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BRIEF COMMUNICATION

Cuprizone does not induce CNS demyelination in nonhuman primates

Zhihong Chen[1,a], Jacqueline T. Chen[1,a], Matthew Johnson[1], Zachary C. Gossman[1], Megan Hendrickson[1], Ken Sakaie[2], Clarissa Martinez-Rubio[3], John T. Gale[1,4] & Bruce D. Trapp[1]

[1]Department of Neurosciences, Cleveland Clinic, Cleveland, Ohio
[2]Neurological Institute, Cleveland Clinic, Cleveland, Ohio
[3]Department of Neurosurgery, Massachusetts General Hospital, Boston, Massachusetts
[4]Center for Neurological Restoration, Neurological Institute, Cleveland Clinic, Cleveland, Ohio

Correspondence
Bruce D. Trapp, Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, Euclid Avenue, NC30, Cleveland, OH 44195. Tel: 216-444-7177; Fax: 216-444-7927; E-mail: trappb@ccf.org

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[a]Equal contribution.

Abstract

Cognitive decline is a common symptom in multiple sclerosis patients, with profound effects on the quality of life. A nonhuman primate model of multiple sclerosis would be best suited to test the effects of demyelination on complex cognitive functions such as learning and reasoning. Cuprizone has been shown to reliably induce brain demyelination in mice. To establish a nonhuman primate model of multiple sclerosis, young adult cynomolgus monkeys were administered cuprizone *per os* as a dietary supplement. The subjects received increasing cuprizone doses (0.3–3% of diet) for up to 18 weeks. Magnetic resonance imaging and immunohistological analyses did not reveal demyelination in these monkeys.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). Increases in cortical[1,2] and hippocampal lesion load[1] as well as decreases in magnetization transfer ratio (MTR) in both gray[3–5] and normal-appearing white matter[3,6,7] have been shown to be associated with a decline in cognitive performance, suggesting that demyelination is an important factor in cognition dysfunction.

Cuprizone, a copper chelator, has been administered to mice to induce demyelination in the CNS. When co-administered with the antibiotic Rapamycin,[8] which is known to suppress spontaneous remyelination, the cuprizone/rapamycin protocol in mice is a robust animal model of CNS demyelination and MS.[8] In addition to demyelination of the white matter, this model also features extensive gray matter demyelination in neocortex, piriform cortex, and hippocampus. Interestingly, once cuprizone is withdrawn from the diet, demyelinated brain regions undergo spontaneous remyelination. Data suggest that cuprizone-demyelinated rodents are cognitively deficient, modeling what has been observed in MS patients.[9] Experimental autoimmune encephalitis (EAE) is a well-established animal model which recapitulates some MS symptoms; however, heterogeneity in the susceptibility of induction results in high variability in the location and extent of demyelination, and remyelination does not occur reliably. An animal model that can accurately inform on the effects of demyelination and remyelination

on complex functions would be invaluable for the development and testing of therapies to improve remyelination and to restore neurological function. Here, we proposed to establish a nonhuman primate model of MS based on the robust cuprizone/rapamycin rodent protocol. Young adult cynomolgus monkeys were administered cuprizone *per os* as a dietary supplement. Magnetic resonance imaging (MRI) and immunohistological analyses were performed to evaluate the brain for demyelination. We have found that cuprizone, at a concentration up to 3% of animal feed and administered for up to 18 weeks, does not induce demyelination in young adult monkey brains.

Materials and Methods

Subjects

One female (Animal #1, 2–3 years old) and one male (Animal #2, 2–3 years old) cynomolgus macaque (*Macaca fascicularis*) were administered cuprizone and rapamycin *per os* as a voluntary dietary supplement. The brain from a third male (5–6 years old), which was used as an organ donor for transplant studies and had no known diseases, was included as a healthy control for histological analysis. All procedures were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic and in accordance with the United States Public Health Services' Policy on Humane Care and Use of Laboratory Animals.

