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An Alternative Cuprizone-Induced Demyelination and Remyelination Mouse Model

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Abstract

The cuprizone model is a well-established and investigated paradigm to study demyelination and remyelination in rodents. Cuprizone is usually administrated by mixing in the powdered or pelleted rodent chow. However, since cuprizone is sensitive to the environment and the consumption of it varies between different animals, the major issue is the discrepancy in demyelination of the animals. This study reports the development of the cuprizone model by gavage administrations in mice. Following testing a series of doses of cuprizone, 400 mg/kg/day was found to be the best dosage to induce dramatic and consistent demyelination after 5 weeks of administration; while remyelination quickly occurred after 9 days of cuprizone withdrawal. The advantage of this alternative model is that the consumption of cuprizone could be well controlled, and the mice were exposed to the same dose of cuprizone. Thus, the variation in demyelination was minimized. This alternative cuprizone dosing regime minimizes the interanimal variability on demyelination and hence provides a consistent model for pharmacological evaluations, in addition to reducing the number of animals used in the experiments.

Keywords

animal model, cuprizone, demyelination, oral administration, remyelination

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Introduction

Demyelination of the central nervous system (CNS) is a hallmark of diseases such as multiple sclerosis (MS). The pathology of active demyelination lesions has previously been analyzed and has been categorized into distinct patterns (Lucchinetti et al., 1999, 2000). Four fundamentally different patterns of demyelination have been defined on the basis of I: myelin protein loss, II: the geography and extension of the plaques, III: the patterns of oligodendrocyte destruction, and IV: the immunopathological evidence of complement activation. Patterns I and II were described as predominantly immune-mediated inflammation, and Patterns III and IV were induced by primary oligodendrogliopathy (Lucchinetti et al., 2000). Although remyelination frequently occurs after demyelinating events, it is often incomplete (Patrikios et al., 2006; Goldschmidt et al., 2009). Thus, one of the challenges in MS research is to understand the mechanism by which remyelination fails and to develop strategies to restore myelination. An animal model is a valuable tool to investigate the mechanisms underlying demyelination and remyelination and to study the cellular responses and interplay during these processes, thus providing a platform to elucidate putative therapeutic targets.

Cuprizone-induced toxicity has been extensively used to identify treatments for demyelinating diseases (Matsushima and Morell, 2001; Kipp et al., 2009; Skripuletz et al., 2011). Cuprizone, oxalic acid

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bis(cyclohexylidene hydrazide), a copper-chelating agent, induces a highly reproducible demyelination in distinct brain regions, among which the corpus callosum (CC) represents the most frequently investigated white matter tract in animal models (Stidworthy et al., 2003; Komoly, 2005). In the cuprizone model, animals are fed with cuprizone that causes cell death of oligodendrocytes resulting in consistent demyelination (Torkildsen et al., 2008). However, because cuprizone does not destroy new oligodendrocytes, significant spontaneous remyelination occurs during and following cuprizone-induced demyelination (Bai et al., 2016). Furthermore, removal of cuprizone from the diet of animals enhances the remyelination (Morell et al., 1998). In current cuprizone models, animals were fed with either powder diet mixed with cuprizone or cuprizone-containing pellets (Liu et al., 2010). It is unknown whether cuprizone in the powdered diet was mixed homogeneously, and the amounts of cuprizone intake were the same in different animals. Besides that, cuprizone can be easily degraded due to exposure to the environment (Gudi et al., 2014). In addition, since cuprizone diet is placed in the cage, some of the cuprizone in the diet may lose its activity leading to further variation in the amounts of cuprizone consumed by the animals. As a result, the cuprizone intake of each animal can differ leading to a discrepancy in demyelination of each animal.

To solve this issue, oral administration was conducted to control the amount of cuprizone consumption. Basoglu et al. (2013) have reported that demyelination was well induced by giving a daily standard dose of cuprizone to rats by oral gavage. This study reports an alternative cuprizone model in mice by oral gavage administrations to reduce the discrepancy in demyelination in the CNS tissue.

Materials and Methods

Reagent

Cuprizone was purchased from Sigma (St. Louis, MO). Black-Gold II myelin staining kit was obtained from Millipore (Temecula, CA; Cat#: AG105). All other chemicals were analytical grade reagents of the highest purity available.

Animals

Eight- to 10-week-old male C57BL/6Bkl mice were obtained from the breeding colony of the Shanghai Sippr-*BK* Laboratory Animal Co. Ltd. (originally from B&K Universal Ltd in UK), and all studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed. Mice were housed five per cage with food and water available ad libitum and were kept on a daily 12 hr light cycle. Temperature and humidity were controlled.

