Neuropathological changes in dorsal root ganglia induced by pyridoxine in dogs

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Research article

Keywords: dog, dorsal root ganglia, H-reflex, pyridoxine, sensory neuropathy

Posted Date: November 12th, 2019

DOI: https://doi.org/10.21203/rs.2.17162/v1

Version of Record: A version of this preprint was published at BMC Neuroscience on March 24th, 2020.
See the published version at https://doi.org/10.1186/s12868-020-00559-3.

Abstract

Background: Pyridoxine (PDX), vitamin B 6 , is an essential vitamin. PDX deficiency induces various symptoms and abuse of PDX supplement also acts as a neurotoxicant that induces severe sensory neuropathy.

Results: To construct a sensory neuropathy model in dogs, excess pyridoxine (PDX) was injected subcutaneously into dogs for seven days and dorsal root ganglia (DRG) were observed at 0, 1 and 4 weeks after the last PDX treatment. During the administration period, body weight loss and proprioceptive loss occurred. After the treatments were completed, electrophysiological recordings showed that the H-reflex of the treated dogs had disappeared at week 0. These phenomena persisted for four weeks exceptional body weight. CV and HE staining revealed that neurons in DRG had significant loss of large-sized neurons 0 weeks, but these neurons were recovered 4 weeks. Iba-1 and GFAP immunohistochemistry showed that reactive microgliosis and satellite glial cells were pronounced at 1 week.

Conclusions: This result suggests that PDX-induced neuropathy model is reversible and can be a good experimental model for research on neuropathy in dogs.

Background

Pyridoxine (PDX) is one of the compounds that can be called vitamin B_6, along with pyridoxal and pyridoxamine. PDX is an essential vitamin, and PDX deficiency induces various symptoms [1]. However, PDX has been identified as a neurotoxicant that induces severe sensory neuropathy in response to chronic abuse of oral PDX supplements [2]. Compelling evidence related to the functional and physiological parameters induced by PDX neurotoxicity has been demonstrated in rodents [3–6], dogs [7–9], and humans [10].

There are many disorders that involve invasion of the peripheral nerve, inducing sensory neuropathy, which is caused by genetic diseases, metabolic imbalance, endocrine disease, toxins, fluoroquinolone toxicity, inflammation, and physical trauma. Accordingly, many trials have been conducted to treat sensory neuropathy; however, more animal models of sensory neuropathy are required [11–13]. Dogs are valuable animal models because they are more similar to humans than rodents. However, fewer neurological studies have been conducted using canine models than rodent models [7].

All sensory axons pass into the Dorsal root ganglion (DRG) and then into the spinal cord itself. Therefore, observation of DRG is useful to obtain information about the sensory tract [14].

In this study, we conducted neurological examination and observed H-reflex and chronological changes in satellite glial cells (SGC) and microglia using glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba–1) in DRG, respectively. Specifically, we evaluated H-reflex and expression of GFAP and Iba–1 to determine if they changed with time in response to subcutaneous

injection of PDX for seven days. To accomplish this, a dog model of sensory neuropathies was established by administering subcutaneous injection of PDX over a short term period. We observed the dogs for four weeks after the last PDX treatment to identifying chronological changes in the effects of PDX on DRG.

Results

Physical examination

Weight loss was observed in the treated group, with significant differences in body weight being observed at 0 and 1 week after the last PDX treatment ($p<0.05$). However, there was no significant difference in the weight between before PDX treatment and 4 weeks after the last PDX treatment.

Postural reaction assessments

All dogs in the treated group developed neurologic disorders. On day 3 or 4 of PDX injection, proprioceptive abnormalities involving the hindquarters were observed based on the postural reaction test (wheel-barrowing, hopping, extensor postural thrust, placing, tonic neck reaction and proprioceptive positioning). At 0 weeks after the last PDX treatment, all dogs experienced hindlimb stiffness when attempting to walk or turn. At 1 week after the last PDX treatment, these phenomena were maintained. At the end of the experimental period (4 weeks after the last PDX treatment) the hindlimb stiffness had disappeared, but the proprioceptive abnormalities were maintained. Except for weight loss and neurological problems, there were no changes in body conditions.

