Magnesium supplement promotes sciatic nerve regeneration and down-regulates inflammatory response

Hung-Chuan Pan¹,₆, Meei-Ling Sheu³, Hong-Lin Su⁴, Ying-Ju Chen², Chun-Jung Chen², Dar-Yu Yang⁵, Wen-Ta Chiu⁶, Fu-Chou Cheng²

¹ Department of Neurosurgery; ² Stem Cell Center, Department of Medical Research, Taichung Veterans General Hospital, Taichung; ³ Institute of Biomedical Sciences; ⁴ Institute of Life Sciences, National Chung-Hsing University, Taichung; ⁵ Department of Neurosurgery, Chang Bing Show Chwan Memorial Hospital, Changhua; ⁶ Department of Neurosurgery, Taipei Medical University, Shuang Ho Hospital, Taipei, Taiwan

Abstract. Magnesium (Mg) supplements have been shown to significantly improve functional recovery in various neurological disorders. The essential benefits of Mg supplementation in peripheral nerve disorders have not been elucidated yet. The effect and mechanism of Mg supplementation on a sciatic nerve crush injury model was investigated. Sciatic nerve injury was induced in mice by crushing the left sciatic nerve. Mice were randomly divided into three groups with low-, basal- or high-Mg diets (corresponding to 10, 100 or 200% Mg of the basal diet). Neurobehavioral, electrophysiological and regeneration marker studies were conducted to explore nerve regeneration. First, a high Mg diet significantly increased plasma and nerve tissue Mg concentrations. In addition, Mg supplementation improved neurobehavioral, electrophysiological functions, enhanced regeneration marker, and reduced deposits of inflammatory cells as well as expression of inflammatory cytokines. Furthermore, reduced Schwann cell apoptosis was in line with the significant expression of bcl-2, bcl-XL and down-regulated expression of active caspase-3 and cytochrome C. In summary, improved neurological function recovery and enhanced nerve regeneration were found in mice with a sciatic nerve injury that were fed a high- Mg diet, and Schwann cells may have been rescued from apoptosis by the suppression of inflammatory responses.

Key words: cytokines, inflammation, magnesium, nerve regeneration, sciatic nerve injury

Neural trauma has been shown to result in endoneurial production of cytokines and their mRNA, including interleukin (IL)-1β, tumor necrosing factor (TNF)-α, IL-6, and Interferon (INF)-γ [1-4]. The production of TNF-α and IL-1 following nerve injury is a critical determinant of later sequelae of injury and inflammation in peripheral nerves, particularly because of the key roles of these cytokines in Wallerian degeneration (WD) [1, 2, 5, 6]. TNF-α and IL-1 are of particular significance to WD. First, they can initiate the cytokine networks of WD as they do in other networks of inflammation. Second, they contribute to macrophage recruitment to inflammatory sites through endothelial cell activation and chemokine production as well as myelin removal [7].
Furthermore, they can indirectly regulate survival and growth of peripheral nervous system neurons and neuropathic pain through the regulation of nerve growth factor (NGF) production [8, 9].

Nerve tissue injury in general leads to a local inflammatory response that is regulated by numerous signal molecules, including cytokines. Their production and local effects may also affect the outcome of nerve trauma. Cytokines act in a network-like fashion and are crucial in nerve regeneration. However, TNF-α has been previously described as a potent cytotoxic molecule during active phases of demyelination in various forms of peripheral neuropathy [10]. In addition, exogenous administration of TNF-α increased Schwann cell apoptosis and viability in axotomized mice [11, 12]. In our previous investigation, we found that attenuation of TNF-α in a sciatic nerve crush injury model prevented Schwann cell apoptosis and further escalated nerve myelination [3]. Furthermore, the partly abolished expression of inflammatory cytokines such as TNF-α, IL-1β, and INF-γ contributed to a decrease in Schwann cell apoptosis with a subsequent increase in nerve regeneration [4, 13]. Thus, the intensity of responsive cytokine expression in peripheral nerve injury modulated WD, attenuated nerve regeneration, and played a cytotoxic role in orchestrating Schwann cell apoptosis. The optimal elicitation of the appropriate expression of cytokines should be finely tuned to allow for removal of myelin debris without exacerbation of the property that is cytotoxic to Schwann cells.

It is known that magnesium (Mg) is crucial for cellular bioenergetics, DNA synthesis, RNA aggregation, protein synthesis, the functioning of ATPase, and plasma membrane integrity. Mg treatment blocks the mitochondrial permeability transition pore, calpain activation, lipid peroxidation, and the production of oxygen-free radicals [14]. The first observation of clinical symptoms of inflammation in Mg-deficient rats was published in the 1930s [15]. Significant elevation of plasma levels of several cytokines and substance P was observed in rats after 3 weeks of Mg deficiency [16]. Furthermore, a rise in plasma concentration of IL-6 was also found in rats that were fed an Mg-deficient diet [17]. The stress-induced overproduction of TNF-α appears as early as 2 days after initiation of Mg-deficient diets [18]. In addition, Mg deficiency upregulates IL-1α and IL-6, and pleiotropic cytokines have been implicated in acute phase response and inflammation [19].