Cuprizone Treatment

For oral gavage, cuprizone powder was mixed in 1% methyl cellulose (MC) and vortexed in order to obtain a homogeneous cuprizone–MC suspension. Mice were fed daily or twice a day (b.i.d.) by gavage with 10 ml/kg volume. Stock solution of 1% MC was prepared weekly and stored at 4°C. Cuprizone and MC suspension were freshly prepared and consumed daily. Cuprizone did not react chemically with MC and was just present in the suspension mixture. The vehicle group received 1% MC without cuprizone by gavage. The mice were randomly divided into groups, and the scientists dosing the mice and analyzing the data were blind to the treatment. All groups of mice were weighed 3 times per week during cuprizone administrations until they were sacrificed. The animal numbers of each treatment groups were from four to seven mice, and these group sizes were determined by power analysis calculations. Tissue samples from the control group, vehicle group, and cuprizone treatment group were processed at the same time within the same experiment.

Tissue Preparation

Mice were euthanized with 4% isoflurane and subsequently perfused with phosphate buffer saline (PBS). Brains were carefully removed and postfixed overnight in 4% paraformaldehyde at 4°C. All samples were then transferred into 30% sucrose to dehydrate at 4°C. Finally, the samples were embedded in optimal cutting temperature compound, frozen on dry ice, and stored in −80°C until processed.

Black-Gold II Staining

The whole brain was frozen-sectioned in coronal plane at 30 µm thickness using a cryostat microtome (Leica CM1950). For each animal, two sections from hindbrain around the bregma −1.82 mm and two sections from forebrain around bregma 0.62 mm were chosen for Black-Gold II staining. Briefly, the sections were washed in H_2O and incubated in Black-Gold II solution at 60°C for 20 min until desired signals developed. After rinsing in H_2O on ice and incubating in 1% sodium thiosulfate solution at 60°C for 6 min, the sections were rinsed in PBS containing 0.01% Tween (PBST), dried at 37°C, dehydrated, and mounted with mounting medium. The sections were scanned by ScanScope XT (Leica Biosystem) at 20× magnification. Digital images of

Black-Gold II staining in CC from forebrain and hindbrain were captured at 5× magnification using ImageScope (Leica Biosystems Imaging, Vista, CA) and put together using Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA) for density quantification.

Quantification and Statistical Analysis

Image-Pro 6.3 software (Media Cybernetics, Bethesda, MD) was used for image analysis. To measure the signal intensity in the CC, the medial CC area, as an area of interest (AOI), was outlined and the area and integrated optical density (IOD) were the two selected parameters used for the analysis (similar to that described by Aparicio et al., 2013). Using the HSI (Hue–Saturation–Intensity) histogram-based model of the "segmentation" command to identify the range of positive stained signal (Lejeune et al., 2008) in order to differentiate positive- and negative-stained myelin. The HIS setting was as follows: hindbrain: H = 234–243, S = 0–255, I = 0–255; forebrain: H = 233–243, S = 0–255, I = 0–255 (Experiment I); hindbrain: H = 233–242, S = 0–255, I = 0–255; forebrain: H = 234–243, S = 0–255, I = 0–255 (Experiment II). The final read-out was expressed as mean density = the ratio of IOD units/AOI area (Aparicio et al., 2013). The average mean density of CC in naïve or vehicle group was considered as "100%" (baseline), and the values of signals in cuprizone group were normalized accordingly, expressed as percentage of mean density of the control group. Column statistical analysis was performed by GraphPad PRISM 6 software (GraphPad Software, Inc., La Jolla, CA) first to see if the data follow a normal distribution. Analysis of variance (ANOVA) followed by appropriate multiple comparison method (Dunnett or Sidek) was used to analyze the difference between the groups. For the difference of the body weight loss between the groups, ANOVA with Dunnett's test was performed, and p value $< .05$ was considered statistically significant. Significance is indicated in the figures by asterisks $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$. The coefficient of variation (CV %) of density of each group was calculated by SD/mean density × 100%. The analysis of each figure in this article represents an independently treated cohort of animals.