Electrophysiological recording

Evaluation of the Direct-evoked muscle potentials (DEMP; M wave) and Reflex-evoked muscle potentials (REMP; H-reflex) in clinically normal dogs revealed the following. The mean M wave amplitudes (5.4 ± 2.3 mV) were much higher than the mean H-reflex amplitudes (0.5 ± 0.3 mV). The mean M wave latencies (2.8 ± 0.3 ms) showed that the M wave was an early response; however, the mean H-reflex latencies (16.2 ± 2.5 ms) showed that the H-reflex was a late response [6]. In the treated group, there were no significant differences in M wave amplitude at 0 weeks, 1 week or 4 weeks after the last PDX treatment when compared with before PDX treatment ($p>0.05$). However, there were significant differences in the H-reflex amplitude at 0 weeks, 1 week and 4 weeks after the last PDX treatment when compared with before PDX treatment ($p<0.05$). At 0 weeks, the H-reflex of the treated dogs had disappeared, and this condition was sustained until 4 weeks after the last PDX treatment. Interestingly, just one dog among three dogs, two times of H-reflex had appeared among 8 times measurement.

Immunohistochemistry

In control dogs, cresyl violet (CV) and Hematoxylin and eosin (HE) stained neurons were abundant in all levels of DRGs (Fig. 1A, 1D, 1G, 2A, 2D and 2G). At 0 weeks after the last PDX treatment, the number of CV and HE stained large-sized neurons was significantly decreased in the cervical, thoracic and lumbar level of DRG, while CV and HE stained small- and medium-sized neurons were intact. In addition, there were no significant differences in neuronal damage between regions of DRG (Fig. 1B, 1E, 1H, 1M, 2B, 2E and 2H). At 1 week after the last PDX treatment, CV stained neurons in large-sized neurons were significantly increased in all levels of DRG and about 77.3% of those in the control group (Fig. 1C, 1F, 1I and 1M). In addition, HE staining in this group revealed that some hematoxylin stained nuclei were aggregated in the DRG (Fig. 2C, 2F and 2I). At 4 weeks after the last PDX treatment, CV and HE stained large-sized neurons were increased in all levels of DRG and about 92.3% neurons were detected in this group when compared to the control group (Fig 2. 1D, 1H, 1L, 1M, 2D, 2H and 2L).

GFAP immunoreactive SGCs were detected in all levels of DRG in the control group, and this surrounded the neurons in the DRG (Fig. 3A, 3E and 3I). At 0 weeks after the last PDX treatment, GFAP immunoreactivity was slightly, but not significantly, increased in the DRG (Fig. 3B, 3F, 3J and 3M). At 1 week after PDX treatment, GFAP immunoreactivity was significantly increased when compared to the control group (Fig. 3C, 3G, 3K and 3M). At 4 weeks after PDX treatment, GFAP immunoreactivity had decreased significantly and was similar to that in the control group (Fig. 3D, 3H, 3L and 3M). In the control group, ionized calcium-binding adapter molecule 1 (Iba–1) immunoreactive microglia were detected in all levels of DRG (Fig. 4A, 4E and 4I). At 0 weeks after the last PDX treatment, Iba–1 immunoreactivity was significantly higher in the DRG and the cytoplasm of the Iba–1 immunoreactive microglia/macrophage was hypertrophied (Fig. 4B, 4F, 4J and 4M). At 1 and 4 weeks after the last PDX treatment, Iba–1 immunoreactivity was decreased in the DRG, but Iba–1 immunoreactivity in these groups was higher than in the control group (Fig. 4C, 4D, 4G, 4H, 4K, 4L and 4M).

Discussion

In this study, we selected PDX for the induction of peripheral neuropathy in dogs, and an animal model of PDX induced peripheral neuropathy was safe for other organs [15–17].

In rat studies, H-reflex was measured to demonstrate that PDX induced sensory neuropathy. Electrophysiological recordings taken during peripheral nerve stimulation in the experimental group were consistent with selective toxicity to sensory, but not motor nerve function. We also confirmed that this procedure was suitable in dog studies with PDX induced sensory neuropathy [7].

Based on previous studies [7, 8], we were concerned that the changes and the relationship with time of neurological examination, electrophysiological recording, and histopathological analysis of dog model of sensory neuropathies, by PDX over a short term period. In previous experiments, body weight decreased during the PDX administration period and was related to food consumption. This phenomenon was the same for the PDX administration period, but the body weight increased with time after PDX administration in this study. We also confirmed that the H-reflex disappeared for four weeks after the last

PDX treatment, even though histological analysis showed that the DRG were recovered. Based on these data, we supposed that the structural recovery of DRG occurs before its functional recovery.