Experimental Mg deficiency in the rat has been shown to induce a clinical inflammatory syndrome characterized by leukocyte and macrophage activation, release of inflammatory cytokines and acute phase proteins, and excessive production of free radicals. The priming of phagocytic cells, the opening of the calcium channel, the activation of N-methyl-D-aspartate (NMDA) receptors, and the activation of nuclear factor-kappa B (NFκB) have been considered as potential mechanisms [20].

Mg deficiency contributes to an exaggerated response to immune stress and oxidative stress as a consequence of the inflammatory response. Inflammation contributes to the pre-atherosclerotic changes in lipoprotein metabolism, endothelial dysfunction, thrombosis, hypertension and the development of metabolic syndromes [20]. Although suppression of the inflammatory response has been shown to rescue Schwann cells from apoptosis to promote nerve regeneration [3, 4, 13], there are insufficient data to support the hypothesis that down-regulation of the inflammatory response by the administration of Mg is highly correlated with a protective effect on peripheral nerve injury. To date, except for a few reports that address the administration of Mg supplements to prevent motor neuron death in neonatal sciatic nerve injury [21], there are scant data concerning the effects of either Mg supplements or deficiency on functional recovery in adult sciatic nerve injury, especially in regard to the relationship of inflammatory response and nerve regeneration. In this study, three groups of animals were given diets supplemented with basal-, low- or high-dose Mg and actual concentrations of Mg in plasma and various tissues were then determined. Subsequently, these three groups of animals were subjected to sciatic nerve crush injury and then given the same respective Mg-supplemented diets (to determine the relationship of nerve regeneration with inflammatory response). Furthermore, the molecular mechanism contributing to these effects was also investigated.

Material and methods

Crush models

Imprinting control region (ICR) mice weighing 30-40 g were used in this study. Permission was obtained from the Ethics Committee of Taichung
Veterans General Hospital for their use. Before the animals were subjected to the crush injury, they were divided into three groups and fed a basal diet (5755 TestDiet containing 0.7 mg/g Mg, Richmond, IN, USA), a low-Mg diet (containing <0.08 mg/g Mg, 5865 TestDiet), or a high-Mg diet (5755 TestDiet containing 0.7 mg/g Mg and supplemented by MgCl₂ -0.5 mg/mL in water) for 3 weeks before experiments and 4 weeks after sciatic nerve injury. The mice were anesthetized with 2% isoflurane during induction followed by a maintenance dose (0.5-1%). The left sciatic nerve was exposed under a microscope using the gluteal muscle splitting method. A vessel clamp (B-3, pressure 1.5 gm/ mm², S&T Marketing, Ltd., Switzerland) was applied 10 mm from the internal obturator canal for 20 minutes, as previously described [22]. The crush site was sutured with 9-0 nylon over the epineuria as the mark. After injury, the animals were fed the same respective diets for another 4 weeks. For determination of the Mg concentrations in plasma and various tissues, six animals in each group were used. To assess the Mg concentration in plasma after crush injury, blood was withdrawn from six animals per group at one-week intervals for four weeks. Neurobehavior, electrophysiology, and histology were assessed in six animals in each group. At three different time points, six animals per group (a total of 18 animals in each group) were used for determination of inflammatory cell infiltration and associated gene expression. Six animals per group were used for determination of inflammatory cytokine levels and blood was withdrawn for determination of activity of macrophages and neutrophils. At three different time points, six animals per group (a total of 18 animals in each group) were used for TUNEL assay. At four different time points, six animals per group (a total of 24 animals in each group) were used for analysis of anti-apoptotic associated gene expression. Various tissues (sciatic nerve, spinal cord, brain, muscle and small intestine) were harvested and Mg concentrations were determined four weeks after injury. Mg concentrations in blood and tissues were determined using a biochemical analyzer (TBA-200 FR, Toshiba Medical Products, Tokyo, Japan).

**Analysis of functional recovery**

A technical assistant who was blinded to treatment allocation evaluated sciatic nerve function 4 weeks after the surgery. The evaluation method included ankle kinematics [23] and sciatic function index (SFI) [24]. In the sagittal plane analysis, the following formula was used in the mechanical analysis of the rat ankle: \( \theta \) ankle=\( \theta \) foot-\( \theta \) leg 90. Several measurements were taken from the footprint: (i) distance from the heel to the third toe, the print length (PL); (ii) distance from the first to the fifth toe, the toe spread (TS); and (iii) distance from the second to the fourth toe, the intermediary toe spread (ITS). All three measurements were taken from the experimental (E) and normal (N) sides. The SFI was calculated according to the equation:

\[
SFI=38.3(EPL-NPL/NPL)+109.5(ETS-NTS/NTS)+13.3(EIT-NIT/NIT)-8.8
\]

The SFI oscillates around 0 for normal nerve function, whereas SFI around -100 represents total dysfunction [24].