Results

Tolerance of the Animal on Different Dosages of Cuprizone

To optimize the consistence of demyelination, a study was carried out using daily administration of cuprizone to animals by oral gavage. Since cuprizone has a variety of biotoxic effects in mice, a dose that consistently led to demyelination in the absence of overt general toxicity was firstly determined. Based on the previously reported information that daily food intake of mouse is about 5 g/30 g body weight (Bachmanov et al., 2002), and usually 0.2% cuprizone was mixed in powder diet, the consumption of cuprizone per day was assessed to be approximately 300 mg/kg. Thus, doses of 100 mg/kg, 300 mg/kg, and 1000 mg/kg cuprizone were selected for gavage administration to determine the most appropriate doses for further studies. The results (Figure 1) showed that the body weight of mice was decreased after all doses of cuprizone. But all mice in 1000 mg/kg group died after 12 days of cuprizone administrations. The mortality was 100% indicating this dosage was too high. However, the mice in the 100 mg/kg and 300 mg/kg groups survived during 5 weeks' administrations and showed essentially normal behavior except the decrease of body weight.

Effect of Oral Gavage Treatment of Cuprizone on Demyelination

To further determine the dose of cuprizone tolerated by the mice and investigate whether the demyelination could be successfully induced by oral gavage cuprizone administrations, the mice were dosed daily by gavage with cuprizone suspension for 5 weeks at 400 mg/kg and 800 mg/kg. Figure 2(a) shows that all mice from 800 mg/kg group died during dosing which indicates this dosage is also too high. However, the mice from 400 mg/kg survived for 5 weeks of administrations and

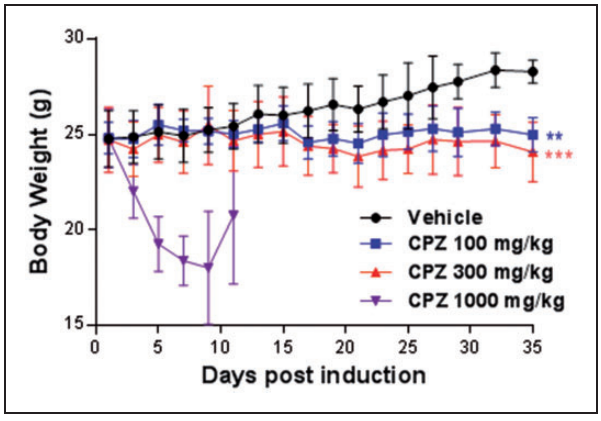

Figure 1. The body weight and survival of mice treated with different gavage dosages of cuprizone. The body weight was measured 3 times/week during 5-week cuprizone administrations ($n = 4$). The body weight showed that CPZ 1000 mg/kg group was toxic to mice, and all mice died after 12 days of cuprizone administrations. The CPZ 100 and CPZ 300 mg/kg groups had no mortality. ANOVA with Dunnett's test was performed to analyze the difference between the groups, and p value $< .05$ was considered statistically significant, $**p < .01$, $***p < .001$. g = gram; CPZ = cuprizone.

