European Review for Medical and Pharmacological Sciences 2013; 17: 2711-2717

Cuprizone-induced demyelination in Wistar rats; electrophysiological and histological assessment

H. BASOGLU, N.T. BOYLU[1], H. KOSE[2]

Department of Biophysics, and [1]Department of Histology and Embryology; Medical Faculty, Adnan Menderes University, Aydin, Turkey
[2]Department of Biophysics, Bezmialem Vakif University, Medical Faculty, Istanbul, Turkey

Abstract. – OBJECTIVES: Multiple Sclerosis (MS) is a disease that affects the Central Nervous System by destructing myelin shield and also can affects the peripheral nervous system. Demyelination is acquired characteristics disease and appears with the degeneration of myelin which protects the axons. Cuprizone (CPZ) model is a toxic demyelination model. The purpose of this study was to develop an MS model by cuprizone exposure to Wistar rats.

MATERIALS AND METHODS: Rats were separated into control and experimental groups and daily cuprizone was administered to experimental groups for 4, 5, 6 and 7 weeks. At the end of the experiments, spinal nerve conduction velocity was measured by EMG detected from the gastrocnemius muscle. After scarification, cerebrum and cerebellum of the animals were taken for histopathological investigation.

RESULTS: Spinal cord nerve conduction velocity (SNCV) of control animals was 76.54 m/s. Whereas SNCV of the rats that were feed with CPZ for 6 weeks was significantly reduced to 46.35 m/s in comparison with the control group. Demyelinated areas and vacuolization were seen on the brain sections of CPZ exposed rats.

CONCLUSIONS: SNCV of the rats were feed with cuprizone began tendency of decrease after 4th weeks. These reductions were observed as maximum at 6th weeks. At 7th week increments were observed at SNCV. These results indicated that 6 weeks of cuprizone feedings could be suitable to bring into existence of MS model in Wistar rats.

Key Words:
Cuprizone, Multiple sclerosis, Nerve conduction velocity, Spinal cord, Wistar.

Introduction

Nerve fibers consist of axons enveloped by special sheath of ectodermal origin. Groups of nerve fibers constitute the tract of the brain, spinal cord and peripheral nerves. Nerve fibers exhibit differences in terms of their enveloping sheaths that related to whether the fibers are part of the central or peripheral nervous system. The sheath cells in central nerve fibers are oligodendrocytes and the Schwann cells in peripheral nerve fibers[1]. Multiple Sclerosis (MS) is an inflammatory autoimmune disease in which myelin sheaths around the axons of the brain and spinal cord are damaged that leading to demyelination[2-4]. The mission of myelin in central nerves system (CNS) is making the conduction of electrical impulse faster by saltatory axonal conduction. The outcome of demyelination for saltatory conduction explains many clinical and laboratory features of multiple sclerosis. Partially demyelinated axons conduct impulses at reduced velocity[2].

There are at least three animal models for demyelination that includes experimental autoimmune encephalomyelitis (EAE), virus induced model of demyelination with Theiler's virus, murine hepatitis virus and toxic models of demyelination with lysolecithin, ethidium bromide and cuprizone[5]. Cuprizone (CPZ) bis-cyclohexanone oxaldihydrazone is a potent cupper chelator. In the CPZ model, animals are fed with CPZ which causes cell death of oligodendrocytes which causes consistent demyelination[6-10]. Furthermore, removal of cuprizone from diet of animals initiates remyelination[7].

As results of some experiments that have been done with CPZ, MS model was achieved at mouse[9,11,12] whereas researchers have published controversial results in rats for MS models. For instance, although Love[13] notified that CPZ model did not work at Wistar rat strain, Adamo et al reported demyelination in the corpus callosum (CC) of Wistar rats[10]. Furthermore, there are some new studies reporting CPZ model in Wistar

Corresponding Author: Hayrullah Kose, MD; e-mail: hayrullahkose@yahoo.co.uk

rats[14-16]. On the other hand Love[13] propounded that developing of central demyelination was possible for the other strains of rats but not in Wistar.