**Electrophysiological study**

Sciatic nerves from individual groups were exposed 4 weeks after operation, and electrical stimulation was applied to the proximal side of the injured site; the evoked compound muscle action potential (CMAP) amplitudes and conduction latency were recorded in the gastrocnemius with an active monopolar needle electrode 10 mm below the tibia tubercle and with a reference needle 20 mm from the active electrode. The stimulation intensity and filtration ranges were 2.5 mA and 20-2,000 Hz, respectively. A similar assessment was performed on the non-injured side. The CMAP data and conduction latency were converted to the ratio of the injured side divided by the normal side to adjust for the effect of anesthesia [22].

**Western blot**

The nerve tissue (10 mm) (9-0 nylon marker in the middle of the nerve) was harvested after treatment at different time points and proteins were extracted. Proteins (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a blotting membrane. After blocking with non-fat milk, the membranes were incubated with antibodies against bcl-2 (anti-mouse; 1:1,000 dilution; BD Biosciences, Franklin Lakes, NJ, USA), bcl-X₇ (anti-rabbit; 1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), active
Magnesium promotes nerve regeneration and regulates inflammation

caspase-3 (anti-mouse; 1:1,000 dilution; Abcam, Cambridge, MA, USA), cytochrome C (anti-rabbit; 1:1,000 dilution; Santa Cruz Biotechnology), Bad (anti-mouse; 1:1,000 dilution; Santa Cruz Biotechnology), Bax (anti-rabbit; 1:1,000 dilution; Santa Cruz Biotechnology), and β tubulin (anti-mouse; 1:10,000 dilution; Santa Cruz Biotechnology) overnight at 4°C. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody and developed using ECL Western blotting reagents. The intensity of protein bands was determined using a computer image analysis system (IS1000, Alpha Innotech Corporation, Santa Clara, CA, USA).

Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)
The isolation of RNA, synthesis of cDNA, and PCR were carried out as previously described [4]. DNA fragments of specific genes and internal controls were co-amplified in a single one-step RT-PCR set-up. The PCR reaction was carried out under the following condition: one cycle of 94°C for 3 min, 28 cycles of (94°C for 50 s, 58°C for 40 s, and 72°C for 45 s), and then 72°C for 5 min. The amplified DNA fragments were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The intensity of each signal was determined using a computer image analysis system (IS1000, Alpha Innotech Corporation). The primer sets used in the study were: 5'-CACCGTCATCTCGTGTGC and 5'-CAGTCCTGTCAGCTCTC for RANTES; 5'-CAGTCCTGCATCGCTTC and 5'-AGTATTCTAG GAAGGGAATAG for MCP-1; and 5'-TCTGGTGG CATCCATGAACT and 5'-GGGAGGATATGCTC for β-actin (as a reference gene). β-actin control was analyzed per plate of experimental gene to avoid plate-to-plate variation. Final RT-PCR data are expressed as the ratio of copy numbers of experimental gene per 10³ or 10⁴ copies of β-actin for samples performed in duplicates.

Quantification of pro-inflammatory cytokines
Six nerve tissues in each group for each single parameter were removed 7 days after sciatic injury. These tissues (10 mm in length) were retrieved and stored at -80°C. Subsequently, each tissue sample was homogenized with Laemmli SDS buffer. The homogenate was centrifuged for 10 minutes at 12,000 g at 4°C. The tissue homogenate, 100 μL in triplicate, was applied to a microtiter plate and allowed to adhere overnight at 4°C. The microtiter plates were washed with phosphate-buffered saline (PBS)-Tween-20 and blocked with 1% BSA in PBS (200 μL) for 1 h. The plates were then treated with respective primary antibodies and allowed to incubate for 6 hours at 37°C. One hundred μL of the respective polyclonal antibodies against TNF-α (anti-goat; 1:100 dilution; R&D Systems, Inc., Minneapolis, MN, USA), IL-1β (anti-goat; 1:200 dilution; R&D Systems, Inc.), IL-6 (anti-goat; 1:300 dilution, R&D Systems, Inc.) and INF-γ (anti-goat; 1:150 dilution, Chemicon, Inc., Billerica, MA, USA) were applied overnight to microtiter plates. After further washing in PBS-Tween-20, the plates were incubated with the respective second antibody conjugated to alkaline phosphate (100 μL) for 1 h. The reaction was developed using p-nitrophenyl phosphate, disodium (3 mM) in carbonate buffer, pH 9.6 [100 mM Na₂CO₃ and 5 mM MgCl₂ (150 μL)] and the reaction was terminated after 30 minutes using 0.5 N NaOH (50 μL). The absorbance of colored product was read at 450 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The relative amount of antigen present was measured from the densitometric reading against a standard curve.