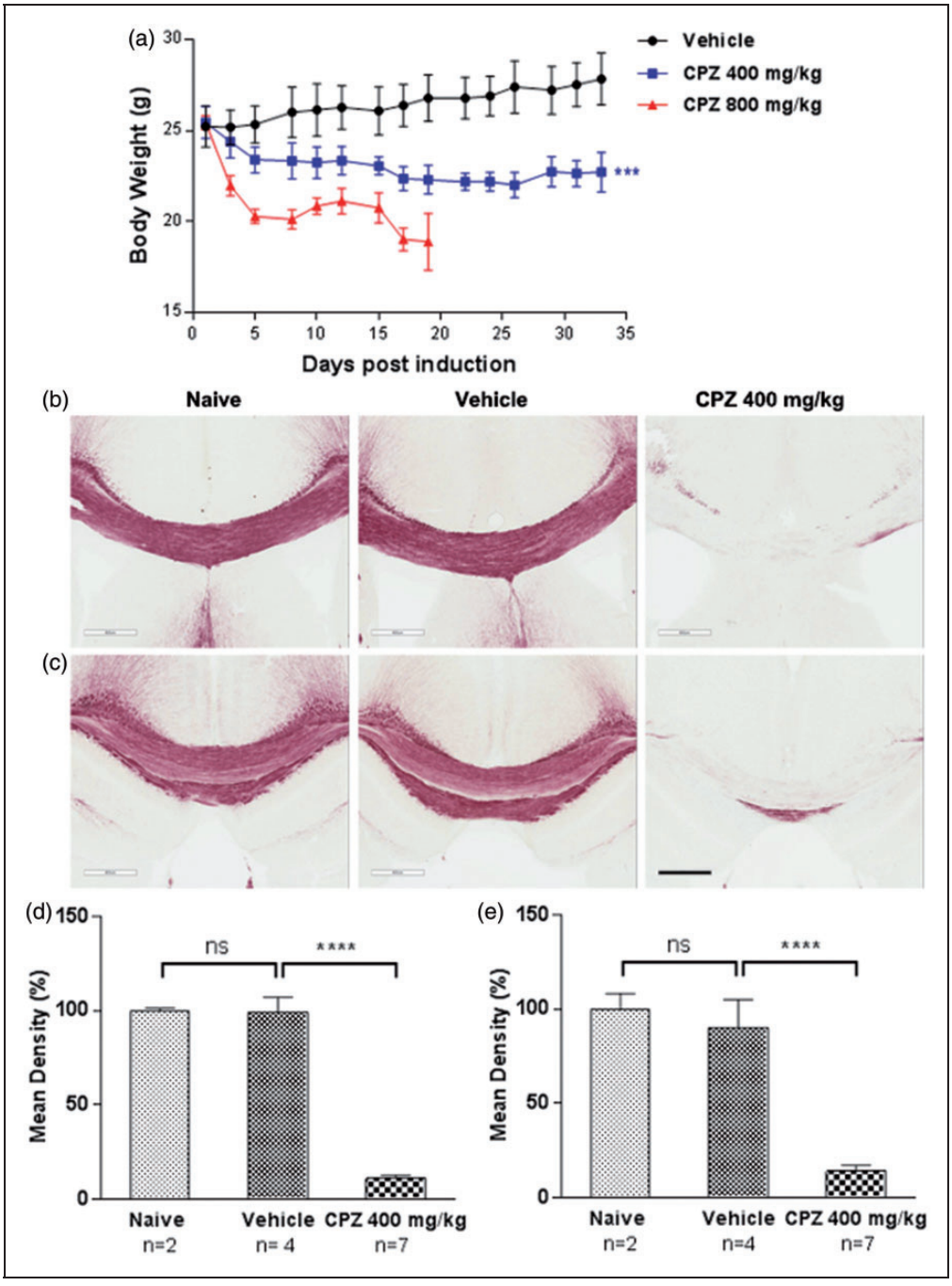

Figure 2. Demyelination induced by cuprizone oral gavage administrations. (a) The body weight was measured 3 times/week during 5-week cuprizone administrations for different dosage groups (vehicle group: $n = 4$; oral gavage groups: $n = 7$). After the cuprizone oral dosing, the body weight of all oral gavage groups decreased. All mice died after 20 days of cuprizone administrations in 800 mg/kg group. ANOVA with Dunnett's test was performed to analyze the difference between the groups, and p value $< .05$ was considered statistically significant, $^{***}p < .001$. Representative images of Black-Gold II staining in the forebrain (b) and hindbrain (c) of corpus callosum region from the mice in each group. Scale bar = 400 μm ((b) and (c)). Quantification of mean density of Black-Gold II staining in corpus callosum region from forebrain (d) and hindbrain (e) reveals prominent demyelination in cuprizone oral dose groups. ANOVA with Dunnett's test was performed to analyze the difference between the groups. Error bars represent standard errors of the mean (SEM), and p value $< .05$ was considered statistically significant, $^{****}p < .0001$, ns indicates no significant difference. g = gram; CPZ = cuprizone.

showed normal behavior except for decreases in body weight which has been reported to be a surrogate marker of the desired *in vivo* demyelination activity of cuprizone (Hiremath et al., 1998).

Images of Black-Gold II staining of medial CC are shown in Figure 2(b). As expected, significant demyelination was observed in both forebrain and hindbrain of the CC in 400 mg/kg cuprizone group. Myelin levels in