In all previous CPZ models in Wistar rats, animals were fed with milled chow containing CPZ. Rats can mix the milled chow by their nose and eat less CPZ side of milled chow, because CPZ is powder and can be gather on the bottom of manger. As a result of this, CPZ intake of each animal can be different which can causes discrepancy in demyelination on each animal. Therefore, we thought that we can manage to give daily standard dose of CPZ to animals by gavage from mouth.

We also made a new approximation in this study. CPZ destroys oligodendrocytes and oligodendrocytes exist at both, brain and spinal cord. We thought that we can do electrophysiological assessment of CPZ induced demyelination by calculating spinal nerve conduction velocity (NCV). Hence we investigated spinal nerve conduction velocity with epidural electrical stimulation of spinal nerves with needle electrodes.

Materials and Methods

In this study, 44 male, one year old Wistar-albino rats weighing between 300-370 g were used. The experimental procedures were conducted in accordance with experimental protocols approved by the Local Experimental Animal Care Committee at the University of Adnan Menderes, Aydin, Turkey. The rats were maintained at approximately 22°C with a 12 hours light-dark cycle, and had free access to commercial food and water. Animals divided into 6 groups. Table I shows the groups of animals with more details.

Cuprizone Administration

In literature, different CPZ dose were reported in order to make animal models of demyelination. The range of CPZ doses were between 0.4% and 2% in powdered rat chow[13,15-18]. In this present study, 1% dose of CPZ from Fluka, Sigma-Aldrich Co. (St Louis, MO, USA) was chosen. At the beginning, average daily chow consumption of the rats was measured as 69±3 gr per kg of live body weight. However 0.69 g of CPZ powder was added in a liter of 1% carboxymethyl cellulose (CMC) filler from Sigma-Aldrich Co. This mixture was vortexed in order to obtain a homogeneous CMC-CPZ suspension. Animals were fed daily by gavage with 69 ml/kg CMC-CPZ mixture that was named as 1% of daily total food intake of animal was CPZ. Stock solution of 1% CMC was prepared weekly and stored in +4°C. CMC and CPZ suspension was prepared and consumed daily. CPZ does not react chemically with CMC mixture, just suspends in it. When weight loss of animals reached at about 20% of total body weight, CPZ application was halted for a day and continued after rest day. None of the animals was needed more than one rest day and just some of the animals lost weight at a maximum of 20% of their body weight at the end of CPZ application period. Weight monitoring and gavage applications to animals were done by same researcher.

Spinal Motor NCV Measurement by Electromyography (EMG)

EMG (electromyography) measurements were taken at the end of the CPZ application periods. All animals were kept under anesthesia during EMG measurements with the combination of ketamine 100 mg/kg and xylazine 10 mg/kg. Rats were electrically stimulated with monopalar needle electrodes (EL400) from two different points in spinal cord; L1-L2 and L5-L6 spinal segment and EMG was recorded from surface of the gastrocnemius muscle by using 4 mm diameter shielded Ag/AgCl surface electrodes, EL254S and MP100 data acquisition

Table I. Groups of animal and application periods of Cuprizone.

Groups	N	Applied materials	Application period (weeks)
Control	6	Nothing was given	_
CMC	6	69 ml/kg 1% CMC	6
Cuprizone	8	1% CPZ in 69 ml/kg 1% CMC	4
Cuprizone	8	1% CPZ in 69 ml/kg 1% CMC	5
Cuprizone	8	1% CPZ in 69 ml/kg 1% CMC	6
Cuprizone	8	1% CPZ in 69 ml/kg 1% CMC	7

system with EMG module Biopac System Inc., Goleta, CA, USA. Compound muscle action potential (CMAP) was taken from gastrocnemius muscle and used to measure Spinal NCV as previously described[19]. Briefly, EMG based motor nerve conduction velocity was calculated with the formula; NCV = $\Delta x / \Delta t$ where x is the distance between distal and proximal stimulus, t is the differences between latency of the distal and proximal stimulus. The distance between two stimuli on the spine of the rat was measured with 150 mm Vernier Caliper. Hence, we obtained motor nerve conductance velocity (MNCV) as m/s. For the determination of latency of CMAP, the length of time between stimulus artifact and the first deflection from the baseline of EMG signals were taken.