In this study, we also observed the chronological changes in SGCs and microglia because these cells contribute to several neuropathological conditions [18, 19]. GFAP immunoreactive SGCs were significantly upregulated at 1 week after the last PDX treatment and decreased 4 weeks after the last PDX treatment. These results were similar to the previous findings that GFAP expression was upregulated in SGCs of the injured DRG from day 3 onwards immediately after sciatic nerve injury because we also observed proprioceptive abnormalities at 3–4 days after PDX treatment.

Iba–1 immunoreactive microglia were significantly increased at 0 weeks after the last PDX treatment and Iba–1 immunoreactive microglia continued to decrease throughout the experiment. This result was supported by our histological findings that neuronal death was prominent at 0 weeks after the last PDX treatment, and that the number of large-sized neurons in the DRG increased thereafter.

Conclusions

We established a reversible dog model of sensory neuropathies by administering subcutaneous injections of PDX over a short term period. However, further studies involving the mechanism of sensory neuropathy of PDX in dogs are necessary.

Methods

Animal model

Eleven beagle dogs approximately 2 years of age were used to evaluate PDX-induced neuropathy. The body weight of the dogs ranged from 7 to 11 kg. Two dogs were in the control group and nine were in the experimental group. Three of the dogs in the experimental group were included in the 0 week group, while three were included in the 1 week group, and three were in the 4 weeks group (based on the time after the last PDX treatment).

All dogs were clinically judged to be in good health and neurologically normal. All experimental dogs had their own admission number (SNU–200908–18) from the Institute of Laboratory Animal Resources, Seoul National University (Korea). During the experiment, all dogs were cared for according to the Animal Care and Use Guidelines of the Institute of Laboratory Animal Resources, Seoul National University.

PDX intoxication

PDX (Sigma, St. Louis, MO) was diluted in a 0.9% sterile aqueous solution of sodium chloride and administered subcutaneously (SC) once a day during the morning for seven days. PDX solution was

prepared immediately prior to each injection. Animals in the control group received vehicle (iso-osmotic sterile aqueous solution of sodium chloride), while animals in the experimental group were administered 150 mg/kg PDX in a volume of 100 mg/ml SC [7].

Physical examination and postural reaction assessments

The body weights of the test dogs were measured every morning during the PDX injection period, as well as 1 weeks and 4 weeks after the last PDX treatment. The body conditions of the test dogs were also checked at those times.

Postural reaction (wheelbarrowing, hopping, extensor postural thrust, placing, tonic neck reaction and proprioceptive positioning) assessments were conducted on all the dogs every morning during the PDX injection period, as well as on one and four weeks after the last PDX treatment.

Electrophysiological recording

All dogs were pre-anesthetized with atropine (0.1 mg/kg of body weight, IM). Anesthesia was induced with diazepam and maintained with isoflurane (Baxter Healthcare, Deerfield, IL) through a semiclosed system. The subcutaneous temperature was maintained at 37–38ºC. Neuropack2 (Nihon Koden, Tokyo, Japan) was used for all recordings. All measurements were conducted in the left hindlimb of each dog.

DEMP, M wave, were recorded for the tibial nerve using 1 Hz, 0.5 ms, supramaximal stimulus. Stimulating electrodes were positioned in the distal tibial nerve and recording electrodes were positioned in the plantar interosseous muscle, with a ground electrode positioned between the stimulating electrode and recording electrode. The recording electrode was a bipolar needle electrode. REMP, H-reflex, were recorded using 1 Hz, 0.5 ms, submaximal stimulus. The stimulating electrode was positioned in the tibial nerve adjacent to the hook and a recording electrode and ground electrode were positioned in the same site of the tibial nerve where the M wave was measured. All measurements were conducted at least eight times [7]. Electrophysiological recordings were conducted before PDX treatment and at 0, 1, and 4 weeks after the last PDX treatment.