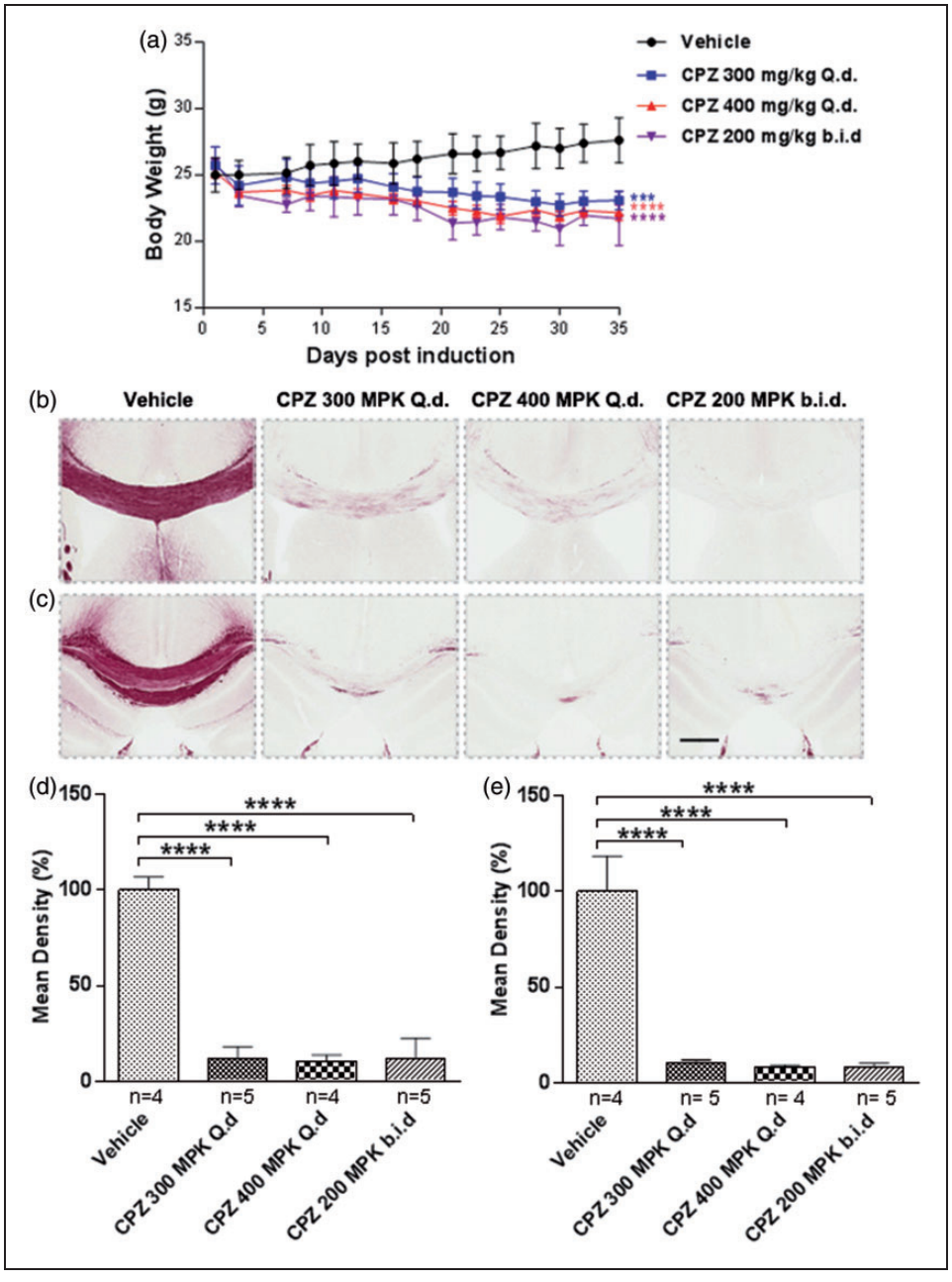

Figure 3. Optimization of cuprizone oral dosage for demyelination induction. (a) The body weight was measured 3 times/week during 5-week cuprizone (CPZ) administrations for different groups of animals (vehicle and 400 mg/kg groups: $n = 4$; 200 mg/kg and 300 mg/kg groups: $n = 5$). After the cuprizone oral dosing, the body weight of all oral gavage groups decreased. ANOVA with Dunnett's test was performed to analyze the difference between the groups, and p value $< .05$ was considered statistically significant, ***$p < .001$, ****$p < .0001$. (b) and (c) Representative images of Black-Gold II staining in the forebrain (b) and hindbrain (c) of corpus callosum region from the mice. Scale bar = 400 μm ((b) and (c)). Quantification of mean density of Black-Gold II staining in corpus callosum region from forebrain (d) and hindbrain (e) reveals prominent demyelination in CPZ oral dose administration groups. ANOVA with Dunnett's test was performed to analyze the difference between groups. Error bars represent standard error of the mean (SEM), and p value $< .05$ was considered statistically significant. ****$p < .0001$. g = gram; CPZ = cuprizone; Q.d. = once a day; b.i.d. = twice day; MPK = mg/kg.

forebrain and hindbrain of medial CC was dramatically reduced to 11% and 14%, respectively (Figure 2(c) and (d)), as compared with the vehicle group as assessed by the density of Black-Gold II staining. Moreover, the CV% of forebrain and hindbrain in this cuprizone group was only 15% and 22%, respectively. These results indicated that administrating the mice with 400 mg/kg cuprizone suspension daily could induce a significant demyelination in CC with consistent levels of demyelination between animals.