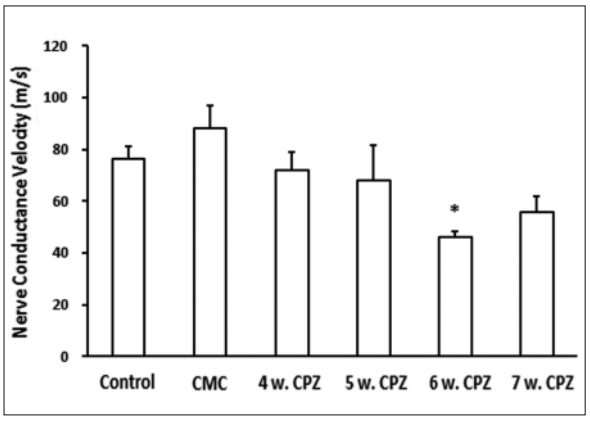

Figure 1. Spinal cord motor nerve conduction velocities of rats. MNCV of the six weeks cuprizone exposed rats was smaller than that of control group (*$p < 0.05$).

Histopathological Tissue Evaluation

At the end of EMG measurements, animals were sacrificed by cervical dislocation. Head was cut below occipital level. Whole brain (cerebrum and cerebellum) was removed and firstly cerebrum and cerebellum were separated. Cerebrum dissected into right hemisphere, left hemisphere and corpus callosum. The tissues were fixed in 10% neutral buffered formaldehyde solution for 48 hours. After routine tissue processing tissues were embedded in paraffin. 5 µm cross sections obtained from paraffin blocks were stained with luxol fast blue (LFB) in order to assess the degree of myelination. Sections were examined by Olympus BX51 microscope and pictures were taken by the Olympus DP 20 digital camera attached to the microscope (Olympus, Tokyo, Japan).

Statistical Analysis

All the data were expressed as mean ± SEM. Statistically significant differences were determined using a one way analysis of variance (ANOVA) and Dunnet Multiple Comparison test to compare relations between control and other groups by using GraphPad Prism 5.0 software. Value of $p < 0.05$ was considered as significant.

Results

Spinal MNCV of groups are seen in Figure 1. Average spinal MNCV of 6 weeks CPZ applied rats was significantly smaller than spinal MNCV of the control group. There is no significant difference among Control, CMC, 4 and 5 weeks CPZ applied groups. High standard errors were seen on motor nerve conduction velocity of 5 weeks CPZ applied rats. Although motor NCV of 7 weeks CPZ applied rats is higher than MNCV of the 6 weeks CPZ applied group, but this differences was not significant.

When the one way ANOVA test applied to all groups, p value was calculated as 0.0087. This indicates that there is significant difference among groups ($p < 0.05$). Dunnet Multiple Comparison test was applied to compare relations between control and other groups and only significant difference was seen between 6 weeks CPZ applied and control group in terms of spinal MNVC.

Myelinated areas in brain corpus collosum and cerebellum were stained dark blue with LFB staining technique in control animals. Highly myelinated areas were stained dense whereas demyelinated or hypomyelinated areas were stained pale. Demyelination was not observed on brain sections of control, CMC and 4 weeks CPZ applied rats. The brain sections of 5, 6 and 7 weeks long CPZ administrated rats were stained pale. These changes were not homogeneous at each animal and differences in staining density were seen on different brain area of the same animals. Figure 2 shows the LFB staining of the CC and cerebellum of the animals. Spongy vacuolization were detected on some of the brain sections obtained from 5 and 7 weeks CPZ applied animals. These spongy vacuolization can be seen in Figure 3. Rare spongy vacuolization were also seen in cerebellum of 5 and 6 weeks CPZ applied animals.