Immunohistochemistry

For euthanasia, cephalic veins of the dogs were selected for intravenous (IV) catheterization. The dogs were anesthetized with a high dose of propofol (5mg/kg of body weight, IV). and tiletamine/zolazepam (10mg/kg of body weight, IV). After confirmation of a deep anesthesia, they were perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) and followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The DRGs of cervical, thoracic, lumbar spinal cords were removed and

postfixed in the same fixative for 12 h, after which they were dehydrated with graded concentrations of alcohol for embedding in paraffin. Next, the paraffin-embedded tissues were sectioned on a microtome (Leica, Wetzlar, Germany) into 3-μm coronal sections and then mounted onto silane-coated slides. To elucidate the PDX-induced DRG damage, the samples were deparaffinized in xylene, rehydrated in a descending ethanol series and stained with hematoxylin and eosin. In addition, CV staining was conducted as previously described [2]. Briefly, CV acetate (Sigma) was dissolved at 1.0% (*w/v)* in distilled water, and glacial acetic acid was then added to this solution. Before and after staining for 2 min at room temperature, the sections were washed twice in distilled water, dehydrated with graded concentrations of alcohol at room temperature, and finally mounted with Canada balsam (Kanto, Tokyo, Japan). Images of all CV stained neurons were taken from the DRG through a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus) connected to a PC monitor. DRG neurons were separated into three categories according to their sizes: small- (area 1000 μm^2), medium- (area 1000–2000 μm^2), and large-sized (>2000 μm^2) neurons. The number of these neurons in all the groups was counted in the DRG using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ). Cell counts were obtained by averaging the counts from the sections taken from each animal. Immunohistochemistry for GFAP and Iba–1 was conducted to elucidate chronological changes in reactive gliosis after PDX treatment. The sections were sequentially treated with 0.3% hydrogen peroxide in PBS for 30 min and 10% normal goat serum in 0.05 M PBS for 30 min, after which they were incubated with diluted rabbit anti-GFAP (1:1000, Chemicon, Temecula, CA) or rabbit anti-Iba–1 antibody (1:500, Wako, Osaka, Japan) overnight at room temperature and subsequently exposed to FITC-conjugated anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) and FITC-conjugated anti-rat IgG (1:600; Jackson ImmunoResearch). The immunoreactions were observed under a BX51 microscope attached to a fluorescent lamp. Analysis of a region of interest (ROI) in the each levels of DRG was performed using an image analysis system. Images were calibrated into an array of 512 × 512 pixels corresponding to a tissue area of 140 × 140 μm (40× primary magnification). Each pixel resolution was 256 gray levels. The intensity of GFAP and Iba–1 immunoreactivity was evaluated by means of a relative optical density (ROD), which was obtained after the transformation of the mean gray level using the formula: ROD = log (256/mean gray level). ROD of background was determined in unlabeled portions and the value subtracted for correction, yielding high ROD values in the presence of preserved structures and low values after structural loss using NIH Image 1.59 software. A ratio of the ROD was calibrated as %.

Statistical analysis

All data are expressed as the mean ± SE or the mean ± SEM. The data were evaluated by a student's *t*-test. Statistical significance was considered at $P < 0.05$. A paired *t*-test was conducted for analysis of the body weights and the M wave and H-reflex amplitudes before and after the pharmacologic treatment. The level of significance was set at $p < 0.05$.

Abbreviations

CV: cresyl violet; DEMP: Direct-evoked muscle potentials; DRG: dorsal root ganglia; GFAP: glial fibrillary acidic protein; HE: Hematoxylin and eosin; Iba–1: ionized calcium-binding adapter molecule 1; PDX: pyridoxine; REMP: Reflex-evoked muscle potentials; ROD: relative optical density; ROI: region of interest; SGC: satellite glial cells

Declarations

Ethics approval and consent to participate

All procedures for animal care were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the by the Institutional Animal Care and Use Committee of Seoul National University (SNU–200908–18).

Consent to publish

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study including the tissue samples of dorsal root ganglion generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

Funding

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF–2013R1A1A2059364, NRF–2014R1A1A1004339) and by the Promising-Pioneering Researcher Program through Seoul National University (SNU) in 2015. The funding body played no role in the design and interpretation of the experiments. The founding sponsors had no role in the study design, performance, data collection and analysis, decision to publish, or preparation/writing of the manuscript.