Optimization of Cuprizone Dosage for Demyelination Induction

The dosage and regimen were further optimized to explore reducing the exposure to cuprizone by assessing twice daily administration instead of a single bolus to reduce the instantaneous exposure peak of cuprizone. The studies of 300 mg/kg, once a day (Q.d.) and 200 mg/kg, b.i.d. (a dosing regimen in which the total amount of cuprizone intake per day is 400 mg/kg) were used in addition to 400 mg/kg Q.d. group.

Treatment with 300 mg/kg Q.d. and 200 mg/kg b.i.d. showed similar effects on body weight (Figure 3(a)) and Black-Gold II staining of CC (Figure 3(b) and (c)) compared with 400 mg/kg Q.d. group. In the 300 mg/kg group, the myelin levels in the forebrain and hindbrain of CC were reduced to 11% and 10%, respectively; while in the 200 mg/kg b.i.d. group, myelin levels in the same two regions were reduced to 11% and 8%, respectively (Figure 3(d) and (e)). On the other hand, the CV% of forebrain and hindbrain myelin levels in the 300 mg/kg group was 54.4% and 16.5%; while in 200 mg/kg b.i.d. group, the CV% was 93.8% and 25.0%. In comparison, the CV% of 400 mg/kg group in this study was 34.0% and 12.4%, similar to previous result. These results showed that a slight dose reduction (300 mg/kg Q.d.) or 200 mg/kg b.i.d. could also successfully induce demyelination of the CC at a level that was consistent with that obtained in the 400 mg/kg group.

The results of these studies presented in Table 1 show that gavage administration of cuprizone induced a significant decrease in myelin in comparison to the control group. Among the different oral dose groups, no obvious difference in the degree of demyelination (88%–92%) was observed expect that the CV% of hindbrain (12.4%–25%) was smaller than that of forebrain (34%–93.8%). This suggested that the variability of demyelination in hindbrain is lower than that in forebrain. Moreover, the CV% of 400 mg/kg group both in forebrain and hindbrain was smaller than that of other groups which indicated that 400 mg/kg daily was the most suitable dosage and regimen in this study.

Effect of Oral Gavage of Cuprizone on Animal Remyelination

To further examine whether the spontaneous remyelination process could occur after cuprizone withdrawal, the mice were fed with normal chow for 9 days following the 5-week oral administrations of 300 mg/kg or 400 mg/kg cuprizone. The mice in both groups rapidly gained similar body weight and reached the same body weight as the vehicle group (Figure 4(a)). Furthermore, the staining of Black-Gold II in the CC (Figure 4(b) and (c)) showed that a significant remyelination occurred after cuprizone withdrawal. In the 300 mg/kg group, the degree of remyelination in the forebrain and hindbrain of the CC was increased from 21% to 54% and from 22% to 53%, respectively. In the 400 mg/kg group, the degree of remyelination in forebrain and hindbrain of the CC was increased from 20% to 82% and from 15% to 69%, respectively, as compared with vehicle group (Figure 4(d) and (e)). The results indicated that the route of oral dose

Table 1. Effects of Different Routes and Dosages of Cuprizone Administrations on Mouse Myelination and the Coefficient of Variation.

Brain region	CPZ concentration	Route	Regimen	Demyelination (%)	Animal number	CV%
Forebrain	300 mg/kg	P.O.	Q.d.	88	5	54.4
	400 mg/kg	P.O.	Q.d.	89	4	34.0
	200 mg/kg	P.O.	b.i.d.	88	5	93.8
Hindbrain	300 mg/kg	P.O.	Q.d.	90	5	16.5
	400 mg/kg	P.O.	Q.d.	86	4	12.4
	200 mg/kg	P.O.	b.i.d.	92	5	25.0

Note. The percentage of demyelination and CV% of forebrain and hindbrain in each treatment group were listed in the table. The study results showed that all doses of cuprizone could induce obviously demyelination by oral administration. While the CV% of 400 mg/kg group was smaller than that of other groups. CPZ = cuprizone; P.O. = per oral; Q.d. = once a day; b.i.d., twice a day; CV%, coefficient of variation.