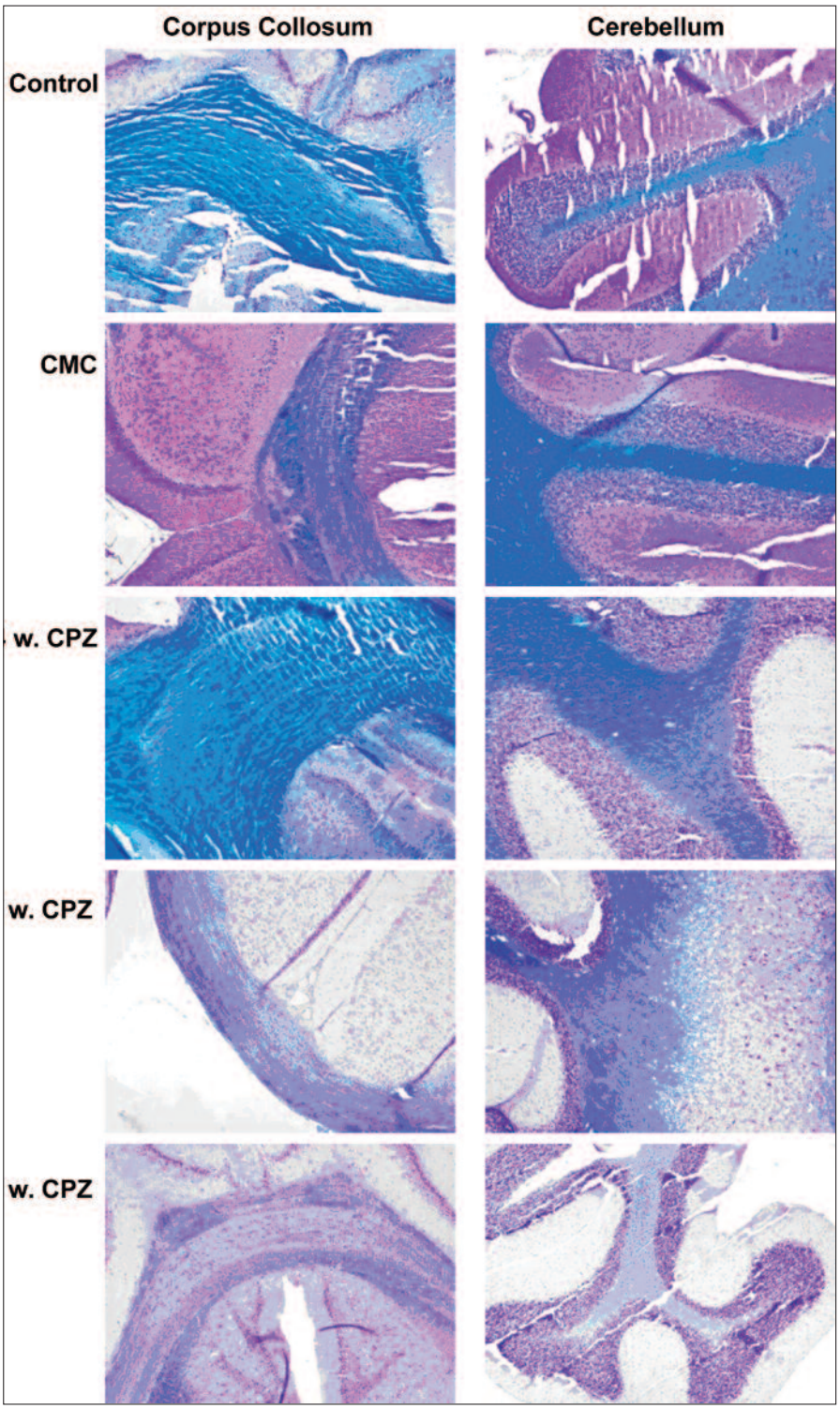

Figure 2. Corpus callosum and cerebellum tissues stained with Luxol fast blue. Control, CMC and 4 weeks CPZ groups display normal myelinated CC. Demyelination, characterized by pale staining is evident on CC of 5 and 6 weeks CPZ groups. Maximal demyelination is observed on the CC of 6 weeks CPZ groups. Cerebellar demyelination is seen only on 5 and 6 weeks CPZ groups (LFB; x40).