Neutrophil and monocyte/macrophage isolation
Blood was collected from the abdominal aorta of pentobarbital-anesthetized mice 7 days after operation. Neutrophils and monocytes/macrophages were purified by dextran sedimentation, centrifugation through Ficoll-Hypaque, and hypotonic lysis of erythrocytes. Cell migration was evaluated with a modified 24-well Transwell, as previously described [25]. Cells (1 × 10⁶) were added to the upper well of the chamber. The lower well received RPMI containing 10% fetal bovine serum. The upper and lower wells were separated by 3-μm pore size polycarbonate filters, and the chamber was incubated for 1 h at 37°C. Migrating cells attached to the lower surfaces were fixed in ethanol for 10 min and stained with crystal violet. Labeled cells were counted microscopically at 400 x magnification within a total
area of 2 mm². For IL-1β analysis, the obtained monocytes/macrophages were incubated with RPMI alone or stimulated with lipopolysaccharide (LPS) (10 ng/mL)/interferon-gamma (IFN-γ) for 24 h. The supernatants were collected and subjected to ELISA for the measurement of IL-1β.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) assay

The nerve tissue (10 mm) obtained after crush injury was embedded, cut longitudinally into 8 μm-thick sections and subjected to TUNEL assay (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions 3, 7, and 14 days after injury. Apoptotic cells were defined as those cells that were TUNEL and S-100 positive. Among longitudinal consecutive resections, five consecutive resections contiguous to a maximum diameter were chosen to be measured. Of 100 squares with a surface area of 0.01 mm² each, 20 squares were randomly selected in an ocular grid and used to count the number of cells. The number of apoptotic transplanted cells was expressed as a percentage of the total number of nuclei counted, with at least 25,000 nuclei for each count [26].

Immunohistochemistry study

Serial 8 μm-thick sections of sciatic nerve were cut on a cryostat, mounted on superfrost/plus slides (Menzel-Glaser, Braunschweig, Germany) and subjected to immunohistochemistry with antibodies against CD68, CD 34 (Chemicon, 1:200 dilution), CD 8, CD3 (Serotec, Dusseldorf, Germany; 1:200 dilution), CD19 (Thermo, Waltham, MA, USA; 1:200 dilution), neutrophil (Abcam, 1:200 dilution), neurofilament (Chemicon, 1:300 dilution), and S-100 for detection of inflammatory cells and nerve fibers, respectively. The immunoreactive signals were observed using goat anti-mouse IgG (FITC) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; 1:200 dilution), anti-mouse IgG (Rhodamine) (Jackson, 1:200 dilution), or 3, 3’-diaminobenzidine brown color. Among longitudinal consecutive resections, five consecutive resections contiguous to a maximum diameter were chosen to be measured. Of 100 squares with a surface area of 0.01 mm² each, 20 squares were randomly selected in an ocular grid and used to count the number of cells. For the determination of neurofilament, six nerves in each group were cut longitudinally into 8 μm-thick sections, and stained with each antibody. The region of maximum diameter of the resected nerve tissue with crush mark was chosen to be examined. Areas of activities (0.2 mm²) appeared dense against background and were measured using a computer image analysis system (Alpha Innotech Corporation, IS1000).

Histological examination

After neurobehavioral and electrophysiological testing, six mice in each group underwent transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) after being re-anesthetized. The bilateral gastrocnemius muscle from the bones was sent for measurement of muscle weight. The nerve was embedded, cut longitudinally into sections 8 μm thick and stained with haematoxylin-eosin (H&E) for the measurement of vacuole number and S-100 for the determination of myelination. The methods for determining the numbers of vacuoles and density against S-100 have been described previously [26]. The left sciatic nerve was harvested from the animals after electrophysiological testing and the nerve tissue was fixed on a plastic plate using stay suturing to keep the nerve straight [27].

Statistical analysis

Data are expressed as mean±SE (standard error). The statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett’s test. For SFI, angle of ankle, and CatWalk analysis, the results were analyzed by repeated-measurement of ANOVA followed by Bonferroni’s multiple comparison method. A p value of less than 0.05 was considered significant.

Results

Alteration of Mg concentrations in plasma and various tissues

Approximately, each ICR mouse consumed 3 g/day and 5 mL/day water during this study.
Magnesium promotes nerve regeneration and regulates inflammation

The estimated Mg daily dietary intakes of low-Mg (ca. 0.24 mg/day), basal (ca. 2.1 mg/day), and high-Mg (ca. 4.6 mg/day) diets of mice are shown in table 1. Significant elevation of plasma Mg concentrations up to 1.51 mM were observed in mice which received high Mg supplementation for 3 weeks. Depletion of Mg in the low Mg diet caused low plasma Mg concentrations down to 0.48 mM when compared to the control (0.92 mM). This trend toward escalation or decrease in plasma Mg concentrations was persistent after crush injury (figure 1A). Mg concentrations in nerve tissues were highly correlated with various Mg diets. In other tissues such as spinal cord, brain, muscle, and intestine, the same trend was also demonstrated (figure 1B).

