurosci Res 1999;15:98-207

Gonsalez MF, Sharp FR, Sagar SM. Axonotomy increase NADPH diaphorase staining in rat vagal motor neurons. Brain Reses Bull 1989;18:417-27

Ilker D, Daiva K, Tomohiro K, Lisa TA, Alice C, David P, Dong T, Richard L, Faith MU. Stimulation of Src familly protein tyrosine kinase as a proximal and mandatory step for SYK kinase dependent phospholipase C-γ2 activation in lymphoma B cells exposed to low energy electormagnetic fields. J Biol Chem 1998;13:4035-39

Janet LW. Enhancement of functional recovery following a crush lesion to the rat sciatic nerve by exposure to pulsed electormagnetic fields. Exp Neurol 1994;125:302-5

John MB, Keith FC, Glen CT. Effect of pulsed electormagnetic stimulation on facial nerve regeneration. Arch Otolaryngol Head Neck Surg 1998;124:383-89

Kim SS, Jun K, Jeong M, Ryu SH, Suh PG, Shin HS. Immunohistochemical localization of eight phospholipase C isozymes in pancreatic islets of the mouse. Exp Mol Med 2001;30:164-68

Lee YH, Bae SS, Seo JK, Choi I, Ryu SH, Suh PG. Interleukin-6 induced tyrosine phosphorylation of phopholipase C-gamma 1 in PC cells. Mol Cells 2000;31:469-74

Paul WF, Daniel HD, Marc DC. Laryngeal synkinesis following reinnervation in the rat. Ann Otol Rhinol Laryngol 1991;100: 797-806

Rusovan A, Kanje M.Stimulation of regeneration of the rat scitic nerve by 50 Hz sinusoidal magnetic field. Exp Neurol 1991;112:312-16

Saika T, Kiyama H, Matsunaga T, Tohyama M. Differential regulation of phospholipase C isoenzyme in the rat facial nucleus following axonotomy. Neuroscience 1994;59:121-29

Sanda C, Charles I, Cedric W, Erich W, Michael C, Barbara B. Electromagnetic field induced changes in lipid second messenger. Lipid Mediator 1996;13:301-24

Tompson GC, Ross CD, Thomson AM, Byers JM. Changes in brainstem calcitonin gene related peptides and the cranial nerve lesion in guinea pig. Brain Res 1995;12:140-48

Wu W. Expression of nitric oxide synthase in injured CNS neurons as shown by NAPDH diaphorase histochemistry. Exp Neurol 1993;120:153-59

Wu W. Inhibition of nitric oxide synthase reduces motoneuron death due to spinal root avulsion. Neurosci Lett 1993;30:121-24

Yu WH. Regulation of nitric oxide synthase expression in motoneurons following nerve injury. Dev Neurosci 1997;19: 247-54