Discussion

It is clear that CPZ application to young adult mice induces demyelination in brain especially at corpus callosum[7,20,21]. Purpose of this study was to evaluate the characteristics of demyelination, induced by CPZ application in Wistar rat strain. In order to give a standard dose of CPZ to one year old adult animals, this is the first study to give CPZ by gavage. In all previous studies, 2-3

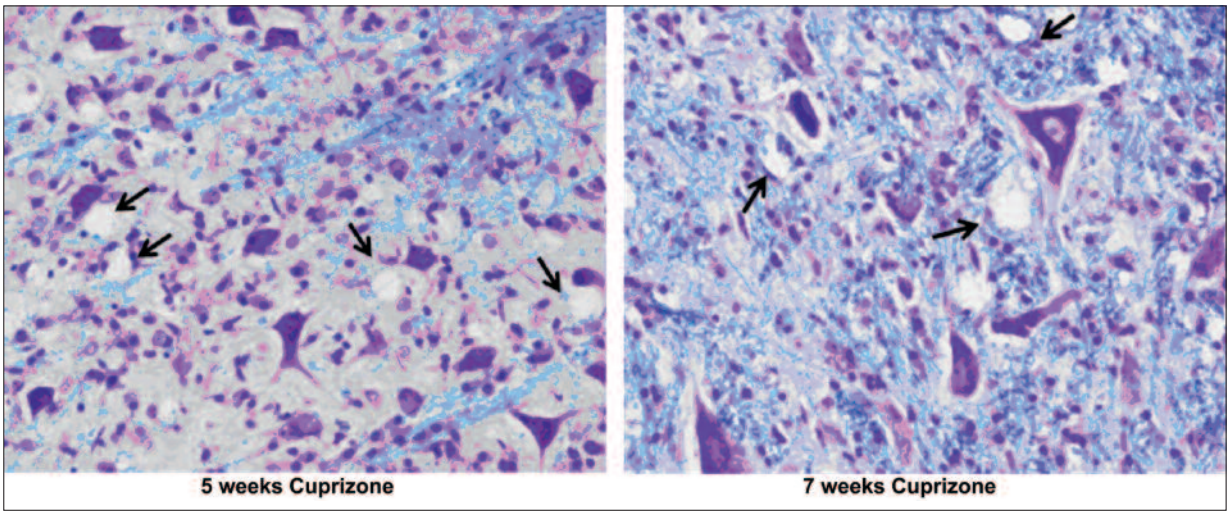

Figure 3. Spongy vacuolation is obvious within the cytoplasm of cell body and those of cytoplasmic processes located at the brain cortex (LFB; x200).

weeks old weanling animals were fed with powdered or milled CPZ containing feed.

Although there are plenty of experiments that have been done with CPZ, it is still unclear the mechanism of CPZ induced demyelination. According to Ventruni[22], CPZ reduces copper content of the brain in mice, and administration of copper-chelated CPZ prevented these changes in the brain. Animals treated with copper-chelated cuprizone did not show any changes. Common idea here, CPZ is a copper chelator that causes demyelination depends on copper deficiency in CNS[6,8]. It has been shown that CPZ induced spongy chances in the brain of experimenting animals[16]. In our study, vacuolation observed only 5 and 7 weeks CPZ applied cerebrum of rats. No vacuolization was observed 4-7 weeks CPZ applied corpus callosum of animals.

Love[13] noticed that CPZ causes intramyelinic edema of the cerebellar white matter, hilum of the dentate nucleus and superior cerebellar peduncle in the Wistar rats. The edema was associated with pyknosis but only rarely degeneration of oligodendrocytes was observed and also prominent axonal regeneration was observed despite the continued administration of CPZ. Here, we represent the first time electrophysiological and histopathological results indicating that 6 weeks CPZ administration causes demyelination. However, if it was considered as axonal degeneration, our 7 weeks CPZ administration result make us thinking that, remyelination starts after 6th week which consistent with the result of Love[13].

On contrary to Love's study[13] indicating Wistar rats is not very good animal model for CPZ induced demyelination, recently at least four CPZ model reported in Wistar rats[10,14-16]. Similar to these studies, histopathological result of our study shows that administration of 1% of daily total food intake was CPZ dose by gavage to Wistar rats causes partial central nervous system damage.