MRI acquisition and analysis

Acquisition

In vivo brain MRIs were performed (3 T Tim Trio, Siemens, Germany) at baseline and at 6 weeks intervals. Animals were sedated with Ketamine (10 mg/kg), Xylazine (0.5 mg/kg), and Atropine (0.05 mg/kg, IM). Anesthesia was maintained with Ketamine (5–20 mg/h, constant infusion). Heart rate and SpO_2 were continuously monitored. Imaging sequences were chosen to minimize scan time and inform on anatomy and myelin. T1-weighted (T1w) (repetition time [TR] = 1860 msec; echo time [TE] = 2.8 msec; inversion time [TI] = 1100 msec; flip angle [FA] = 10°; voxel size = 0.9 mm isotropic) and a pair of gradient recalled echo volumes with/without a magnetization transfer prepulse (MT_{ON} and MT_{OFF}: TR = 24 msec; TE = 3.81 msec; FA = 10°; voxel size = 1 mm isotropic) for MTR calculation were acquired. Higher resolution imaging (0.6 mm isotropic voxels, only acquired if the animal's physiological metrics were stable) included T1w (TR = 2300 msec; TE = 3.8 msec; TI = 900 msec; FA = 9°) and the MT_{ON} and MT_{OFF} pair (TR = 25 msec; TE = 4.3 msec; FA = 10°).

Analysis

T1w MRI was corrected for intensity nonuniformity[10] and registered[11] to a cynomolgus MRI standard space.[12] Four regions of interest (ROIs: lateral ventricles, neocortex, hippocampus and corpus callosum) for MTR analysis were defined on the baseline T1w MRI. ROIs were transformed to MT_{OFF}-space and manually corrected. MT_{ON} images were registered to MT_{OFF} and MTR was calculated ($100 \times [MT_{OFF} - MT_{ON}]/MT_{OFF}$). The MTR measurement noise was estimated as the average of the standard deviations of the baseline MTR values for each brain tissue ROI.

Cuprizone administration

Dosing consisted of a mixture of treat enrichment with cuprizone (Sigma, St. Louis, MO, US) and rapamycin (LC Laboratories, Woburn, MA, US). Rapamycin was used to suppress spontaneous remyelination; the 5 mg/kg daily dose remained unchanged throughout the study. Cuprizone and rapamycin were formulated as a powder and mixed in a small stainless steel bowl with a variety of treats, including jelly, jam, peanut butter, applesauce, pudding, honey, and pie filling. The treat was changed frequently and fruit and other items were sometimes added as incentive for the animal to retrieve the treat from the container. The bowl was left in the animal's cage early each morning and retrieved once the treats were fully consumed, which occurred before their regular daily biscuits were given. The complete consumption of the treat was visually confirmed by the investigators. Animal #1 was started on an initial dose of cuprizone (0.3% w/w of the animal feed) for the first 6 weeks of the study. After the 6-week follow-up MRIs revealed no abnormalities, the daily dose of cuprizone was increased to 0.6% for another 6 weeks. An additional dose increase of cuprizone (2%) was given for an additional 6 weeks after the 12-week follow-up MRIs revealed no abnormalities. After 18 weeks of dosing this animal underwent a final MRI and was euthanized. Animal #2 was started at a dose of cuprizone (2% w/w feed) and rapamycin (5 mg/kg) for 6 weeks. After the 6-week follow-up MRIs revealed no abnormalities, the daily dose of cuprizone was increased to 3% for an additional 6 weeks. After 12 weeks of dosing this animal underwent a final MRI and was euthanized. Animals are monitored frequently for deleterious neurologic deficits, but none was observed. This monitoring included assessing the ability of the animal to obtain treats using both hands, bilateral symmetry of movements within their cage, the ability to coordinate and track normally with the eyes, as well as observations of social behavior when pair-housed.

Immunohistochemistry

Procedures were performed as previously described.[13] The primary antibodies used were rat-anti-PLP (1:200) and mouse-anti-Iba-1 (1:500, CCF Hybridoma Core, Cleveland Clinic, Cleveland, OH, US).

Statistical analysis

The change in MTR between baseline and follow-up was assessed using R (R Core Team 2013) and lme4 (Bates et al. 2014) to perform a linear mixed-effects analysis. Time-point was considered as a fixed effect and region within subject was considered as a random effect.

Results

Cuprizone does not induce brain MRI abnormalities

No regions of hypointensity were found on T1w MRIs at baseline or at any other time-point (Fig. 1), suggesting an absence of focal demyelination. MTR, with higher specificity to myelin density compared to conventional MRI, did not decrease between baseline and the final imaging visit (after 12–18 weeks of cuprizone/rapamycin treatment) within the brain tissue ROIs, suggesting an absence of demyelination.