Authors' Contributions

SY and WK wrote the paper. MSK, THK and YK performed the experiments. JOA and JHC analyzed the data. IKH and JYC wrote the paper, conceived and designed the experiments. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

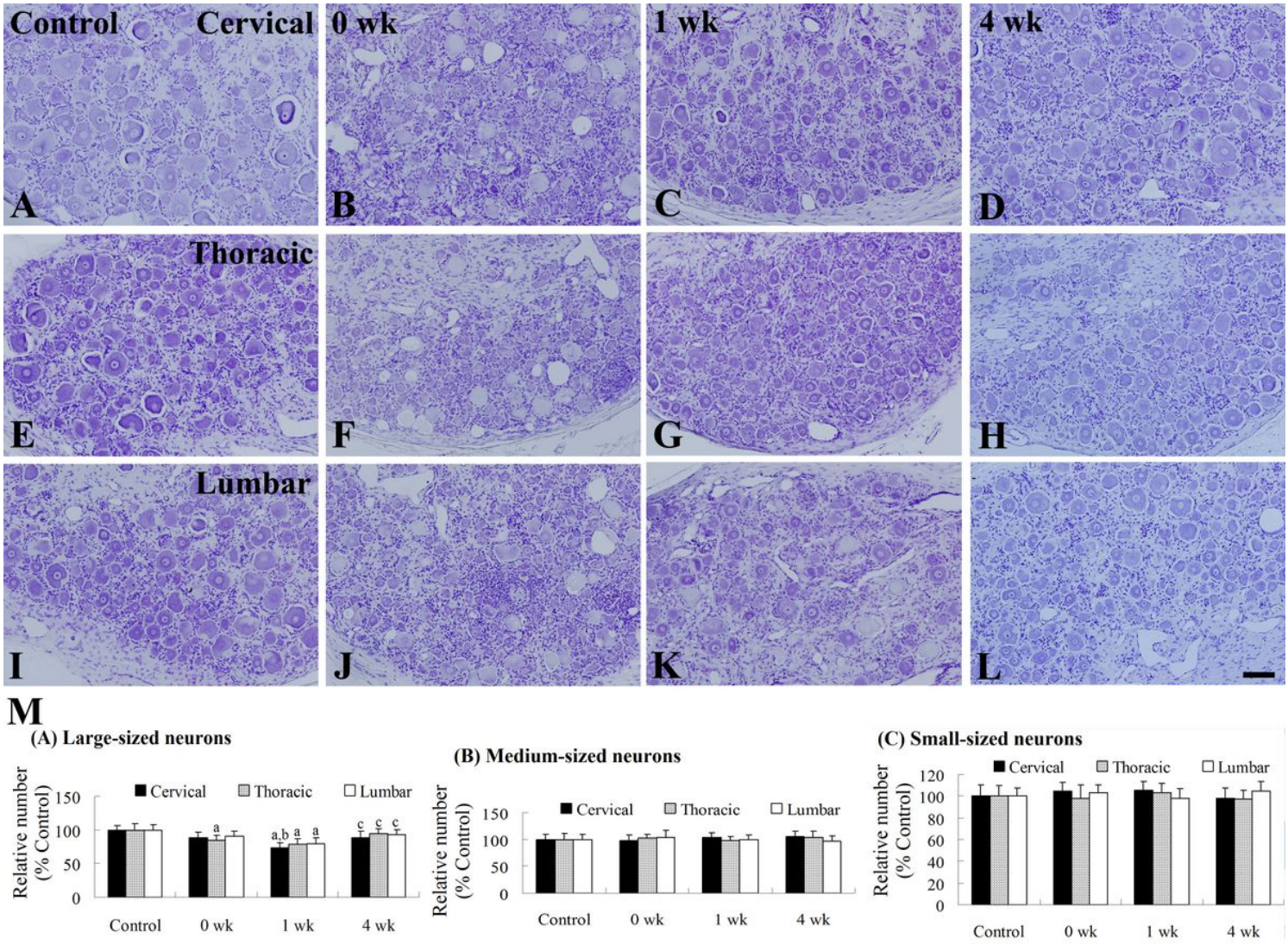

Figure 1

Cresyl violet (CV) staining of the DRG in control (A, E and I) and pyridoxine-treated (B-D, F-H and J-L) groups. In the control group, neurons in all levels of DRGs were well stained with CV. Note that CV stained large-sized neurons decreased in all levels of DRG. Scale bar = 100 μm. M: Relative numerical analysis of CV stained neurons with their sizes (n = 2-3 per group; aP <0.05, significantly different from the control group; bP <0.05, significantly different from the group 0 weeks after the last pyridoxine treatment; cP <0.05, significantly different from the group 1 week after last pyridoxine treatment). The bars indicate the means ± SEM.