Oligondendroglial response to CPZ is not age dependent because the rate and extent of de-and remyelination in affected adults is similar to that found in intoxicated neonates[23]. On the contrary, Matsushima and Morell[8] reported that the age of the animal and period of time of exposure to CPZ are critical consideration. Irvine and Blackmore[17] informed that the distribution of demyelination was similar in young and aged mice but its extent was marginally greater in the young adult mice. Moreover, axon number was reduced in both young adult and aged mice but there was significant loss of axons in the aged animals at the demyelination stage. These results make us to think that same effect can be seen on one year old adult rats that we used in this study.

Our histopathological results reveal that demyelination can be seen on corpus callosum. Furthermore, local demyelinated areas and vacuolization were seen on the sections obtained from cerebellum of rats. Local demyelinated areas and vacuolization were clearly seen on 5 and 7 weeks long CPZ administrated rats. Vacuolization was not detected on cerebellum of 6 weeks long CPZ administrated rats but demyelinated ar-

eas were extended than those of 5 and 7 weeks long CPZ administrated rats. This indicates that some of the oligodendrocytes still produce myelin sheath at least partially. These findings suggest the existence of remyelination on 7 weeks long CPZ administrated rats. Similarly Mason et al[24] reveals that both demyelination and remyelination can be seen on long term CPZ administrated mice. This relapsing-remitting[4] event is a common characteristic of multiple sclerosis.

In human and in animals, it is common to measure reliably NCV with EMG that evoked by surface stimulus while it is one of the best diagnostic parameters in Neurology clinics. An average motor NCV in; rat spinal cord 72, human spinal cord 100, dogs 70 and approximately 55 m/s rabbit[19,25-27]. In this study average spinal NCV of normal rats is calculated as 76.54±4.76 m/s. CPZ shows its neurotoxin properties by effecting oligodendrocytes[6,7]. Oligodendrocytes located in both brain and spinal cord. Degeneration in spinal cord can affect NCV and, therefore, spinal NCV was investigated in this study. Our previous study[19] was in correlation with the NCV of control group in this present study. But the mean NCV from spinal cord of six weeks CPZ fed animals was 46.35±1.95 m/s that supports the CPZ induced demyelination.

To our knowledge this is the first study demonstrating the relationship between spinal NCV and CPZ induced demyelination. Although partial degeneration could be seen on corpus callosum of 7 weeks long CPZ administrated rats, spinal NCV of 6 weeks long CPZ administrated rats was the lowest one. These results make us to think relapsing/remitting type of MS.

Conclusions

These data indicate that CPZ can induce demyelination on Wistar rats and spinal NCV can be a parameter for animal model of MS.

Acknowledgements

This study was supported by Adnan Menderes University, Department of Scientific Research Projects with the code of SAE-08001. The authors would like to thank to Adnan Menderes University.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Junqueira LC, Carneiro L, Kelley RO. Basic Histology, 8th ed. Norwalk CT, Appletion & Lange 1995.
2) Compston A, Coles A. Multiple sclerosis. Lancet 2002; 359: 1221-131.
3) Hurwitz BJ. The diagnosis of multiple sclerosis and the clinical subtypes. Ann Indian Acad Neurol 2009; 12: 226-230.
4) Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, Fujihara K, Havrdova E, Hutchinson M, Kappos L, Lublin FD, Montalban X, O'Connor P,Sandberg-Wollheim M, Thompson AJ, Waubant E, Weinshenker B, Wolinsky JS. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol 2011; 69: 292-302.
5) Rodriguez M. Effectors of demyelination and remyelinition in the CNS: Implications for multiple sclerosis. Brain Pathol 2007; 17: 219-229.
6) Torkildsen O, Brunborg LA, Myhr KM, Bo L. The cuprizone model for demyelination. Acta Neural Scand 2008; 117(Suppl 118): 72-76.
7) Morell P, Barret CV, Mason JL, Toews AD, Hostettler JD, Knapp GW, Matsushima GK. Gene expression in brain during cuprizone-induced demyelination and remyelination. Mol Cell Neurosci 1998; 12: 220-227.
8) Matsushima GK, Morell P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. Brain Pathol 2001; 11: 107-116.
9) Franco-Pons N, Torrente M, Colomina MT, Vilella E. Behavioral deficits in the cuprizone-induced murine model of demyelination/remyelination. Toxicol Lett 2007; 169: 205-213.
10) Adamo AM, Paez PM, Escobar-Cabrera OE, Wolfson M, Franco PG, Paquini JM, Soto EF. Remyelination after cuprizone-induced demyelination in the rat is stimulated by apotransferrrin. Exp Neurol 2006; 198: 519-529.
11) Skripuletz T, Lindner M, Kotsiari A, Garde N, Fokuhl J, Linsmeier F, Trebst C, Stangel M. Cortical demyelination is prominent in the murine cuprizone model and is strain-dependent. Am J Pathol 2008; 172: 1053-1061.
12) Lindner M, Heine S, Haastert K, Garde N, Fokuhl J, Linsmeier F, Grothe C, Baumgartners W, Stangel M. Sequential myelin protein expression during remyelination reveals fast and efficient repair after central nervous system demyelination. Neuropathol Appl Neurobiol 2008; 34: 105-114.
13) Love S. Cuprizone neurotoxicity in the rat: morphologic observations. J Neurol Sci 1988; 84: 223-237.
14) Franco P, Silvestroff L, Soto E, Pasquini J. Thyroid hormones promote differentiation of oligodendrocyte progenitor cells and improve remyelination after cuprizone-induced demyelination. Exp Neurol 2008; 212: 458-467.