### Neurobehavior study

High-dose Mg supplementation induced a significant improvement in SFI compared with findings obtained in the basal diet group (p<0.001), and low-dose Mg supplementation further exacerbated SFI (p<0.001) (figure 2A). The angle of ankle also showed a similar trend (figure 2B). The ratios of CMAP (Lt/Rt) in the basal, low-dose Mg, and high-dose Mg groups were 22±1.8%, 15±2.3%, and 49±3.2%, respectively (p<0.001). The ratios of conduction latency in the basal, low-dose Mg, and high-dose Mg groups were 3.05±0.19, 3.3±0.15, and 1.71±0.1, respectively (p<0.001). The ratios of muscle weight in the basal, low-dose Mg, and high-dose Mg groups were 49±1.4%, 40±0.11%, and 66±1.2%, respectively (p<0.001). Hence, the parameters of CMAP, conduction latency, and muscle weight were decreased by low-dose Mg supplementation and then restored and augmented by a high-dose of Mg (figure 2C).

### Early and late nerve regeneration augmented by Mg supplementation

High-dose Mg supplementation resulted in significantly greater enhancement of neurofilament expression (from 441.5±34.6 to 1115±24.8 relative density/mm²) (p<0.001), whereas low-dose Mg supplementation abrogated the expression to 211.3±12.1 relative density/mm² (p<0.001) (figure 3A, B). The parameters of late nerve regeneration were represented as the intensity of myelination as evidenced by the increased expression of S-100 (from 561±28.9 to 903±30.4 relative density/mm²; p<0.001) after high-dose Mg supplementation and decreased expression after low-dose Mg supplementation (358±19.1 relative density/mm²) (p<0.001) (figure 3A, B).

### Inflammatory response after Mg supplementation

The immunohistochemical results in the basal-dose Mg group showed an accumulation of inflammatory cells starting at 3 days (28.3±0.8/0.05mm²), reaching a plateau at 7 days (32.3±1.1/0.05mm²) (p<0.001) and declining at 14 days (19.2±0.6/0.05mm²) (p<0.001). The administration of high-dose Mg supplements suppressed the macrophage deposits (12±1/0.05mm², 15±1.1/0.05mm², and 11±1.5/0.05mm²) at 3, 7, and 14 days, respectively (p<0.001). In contrast, the administration of low-dose Mg supplements stimulated the macrophage deposition (36.2±0.94/0.05mm², 47.8±2.1/0.05mm², and 25.2±1.1/0.05mm²) at 3, 7, and 14 days, respectively (p<0.001) (figure 4A, B). Significant induction of MCP-1 and RANTES was observed in groups treated with low-dose Mg supplementation. Furthermore, the alteration of MCP-1 and RANTES was highly associated with deposits of macrophages (figure 4C).

### Table 1. Estimated total Mg intakes (mg/day) based on Mg contents in the diets and water in the three studied groups.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Food intake (g/day)</th>
<th>Mg in food (according to TestDiet) (mg/day)</th>
<th>Water intake (mL/day)</th>
<th>Mg in water (mg/mL)</th>
<th>Estimated total Mg intake (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Mg diet</td>
<td>~3</td>
<td>0.24</td>
<td>~5</td>
<td>0</td>
<td>0.24</td>
</tr>
<tr>
<td>Basal Mg diet</td>
<td>~3</td>
<td>2.1</td>
<td>~5</td>
<td>0</td>
<td>2.1</td>
</tr>
<tr>
<td>High Mg diet</td>
<td>~3</td>
<td>2.1</td>
<td>~5</td>
<td>2.5*</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* High Mg diet was supplemented by MgCl₂ in water (0.5 mg/mL).
Figure 1. Plasma and tissue concentrations of Mg altered by various Mg diets.

A) Concentrations of plasma Mg at different time intervals.
B) Mg concentrations in various tissues at 4 weeks after sciatic injury.

N=6; ** p < 0.01; *** p < 0.001 (relative to basal Mg diet).

The elevated production of IL-1β, IL-6, TNF-α and IFN-γ was decreased in the high-dose Mg groups and further enhanced in the low-dose Mg groups (figure 5A). To further explore the inflammatory response in systematic circulation following treatment with various doses of Mg, neutrophils and monocytes/macrophages were isolated from circulating blood 7 days after crush injury. Monocytes/macrophages obtained from injured animals treated with basal-dose Mg supplements showed more elevated cell migration than those in the sham group. Up-regulated cell migration was enhanced by low-dose Mg supplements and inhibited by high-dose Mg supplements. In contrast, neutrophils showed similar levels of cell migration, but the level
Magnesium promotes nerve regeneration and regulates inflammation

Figure 2. Illustration of neurobehavioral and electrophysiological studies. 
A) Representative illustration of SFI and (B) angles of ankle at different time points for the three studied Mg diets. C) Quantitative analysis of ratio (Lt/Rt) of muscle weight, CMAP, and conduction latency.
Lt: left; Rt: right; SFI: sciatic nerve function index; MW: muscle weight; CMAP: compound muscle action potential.
N=6; * p <0.05; ** p <0.01; *** p <0.001 (relative to basal Mg diet).

of expression was much weaker than that in macrophages (figure 5B). Monocytes/macrophages obtained from injured animals spontaneously released higher levels of IL-1β than the sham-operated group. A high dose of Mg inhibited IL-1β release and a low dose enhanced the release (figure 5C). After stimulation with LPS/IFN-γ, the four groups of monocytes/macrophages produced elevated levels of IL-1β. The trend toward IL-1β release after stimulation was similar to that in groups without stimulation (figure 5D).