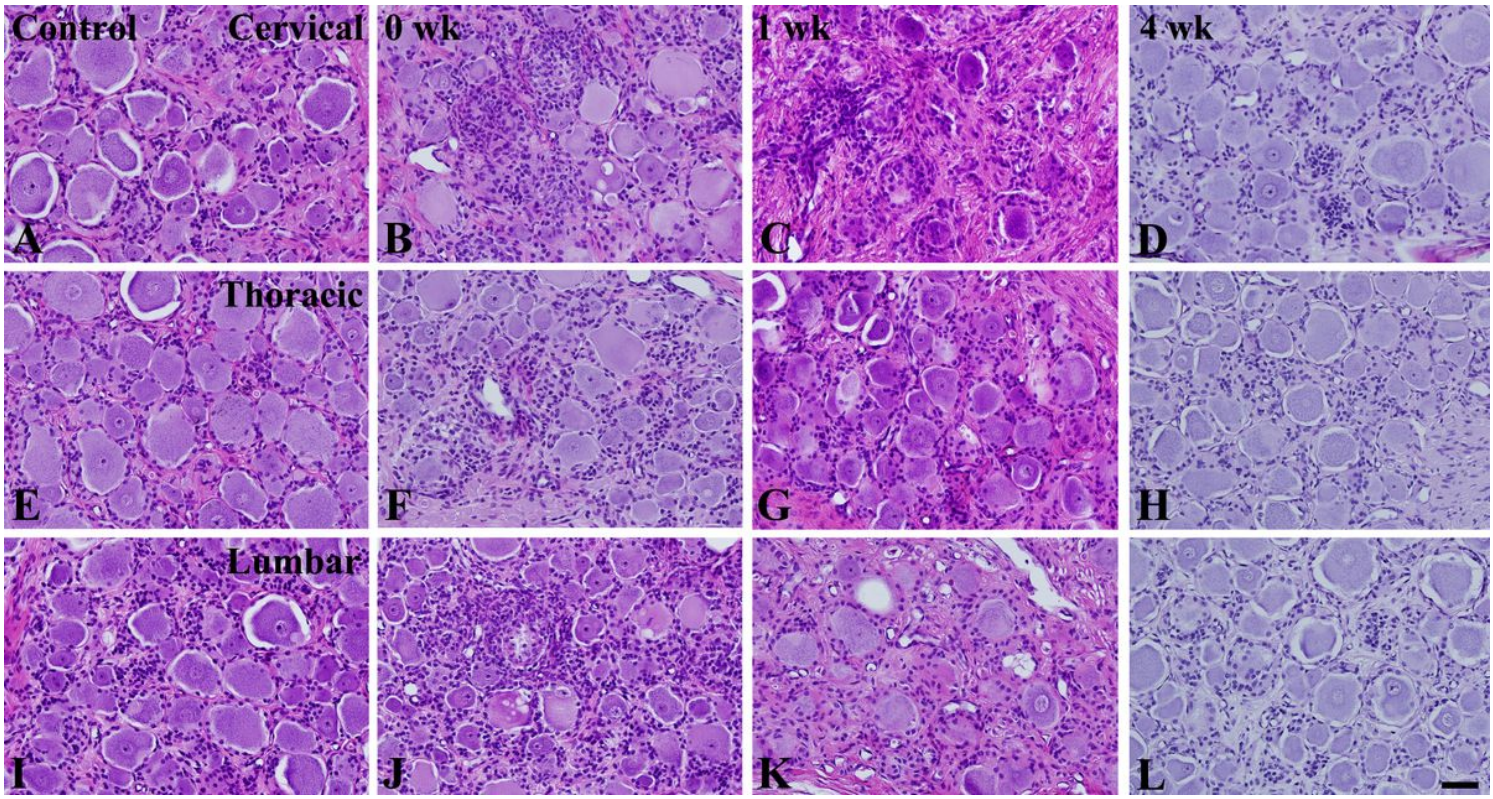

Figure 2

Hematoxylin and eosin (H&E) staining of the DRG in control (A, E and I) and pyridoxine-treated (B-D, F-H and J-L) groups. In the control group, neurons in all levels of DRGs are well stained with H&E. Note that some hematoxylin stained nuclei were aggregated 0 weeks after the last pyridoxine treatment and thereafter it is decreased with time after last pyridoxine treatment. Scale bar = 100 μm.