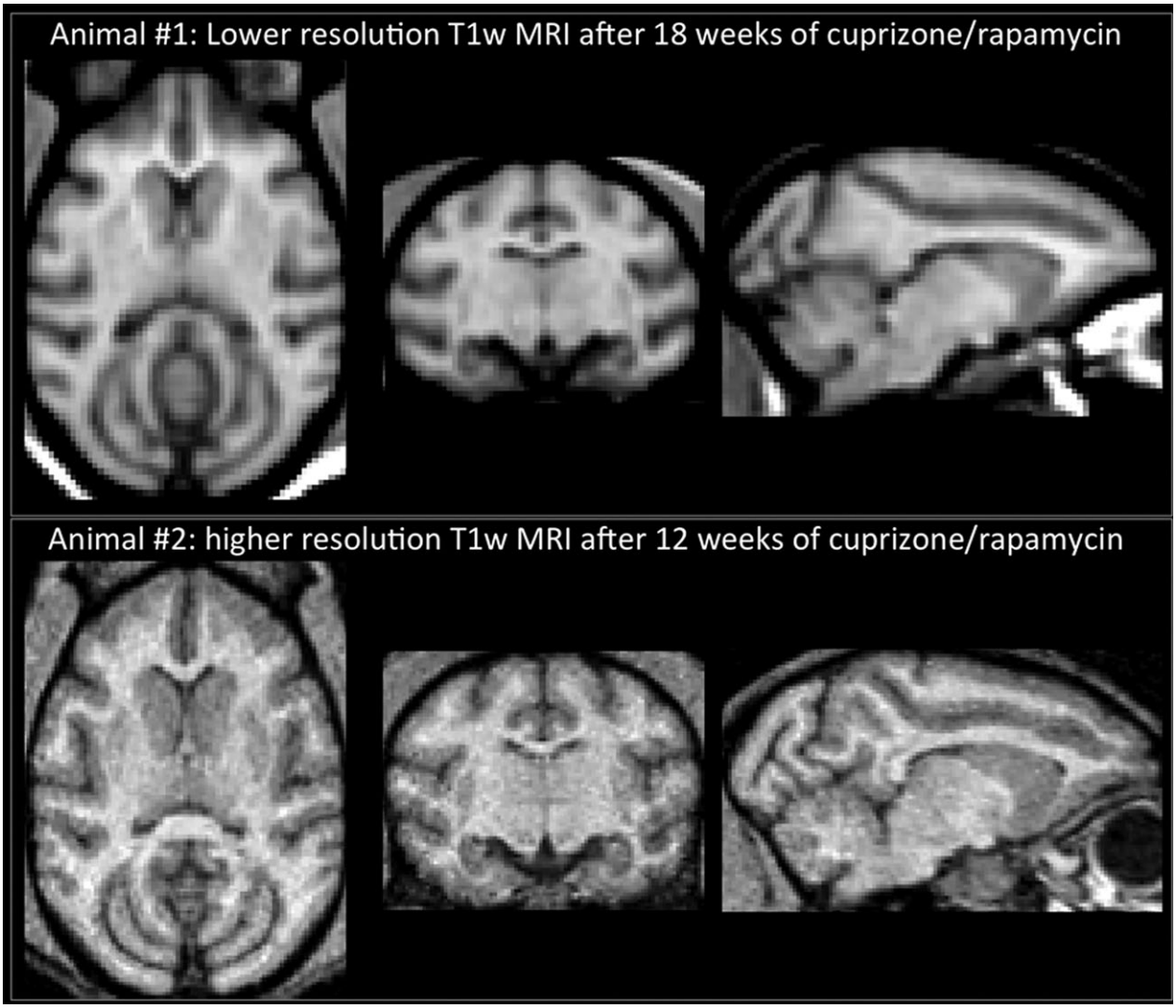

Figure 1. No regions of hypointensity found on T1w MRIs after >12 weeks of cuprizone/rapamycin treatment. Top row: Axial, coronal, and sagittal views of T1w MRI obtained on Animal #1 after 18 weeks of cuprizone/rapamycin treatment (no high resolution images were acquired at this time-point). Bottom row: High-resolution T1w MRI obtained on Animal #2 after 12 weeks of cuprizone/rapamycin treatment. No foci of hypointensity suggesting demyelination are apparent.