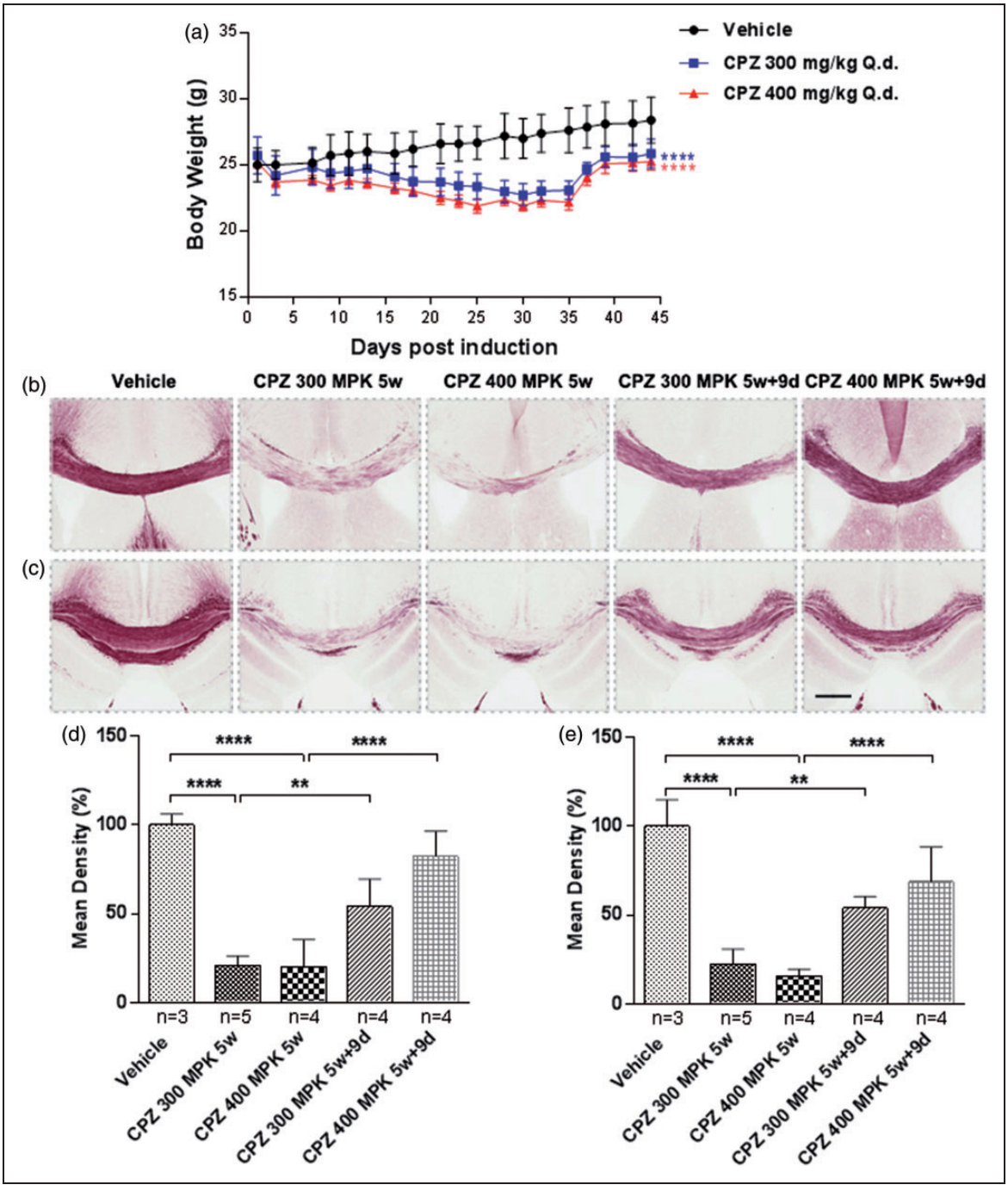

Figure 4. Remyelination after cuprizone withdrawal. (a) The body weight was measured 3 times/week during 5-week cuprizone administrations which was then followed by 9 days of cuprizone withdrawal (vehicle group: $n = 3$; 400 mg/kg group: $n = 4$; 300 mg/kg group: $n = 5$; cuprizone withdrawal groups: $n = 4$). During the cuprizone oral dosing, the body weight of oral gavage group decreased. After cupriozne withdrawal, cuprizone mice gained body weight rapidly. ANOVA with Dunnett's test was performed to analyze the difference between the groups, and p value $< .05$ was considered statistically significant, ****$p < .0001$. (b) and (c) Representative images of Black-Gold II staining in the forebrain (b) and hindbrain (c) of corpus callosum region from mice in each group show the demyelination and remyelination. Scale bar = 400 μm ((b) and (d)). (d) and (e) Quantification of mean density of Black-Gold II staining signals in corpus callosum region from forebrain (d) and hindbrain (e) reveals successful remyelination after cuprizone withdrawal. ANOVA with Sidek's test was performed to analyze the difference between groups. Error bars represent standard error of the mean (SEM), and p value $< .05$ was considered statistically significant. **$p < .01$, ****$p < .0001$. g = gram; CPZ = cuprizone; Q.d. = once a day; MPK = mg/kg; w = weeks; d = days.