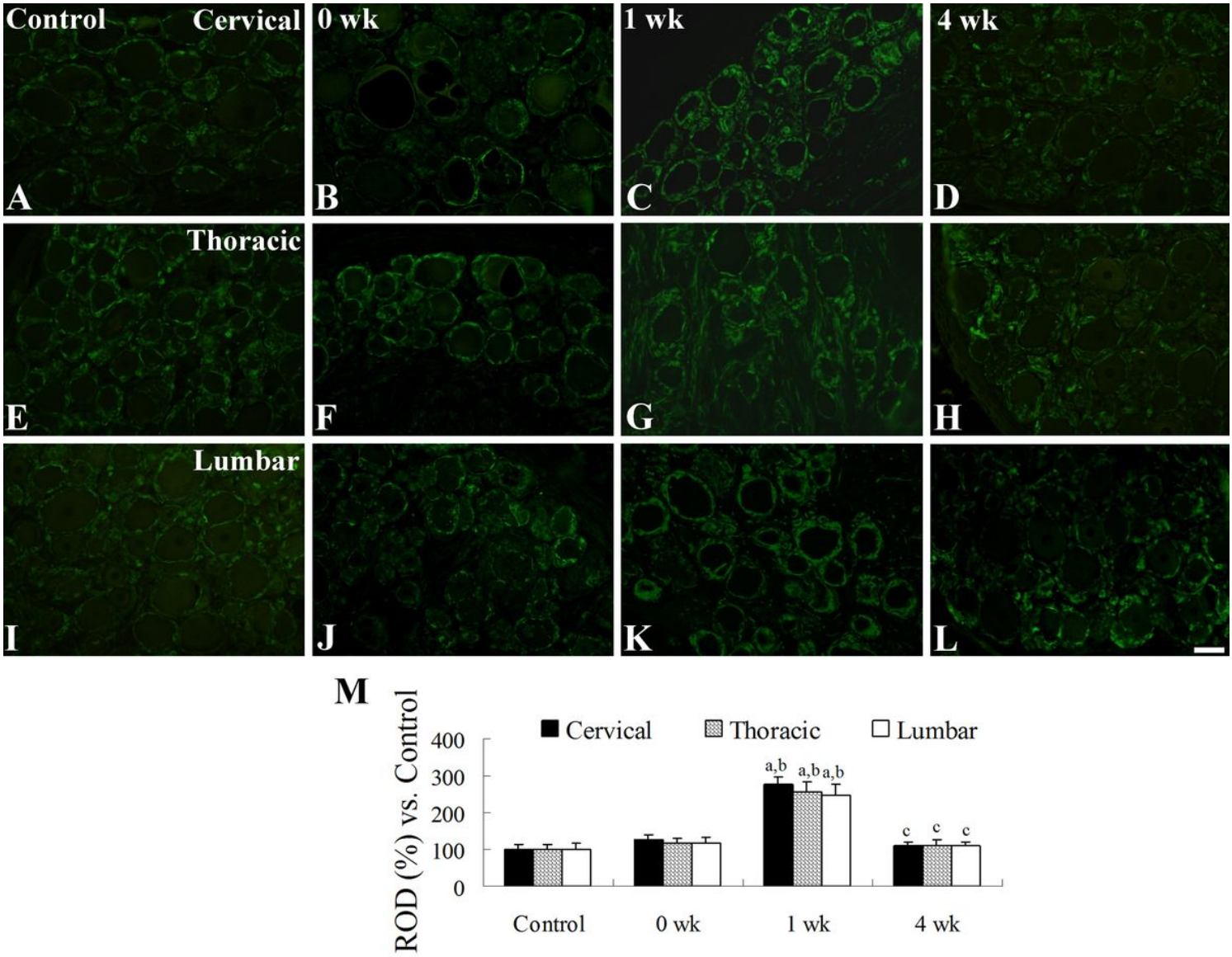

Figure 3

GFAP immunostaining of the DRG in control (A, E and I and) and pyridoxine-treated (B-D, F-H and J-L) groups. In the control group, GFAP immunoreactivity is detected in the DRG and GFAP immunoreactive satellite glial cells are abundant 1 week after the last pyridoxine treatment, after which they decreased with time. Scale bar = 100 μm. M: Relative optical density (ROD) as a % of each level of the control group in the DRG (n = 2-3 per group; aP <0.05, significantly different from the control group; bP <0.05, significantly different from the group at 0 weeks after the last pyridoxine treatment; cP <0.05, significantly different from the group 1 week after the last pyridoxine treatment). The bars indicate the means ± SEM.