Cells rescued from apoptosis in crushed nerve

Following nerve crush injury, a basal dose of Mg administration induced Schwann cell apoptosis of 0.37±0.031% at 3 days, which reached a plateau of 7.3±1.4% at 7 days (p<0.001) and declined to 4.4±0.3% at 14 days (p<0.001). High-dose Mg supplementation attenuated the apoptosis to 0.1±0.01%, 3.01±0.27% (p<0.001), and 1.4±0.11% (p<0.001) at 3, 7, and 14 days, respectively. Low-dose Mg supplementation
augmented the cell apoptosis to 6.6±0.24% (p<0.001), 11.6±0.5% (p<0.001), and 5.9±0.24% (p<0.001) at 3, 7, and 14 days, respectively (figure 6A, B). In Western blotting analysis, Mg depletion significantly enhanced Schwann cell apoptosis by decreasing Bcl-2 and Bcl-XL expression levels, and subsequently increased active caspase-3 and cytochrome C compared with those of the control group. However, supplementation of Mg also enhanced the expression of bcl-2 and bcl-XL, and decreased active caspase-3 and cytochrome C when compared with values obtained in controls (figure 7A, B).

Discussion

Various doses of Mg in the diet profoundly influenced the alteration of Mg concentration in plasma and tissues. In addition, Mg deficiency markedly enhanced the inflammatory response, and Mg supplementation counteracted the inflammatory response. Suppression of the inflammatory response was highly associated with improvement of neurological function, including neurobehavior and nerve myelination, whereas augmentation of the inflammatory response was highly associated with deterioration of neurological function.
Magnesium promotes nerve regeneration and regulates inflammation

Figure 4. Determination of inflammatory cell and associated cytokine levels. The nerve tissues were retrieved at 3, 7, and 14 days after sciatic injury and were subjected to immunohistochemistry studies with antibodies against CD68 and RT-PRC for determination of MCP-1 and RANTES at 7 days after treatment with three Mg diets. A) Deposits of macrophages at different time points. B) Quantitative analysis of inflammatory cells. C) Quantitative analysis of MCP-1 and RANTES. * p < 0.05; ** p < 0.01; *** p < 0.001 (relative to basal Mg diet). N=6. Bar length=100 μm.

function. The inflammatory response in the injured nerve paralleled the increased cell apoptosis and immune cell deposits. The phenomenon of reduced cell apoptosis was in line with increased expression of bcl-2 and bcl-X_L and was found to be inversely correlated with expression of active caspase-3 and cytochrome C. Thus, either high Mg diet before and/or continuous supplementation of Mg after sciatic injury may rescue cells from apoptosis by suppressing the inflammatory responses. The anti-inflammatory effect was consistent with the significant improvement in neurological function.

The alteration of plasma Mg in mice given various Mg diets was similar to those found in previous reports [28-30]. In addition, our data indicate that these diets significantly influenced the concentrations of Mg in plasma and tissues either 3 weeks before or 4 weeks after sciatic nerve injury. However, it is difficult to compare these plasma and tissue Mg concentrations among various species of C57/BL6, ICR and other strains of mice. In the rat model, SD rats are suitable for use in sciatic nerve injury models. In the present study, we initially used C57/BL6 mice in our sciatic nerve injury model as they are frequently used in the
Figure 5. Determination of inflammatory response in tissues and plasma. The left sciatic nerve of rats was injured by crushing. Seven days after injury, (A) the injured nerves (10 mm) were retrieved for the determination of TNF-α, IL-1β, IL-6, and IFN-γ levels. B) Blood was withdrawn for assessment of the migratory ability of neutrophils and monocytes/macrophages. (C, D) Determination of IL-1β secretion in monocytes/macrophages either stimulated with or without LPS. N=6; * p < 0.05; ** p < 0.01; *** p < 0.001 (relative to basal Mg diet).

study of Mg deficiency in animals. However, after sciatic nerve injury, many of the mice (about 60-80%), which were kept in the same cage, were observed biting other mice. Therefore, the experiments were repeated using a less temperamental strain of mice (ICR).

Increased nerve regeneration was accompanied by an improvement in sciatic nerve function index, increased compound muscle action potential, reduced nerve conduction latency, and increased muscle weight [4]. Based on these data, the administration of high Mg supplementation improved the neurobehavior of rats, and the low Mg diet further aggravated the neurological dysfunction.