Histopathological examination confirms that cuprizone does not induce demyelination in brains of cynomolgus monkeys

To confirm whether demyelination occurred in cuprizone-fed monkeys, brain sections from the frontal lobe and hippocampus were stained with anti-PLP antibody to visualize myelin (Fig. 2). Compared with the healthy control, cuprizone did not induce obvious demyelination in the frontal cortex, corpus callosum, or the hippocampus. In areas where myelin staining appears to be less dense than others, close examination in searching for frank pathology such as disintegrated myelin sheaths or myelin debris did not yield any findings (not shown). The same brain regions were also stained with anti-Iba1 antibody to visualize microglia. The microglia in cuprizone-fed monkeys appeared to be in a mildly activated state with ramified morphology and increased intensity of Iba-1 staining (Fig. 2). Amoeboid macrophages were not detected within the sections we examined. Therefore, we conclude that an extended period of cuprizone feeding does not induce demyelination in cynomolgus monkeys.

Discussion

Cuprizone causes widespread demyelination in rodent brains. When adult C57Bl/6 wild-type mice are fed a 0.3% (w/w) cuprizone feed for 6-week *ad libitum*, demyelination can be observed in various brain regions. If mice are kept on this diet for an additional 6 weeks, demyelination can be both extensive and severe; hippocampal myelin can be reduced by up to 95%.[8] There is also a difference in the pattern of demyelination between mouse strains, such that SJL mice did not readily demyelinate at the midline within the corpus callosum but showed greater demyelination immediately lateral to midline, differing from that of C57Bl/6.[14] Studies on cuprizone-induced demyelination in