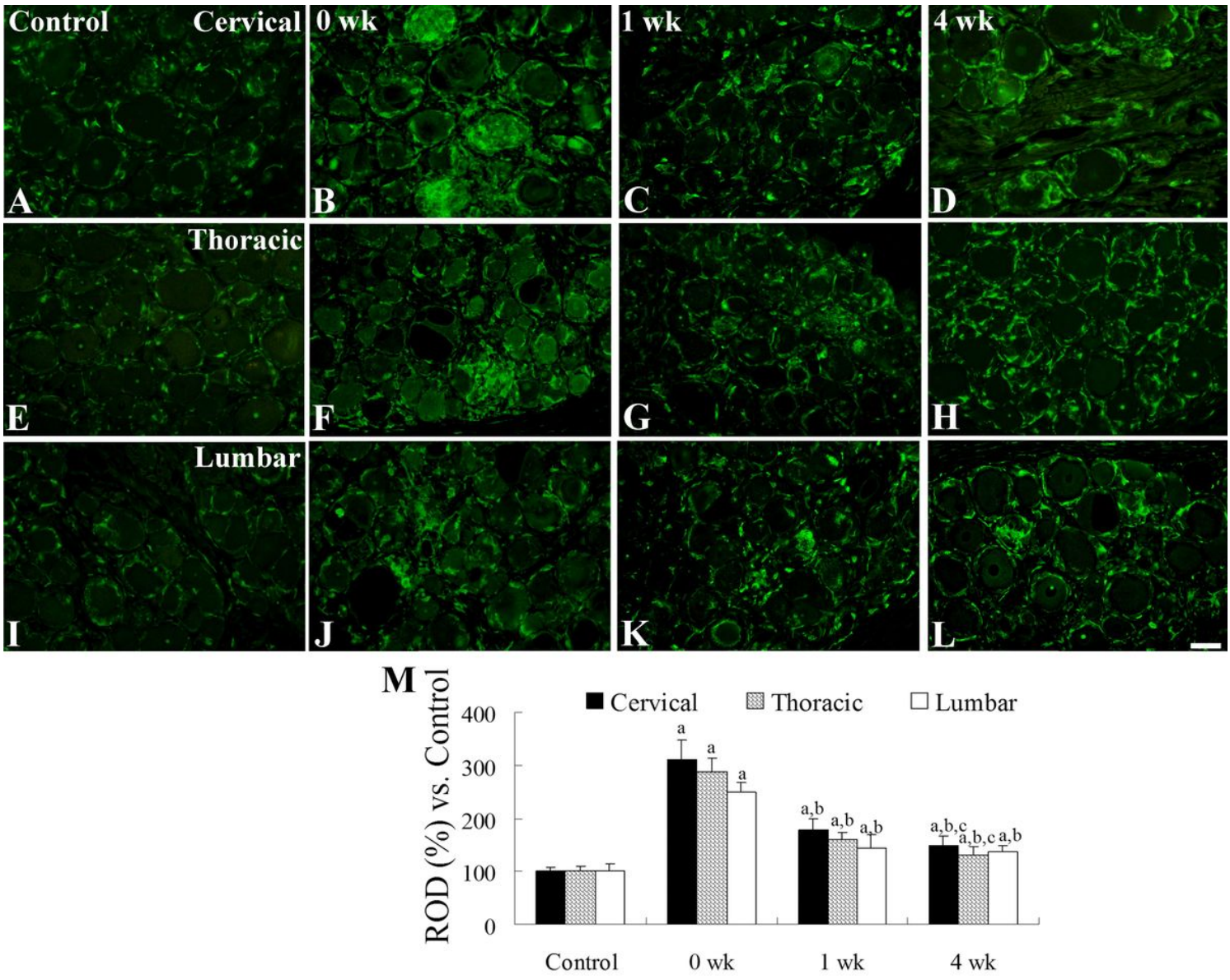

Figure 4

Iba-1 immunostaining of the DRG in control (A, E and I and) and pyridoxine-treated (B-D, F-H and J-L) groups. In the control group, Iba-1 immunoreactivity is detected in the DRG and Iba-1 immunoreactive microglia peaked 0 weeks after pyridoxine treatment, after which they decreased in the DRG. Scale bar = 100 μm. M: Relative optical density (ROD) as a % of each level of the control group in the DRG (n = 2-3 per group; aP <0.05, significantly different from the control group; bP <0.05, significantly different from the group 0 week after last pyridoxine treatment; cP <0.05, significantly different from the group 1 week after last pyridoxine treatment). The bars indicate the means ± SEM.

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