Axon degeneration took place dramatically 3 to 7 days after nerve crush injury. Evidence indicates that increased expression of neurofilament reflects early regenerative potential [31]. Nerve regeneration is also related to Schwann cell proliferation in the distal end of nerves as indicated by increased expression of S-100 [27]. Based on the early expression of neurofilament and late myelination marker, treatment with a high Mg diet may promote greater nerve regeneration, whereas Mg depletion exacerbates neurological dysfunction. In addition, increased myelination was positively correlated to the integrity of nerve tissue and this reflected the strength of nerve regeneration in the later phase [22]. In this study, relative densities of NF and S-100 increased 168% and 39%, respectively, in mice consuming high Mg diets when compared with those found in the basal group. However, relative densities of NF and S-100 decreased by 51% and 36%, respectively, in mice
Magnesium promotes nerve regeneration and regulates inflammation

Figure 6. Determination of apoptosis levels in crushed nerve. Crushed nerve tissues were retrieved 7 days after injury and were subjected to apoptotic assay by TUNEL.

A) TUNEL positive cells after treatment with three Mg doses at different time points.

B) Quantitative analysis of TUNEL positive cells after treatment with three Mg doses at different time points.

N=6; * p<0.05; ** p<0.01; *** p<0.001 (relative to basal Mg dose).
Bar length = 50 μm. The vertical axis represents the percentage of positive TUNEL assays.

An over-activated inflammatory response is a detrimental stress on the nerve tissues and is a potential cytotoxic factor in regulation of nerve regeneration. Macrophages play a central role in the pathogenic response in peripheral nerves, and the expression of inflammatory cytokines...
Figure 7. Western blot analysis of the retrieved nerve in animals treated with various Mg doses. A) Illustrative examples of Western blot analysis for bcl-2, bcl-X\textsubscript{L}, active caspase-3 and cytochrome C at different time intervals. B) Graph shows the quantitative results of Western blot analysis with respect to \(\beta\)tubulin. 

N=6; ** \(p<0.01\); *** \(p<0.001\) (relative to basal Mg diet).

Parallels the number of macrophage deposits [4]. Clearance of macrophage deposits and inhibition of inflammatory cytokines augmented the regeneration in peripheral nerve crush injury. Experimental Mg deficiency is known to have a profound effect on the process of inflammation [32, 33]. The underlying mechanisms of inflammatory response induced by Mg deficiency have not been clearly elucidated. Several triggering events highlight possible mechanisms, including (i) cellular entry of calcium and priming of phagocytic cells; (ii) opening of calcium channels and activation of NMDA receptors; (iii) release of neurotransmitters such as substance P; and (iv) membrane oxidation and activation of nuclear factor kappa B (NF\(\kappa\)B). Furthermore, Mg depletion caused the elevation of inflammatory cytokines in nerve tissue, which was abrogated by Mg supplements. The alterations of inflammatory cells distributed in nerve tissue paralleled the alterations in the expression of inflammatory cytokines, and these events were correlated with the administration of various doses of Mg. These results were consistent with those of our previous report that showed Mg modulated the regulation of Ca\(^{++}\), the priming of inflammatory cells and possibly the release of inflammatory cytokines. Monocyte chemoattractant protein 1 (MCP-1) and RANTES are the important regulators of macrophage response that leads to rapid myelin breakdown and clearance in Wallerian degeneration. Inhibition of MCP-1 and RANTES suppresses macrophage deposits and myelin clearance [4]. The expression of MCP-1 and RANTES was down-regulated in those groups treated with high-dose Mg supplementation based on one reference gene (\(\beta\)-actin). These findings indicate that a high dose of Mg possesses an anti-inflammatory effect and depletion of Mg exacerbates the inflammatory response in the injured nerve.

The efficacy of stimulation-induced IL-1\(\beta\) release was attenuated by a high dose of Mg and augmented by a low dose of Mg, indicating a high Mg dosage rendered monocytes/macrophages insensitive to stimulation of IL-1\(\beta\) release.

The number of resident peritoneal macrophages was greater in Mg-deficient rats and these cells presented the morphological characteristics of
activated cells. The recruitment of phagocytic cells and their activity are enhanced in Mg-deficient animals [17]. In addition, a significant elevation of pro-inflammatory cytokines, such as TNF-α, IL-1, II-6 and INF-γ, has been reported in Mg-deficient animals [16-19]. In this study, a significant increase in deposits of macrophages on the injured nerve in Mg-deficient rats and an alleviation of such deposits were found in animals treated with a high dose of Mg. The distribution of macrophage deposits was highly correlated with the expression of macrophage migration genes such as MCP-1 and RANTES, and this result was consistent with that of our previous report [4]. Furthermore, the measurement of pro-inflammatory cytokines retrieved from the crushed nerve after each treatment also revealed the same trends. Hence, the intensities of macrophage deposits and their associated inflammatory cytokines were highly correlated with the concentration of Mg in the nerve tissue. These results further confirmed the hypothesis that Mg depletion augments the inflammatory response and Mg supplementation abrogates it.