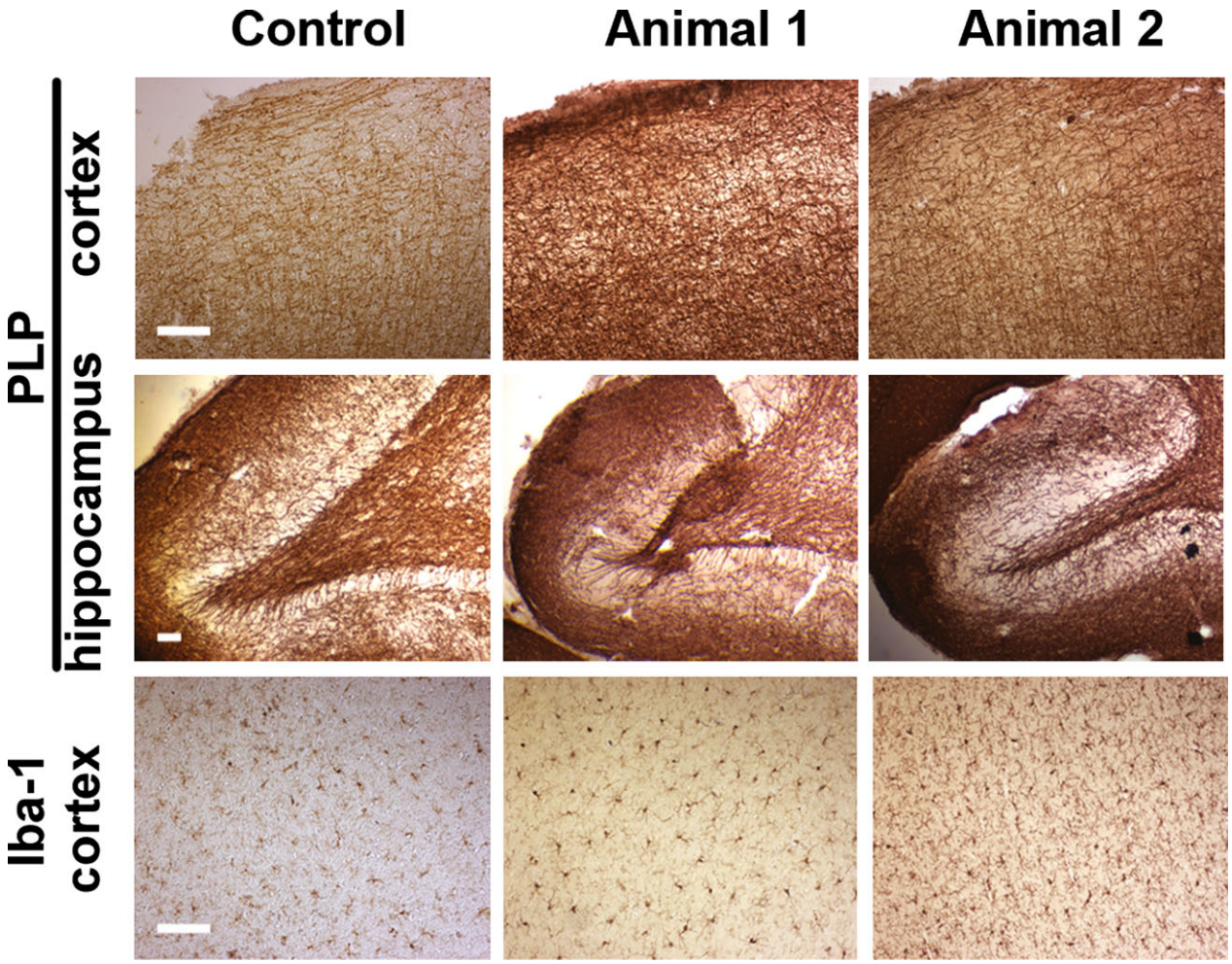

Figure 2. Immunohistochemistry micrographs showing myelin staining (PLP) in the cortex, corpus callosum, and hippocampus of control and cuprizone-fed monkeys and microglia staining (Iba-1) in the cortex. Scale bar = 50 μm across each row.

other species are scarce, and demyelination in nonhuman primates associated with this dietary toxin has never been reported. In this study, we attempted to establish a demyelinating disease model in nonhuman primates. Such a model would be invaluable for the development and testing of therapies to improve remyelination and to restore cognitive function. We provided young adult cynomolgus monkeys with daily cuprizone/rapamycin as a voluntary dietary supplement for up to 18 weeks at increasing doses (from 0.3% to 3% feed). Our results show that demyelination in these monkeys did not occur as a result of cuprizone feeding, despite the escalating concentrations of cuprizone over an extended period of time. The mechanism of cuprizone-induced demyelination is still not well understood, however, this differential response between mice and monkeys can possibly be explained by their differences in metabolism and immune responses.[15]

It was recently reported that myelination is not complete in young primates until the age of sexual maturity.[16] Indeed, we have observed that in some areas of the cortex of cuprizone-fed monkeys, myelin density appeared to be thinner than in others (not shown). However, this differential distribution of myelin was not significantly different between the cuprizone-treated monkeys and the healthy control.

Our study reveals that the cuprizone/rapamycin protocol, which produces robust brain demyelination in mice, does not yield a nonhuman primate model of MS. However, we acknowledge that there are limits to our study, such as the small sample size, the relatively young age, and the single species of nonhuman primates that were used. Recently, spontaneous demyelination was found in a colony of Japanese Macaques in the Oregon National Primate Research Center, and viral infection was suspected to be the cause.[17] This raises the possibility that viral infections can be used in developing demyelinating models in highly evolved nonhuman primates.

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Conflict of Interest

Dr. Trapp reports grants from National Multiple Sclerosis Society during the conduct of the study; personal fees from EMD Serono, Genzyme, Norvartis, Renovo Neural, Biogen Idec, and Endece outside the submitted work. Dr. Chen reports grants from National Multiple Sclerosis Society, during the conduct of the study. Dr. Sakaie reports grants from National Multiple Sclerosis Society, during the conduct of the study; grants from Novartis, outside the submitted work. Ms. Hendrickson reports grants from National Multiple Sclerosis Society, during the conduct of the study. Mr. Johnson reports grants from National Multiple Sclerosis Society, during the conduct of the study. Dr. Chen reports grants from National Multiple Sclerosis Society, during the conduct of the study. Mr. Gossman reports grants from National Multiple Sclerosis Society, during the conduct of the study. Dr. Gale reports grants from National Multiple Sclerosis Society,during the conduct of the study.

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