15) Silvestroff L, Bartucci S, Pasquini J, Franco P. Cuprizone-induced demyelination in the rat cerebral cortex and thyroid hormone effects on cortical remyelination. Exp Neurol 2012; 235: 357-367.

16) Kanno T, Sasaki S, Yamada N, Kawasako K, Tsuchitani M. Hexachlorophene and cuprizone induce the spongy change of the developing rat brain by different mechanisms: the role of 2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase). J Vet Med Sci 2012; 74: 837-843.

17) Irvine KA, Blakemore WF. Age increases axon loss associated with primary demyelination in cuprizone-induced demyelination in C57BL/6 mice. J Neuroimmunol 2006; 175: 69-76.

18) Song SY, Kato C, Adachi E, Moriya-Sato A, Inagawa-Ogashiwa M, Umeda R, Hashimoto N. Expression of an Acyl-CoA Synthetase, lipidosin, in astrocytes of the murine brain and its up-regulation during remyelination following cuprizone-induced demyelination. J Neurosci Res 2007; 85: 3586-3597.

19) Kose H, Basoglu H. Analysis of spinal nerve conduction measurement with epidural stimulation in rats. J Neurol Sci (Turkish) 2010; 27: 112-120.

20) Jurevics H, Hostettler J, Muse ED, Sammond DW, Matsushima GK, Toews AD, Morell P. Cerebroside synthesis as a measure of the rate remyelination following cuprizone-induced demyelination in brain. J Neurochem 2001; 77: 1067-1076.

21) Liebetanz D, Merkler D. Effects of comissural de- and remyelination on motor skill behaviour in the cuprizone mouse model of multiple sclerosis. Exp Neurol 2006; 202: 217-224.

22) Ventruni G. Enzymic activities and sodium, potassium and copper concentrations in mouse brain and liver after cuprizone treatment in vivo. J Neurochem 1973; 21: 1147-1151.

23) Politis MJ, Schaumberg HH, Spencer PS. Neurotoxicity of selected chemicals. In: Schaumberg HH, editor(s). Experimental and Clinical Neurotoxicology, 1st ed. Williams and Wilkins, 1980.

24) Mason JL, Langaman C, Morell P, Suzuki K, Matsushima GK. Episodic demyelination and subsequent remyelination within the murine central nervous system: changes in axonal caliber. Neuropathol Appl Neurobiol 2001; 27: 50-58.

25) Berne RM, Levy MN. Physiology. St. Louis, Missouri. Mosby-Year Book inc 1993.

26) Sekiguchi M, Aoki Y, Konno S, Kikuchi S. The effect of Cilostazol on nerve conduction velocity and blood flow. Spine 2008; 33: 2605-2611.

27) Jia X, Zhen G, Putten A, Zhang J, Chen T. Improved long-term recording of nerve signal by modified intrafascicular electrodes in rabbits. Microsurgery 2008; 28: 173-178.