Sciatic nerve crush injury produced an immediate spike in macrophage invasion. Macrophage recruitment was indicated by a statistically significant rise in cell numbers as early as 24 hours after treatment, which reached a peak at 7 days and declined to normal levels within 21 days [34]. Other studies have reported macrophage recruitment that began at 24 hours after axon injury and peaked at 14-21 days [35]. The macrophages in degenerating peripheral nerves are mainly of haematogenous origin [36, 37]. In this study, crush injury triggered macrophages to initiate an increase at 3 days, followed by a plateau at around 7-14 days and a decline to normal values at around 21 days. Mg depletion augmented the macrophage deposits and Mg supplements alleviated the accumulation of macrophages. The intensity of the distribution of macrophages in the crushed nerve was consistent with the macrophage migratory ability in the blood. Thus, the modulation of macrophage phagocytic ability by either Mg depletion or supplement significantly contributed to the distribution of macrophages in the crushed nerve. The up- or down-regulation of macrophage activity in the crushed nerve was responsible for the subsequent expression of the inflammatory cytokines.

Macrophages are able to produce the pro-inflammatory cytokines TNF-α, IL-1, and IFN-γ and synthesize neurotrophic factors such as IL-6 and LIF [1, 38, 39]. In this study, upregulation of the inflammatory cytokines TNF-α, IL-1, IFN-γ, and IL6 was demonstrated in animals treated with a low dose of Mg, and suppression of these cytokines was observed in animals given a high dose of Mg. The expression of the inflammatory cytokines was in line with the amount of macrophage deposits in crushed nerve as well as the migratory ability of macrophages in the blood. Taken together, these results demonstrate that Mg supplementation or depletion primed the phagocytic ability in the blood and subsequently altered the deposits of macrophages in crushed nerve. The intensity of macrophage deposits was responsible for the expression of inflammatory cytokines in injured nerve, which in turn determined the potentiation of nerve regeneration.

Attenuation of Schwann cell apoptosis contributed to significant nerve regeneration. The ratio of cell apoptosis was highly correlated with expression of active caspase-3 and cytochrome C, which was attenuated by high-dose Mg supplementation and augmented by low-dose Mg supplements. Taken together, these findings imply that a high dose of Mg exerted an anti-apoptotic effect on Schwann cells through significant increases in the expression of bcl-2 and bcl-XL, which abolished the downstream expression of active caspase-3 and cytochrome C. Pro-inflammatory cytokines have been previously described as potent cytotoxic molecules during the active phase of demyelination in various forms of peripheral neuropathy [3, 4, 10]. TNF-α generation and secretion are integral processes in the series of disease-defining events that take place during Wallerian degeneration and also play a central role in regulating the cytokine network through this process [2]. Furthermore, administration of TNF-α to the sciatic nerves of neonatal axotomized mice has been shown to increase Schwann cell apoptosis in the distal segment of injured nerve [12] while depletion of TNF-α decreased Schwann cell apoptosis [3, 4]. Apoptosis involves activation of a cascade of proteolytic enzymes called caspases [40]. Members of the Bcl-2 protein family are key regulators of apoptosis and are categorized according to their ability to promote (e.g. Bak, Bax, Bik) or inhibit (e.g. Bcl-2, Bcl-XL, Bcl-w) apoptosis [41]. Expression of Bcl-2 and Bcl-XL also inhibits cytochrome
C release but may not interact with pro-caspase-9 and therefore reduces caspase-3 activation [42]. In this study, Mg depletion increased Schwann cell apoptosis by decreasing Bcl-2 and Bcl-\(\chi\)L expression levels and subsequently increasing active caspase-3 and cytochrome C. Administration of a high dose of Mg rescued the Schwann cells from apoptosis by upregulation of the expression of Bcl-2 and Bcl-\(\chi\)L. However, fractionation of cytochrome C needs to be clarified either from the cytosolic or mitochondria cytochrome C. This further confirmed that Mg supplementation exerted anti-apoptotic effects against Schwann cell apoptosis.

**Conclusion**

Mg depletion induced the release of inflammatory cytokines with a subsequent cascade of production of macrophage deposits which were detrimental to nerve regeneration. Mg supplementation suppressed the inflammatory response and rescued Schwann cells from apoptosis mainly through upregulation of the anti-apoptotic pathway of Bcl-2 and Bcl-\(\chi\)L expression. In conclusion, Mg supplementation may down-regulate inflammatory responses and promote sciatic nerve regeneration.

**Acknowledgements**

We wish to thank Ms. Shu-Zhen Lai, Ms. Lin-Lan Yang, and Ms. Mu-Jung Liu for their help in preparing the manuscript and the Biostatistics Task Force of Taichung Veterans General Hospital for their assistance with the statistical analysis.

**Disclosure**

This study was supported by grants from Taichung Veterans General Hospital, Providence University (TCVGH-NCHU987611), and the National Science Council (NSC-99-2314-B-075A-005-MY2), Taiwan, R.O.C.

None of the authors has any conflict of interest to disclose.

**References**

Magnesium promotes nerve regeneration and regulates inflammation