cuprizone suspension could successfully induce significant demyelination in medial CC with less variability between animals and that spontaneous remyelination occurred quickly following the withdrawal of cuprizone treatment.

Discussion

Cuprizone-induced toxicity has been described as a useful animal model to study demyelination and remyelination. The history of the cuprizone model spans several decades. Blakemore (1972) described a massive depletion of oligodendrocytes as a primary reason for cuprizone-induced demyelination. His study showed that remyelination occurred spontaneously after removal of the cuprizone diet (Blakemore, 1973a) and established cuprizone administration in food as a model to study demyelination and remyelination in the animal brain (Blakemore, 1973b). Thereafter, Hiremath et al. (1998) determined some crucial points for this model, such as age of animals (8–10 weeks), dosage (0.2% [w/w] in powder standard rodent chow) and the duration of treatment (5–6 weeks). They also showed that the extent of demyelination in the CC, as detected by histological staining, could be easily scored (Hiremath et al., 1998), providing investigators with a reproducible and well predictable animal model for demyelination and remyelination.

However, a variety of different protocols have been subsequently developed that use diverse routes of cuprizone administration, concentrations and amounts of cuprizone used, and time points of the investigations, which led to difficulties in the interpretation of the data (Gudi et al., 2014). Cuprizone was usually administrated by mixing in the powdered rodent chow, but the powdered chow needs to be prepared freshly daily and is easily contaminated by mouse excretion. Cuprizone-containing pellets are also used. However, since cuprizone is a heat-sensitive compound (Gudi et al., 2014), it is critical to pay attention to control the temperature conditions when preparing the pellets. More importantly, the major challenge of this model was the variable consumption between individual animals in addition to cuprizone degradation as a result of exposure to environment. In this study, we successfully established an alternative cuprizone model by oral gavage dosing of cuprizone to mice. In order to give a standard dose to each animal, cuprizone was freshly suspended in MC solution and administrated by oral gavage. The advantage of this route is that the consumption of cuprizone could be well controlled by dosing to each animal according to body weight, ensuring that the mice were exposed to the same dose of cuprizone. Therefore, the variability of demyelination among animals could be reduced.

Following testing a series of doses of cuprizone for tolerability and the extent of demyelination, 400 mg/kg was identified as the best dosage in this oral gavage model. The Black-Gold staining showed that oral gavage cuprizone dosed daily for 5 weeks could induce significant demyelination in the CC region and, in addition, fast and robust remyelination occurred following the withdrawal of the cuprizone treatment. The oral gavage cuprizone model not only induced demyelination in the CC region, but also, more importantly, could potentially reduce the variability of demyelination in different experiments and individual animals within a given experiment. The level of demyelination was very consistent across the separate cohorts with the 400 mg/kg cuprizone treatment. The myelin levels in the forebrain and hindbrain of the CC were reduced to 11%, 10%, 20% and 14%, 8%, 15%, respectively in three independent experiments. Given that this cuprizone dosing regime significantly reduces the interanimal variability on demyelination, this alternative model is more reliable to support pharmacological studies. Moreover, the number of animals required can be minimized resulting in the use of fewer animals to answer scientific questions. Establishing a reliable and consistent model and reducing the animal use were the two major objectives for developing the oral gavage cuprizone model.

In summary, an alternative cuprizone model was developed and refined by using oral gavage in mice. By utilizing this method, demyelination was induced, and the variation of demyelination was minimized. Thus, the numbers of animals used for the experiments can be reduced potentially. This alternative cuprizone mouse model will be very valuable for investigating human diseases such as MS for demyelination and remyelination studies or provide an alternative modeling choice for compound testing during drug discovery and development.

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Author Contributions

W. Z. initially developed the oral dose cuprizone model and carried out extensive studies. W. Z. and A. L. L. generated the data and drafted the manuscript. J. Q. L. participated in the study; W. D. Z. and D. T. edited the manuscript. J. F. W. directed the study and edited the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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