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Cuprizone Induced Demyelination in Male Wistar Rats with Timeline Changes Admistered through Oral Gavage

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ABSTRACT

Background: Cuprizone model of demyelination is a widely studied and successful model to mimic the early stages of Multiple Sclerosis, which is a chronic demyelinating disease of the central nervous system. It is usually given as powder mixed in rat chow. The ingestion of this might not prove accurate because of variable feed that the animal can take. Therefore, for precise dosage, an oral gavage route is better technically and better reproducible. This study aims to record the changes occurred during 3 weeks and 5 weeks.
Materials and methods: The animals were divided into 2 groups each with 6 animals. Control was given 1.5 ml Hydroxypropyl cellulose (HPC) and Cu group was given 450 mg/ Kg b.w of Cuprizone dissolved in 1.5ml of HPC, per day orally for 5 weeks and the study resuts were analysed in two phases. Phase I, 3 animals from each group studied for 3 weeks and phase II, remaining animals from both groups studied for 5 weeks.
Results: significant decrease in body weight and increase in brain organ weight was seen in Cu group and serum MBP (Myelin Basic Protein) and immunoflourescence of GST[pi] (Glutathione S Transferase - pi) was reduced in 3 weeks, showing the beginning of demyelination changes. Body weight and brain weight percentage was also analysed in both the groups in both the phases. Serum copper levels was also analysed and found to be reduced in Cuprizone group than control group.
Conclusion: Significant changes of demyelination were evident after partial scarifice by the end of 3 weeks, which proved the beginning of demyelination changes and Further chronic changes were ascertained by the end of 5 weeks. Through this study, a Fixed dose of cuprizone, can be given through oral gavage, and further changes of Demyelination changes can be studied, which can prove as a useful model for initial Stages of multiple sclerosis..

Keywords:
Cuprizone, demyelination, corpus callosum, Myelin basic protein (MBP), brain, body weight, multiple sclerosis.

1.Introduction

MS is a chronic inflammatory demyelinating disease of the central nervous system (CNS) [1]. In majority of the patients, the disorder usually starts with a relapsing and remitting phase which later progresses to preogressive phase with a gap of 10 to 15 years after the first onset of symptoms usually. In about 10 - 15% of the patients, they directly develop the progressive form of MS, which is then called primary progressive MS [2]. Progressive form of MS is usually seen in old age, whereas relapsing remitting stage of MS presents at an younger age, which suggests that some age related changes of the brain might play a role in the slow and steady increase of neurological disability in the progressive phase [3]. The pathology of MS is characterized by neuronal inflammation, progressive demyelination, astrocytes inflitration (astrogliosis), axonal loss due to progressive demyelination and finally neuronal cell death [4]
Reversible demyelination induced by cuprizone in the central nervous system is a distinguished characteristic feature seen in relapsing remitting stage of multiple sclerosis (RRMS). Many models of demyelination that are done in animal studies are available. These models induce experimental autoimmue encephalomyelitis (EAE). The different models are currently under use

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are virus models, like Theiler's virus and murine hepatitis virus and toxic models using lysolecithin, ethidium bromide and cuprione [5]. Cuprizone (CPZ) is a widely used and popular model, where oligodendrocyte cell death is caused until the animal is fed with cuprizone and once stopped leads to spontaneous remyelination,mimicking relapsing remitting stage of multiple sclerosis[6]. Cuprizone is a primary copper chelating agent, particularly causing apoptosis of oligodendrocytes, which leads to consistent demyelination, which is particularly evident in the corpus callosum of the brain of rodents [7]. Cuprizone model can better assess some important pathologies related to MS, namely, myelin and axonal degeneration and subsequent regeneration. Consistent demyelination can be achieved in a cuprizone dose for 5 - 6 weeks, when continued more than 10 weeks, it will lead to chronic demyelination. Once Cuprizone adminitration is withdrawn, spontaneous remyelination occurs in a 5 - 6 weeks cuprizone administration program

2. Materials And Methods

Chemicals:

The chemicals, Cuprizone (purity >99%) required for this study was procured from Sigma - Aldrich. Hydroxypropyl cellulose was procured from Research Department, Saveetha Medical College.

Animals:

Animals for the study were procured from Biogen Laboratory Animal Facility, Bangalore, India. The animals were maintained in air conditioned animal room with a 12 hour light and dark cycle and diet and water was provided ad libitum throughout experimental period. The study was conducted between september to october 2020, after receiving proper Institutional ethical clearance. [SU/CLAR/RD/010/12/2020]. Male rats with weight ranging from 150 - 200 gm, were chosen for the study to avoid the hormonal changes that might occur due to estrous cycle.

GST [pi] Immunofluorescence:

The tissue was sectioned using a cryostat microtome used for immunofluorescent labeling. The free-floating sections were washed with PBS for 10 min × 3. Then the sections were incubated with antiserum for 1h at room temperature. Subsequently, a primary antibody rabbit polyclonal anti-GST-pi (1:500; Enzo Life Sciences) was added into the blocking solution and incubated overnight at 4°C, followed by incubation at room temperature for 30 min. After rinsed for 10 min × 3 in PBS, sections were incubated with Alexa Fluor 594–conjugated anti-rabbit antibody (1:400)in PBST at room temperature for 90 min. After rinsed in PBST, the sections were covered using Fluoro shield Mounting Medium with DAPI (Thermo Fischer). The slides were preserved under 4°C and shielded from light. Immunofluorescence was observed and recorded using a fluorescence microscope (Zeiss Instruments, Germany). The cell density was expressed as the number per square millimeter.

Experimental design:

After acclimatisation for a period of 1 week, the experiment was carried out for 5 weeks (35 days), animals were divided into 2 groups with 6 animals in each group
Control - 1% HPC, 1.5.ml through oral gavage once for 35 days
Cu group - Cuprizone in 1.5 ml of HPC, 450 mg per Kg b. w dosage, dissolved in1% HPC

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Phase I study - to assess the initiation and extent of demyelination, 3 animals from Control and Cu group were sacrificed by the end of 3 weeks
Phase II study - to assess the completion of demyelination after 5 weeks, 3 animals from Control and Cu group were sacrificed by the end of 5 weeks

3. Data analysis:

The values were analysed using MS Excel and the results were expressed in mean± SE and the results tabulated and shown in graphs.

4. Results:

A. Phase I (Duration - 3 weeks)
Effects of Cuprizone on Serum MBP and GST [pi] expression by immunofluorescence method in the corpus callosum region. Organ and body weight changes were studied.

B. Phase II (Duration - 5 weeks)
Effects of Cuprizone on serum copper and organ and body weight changes were studied.
Phase I - Sacrifice at the end of 3 weeks
Successful induction of demyelination was assessed by serum MBP levels of Control and Cu group and GST [pi] expression by immunofluorescence method in the corpus callosum region
Cuprizone, 450 mg per Kg b.w was given in 1.5 ml of 1% of HPC solution through oral gavage for 3 weeks for Cu group and 1.5ml of 1% HPC solution was given for Control

Serum MBP levels:
The serum MBP levels of Control and Cu group showed considerable differences with serum MBP levels getting significantly reduced in Cu group

Table I: Effects of cuprizone on serum MBP levels expressed in mean±SE (N= 3)

Grouping	Serum MBP levels (in pg/dl)
Control (control)	49.33±1.45
Cu group (cuprizone)	13.33±2.18

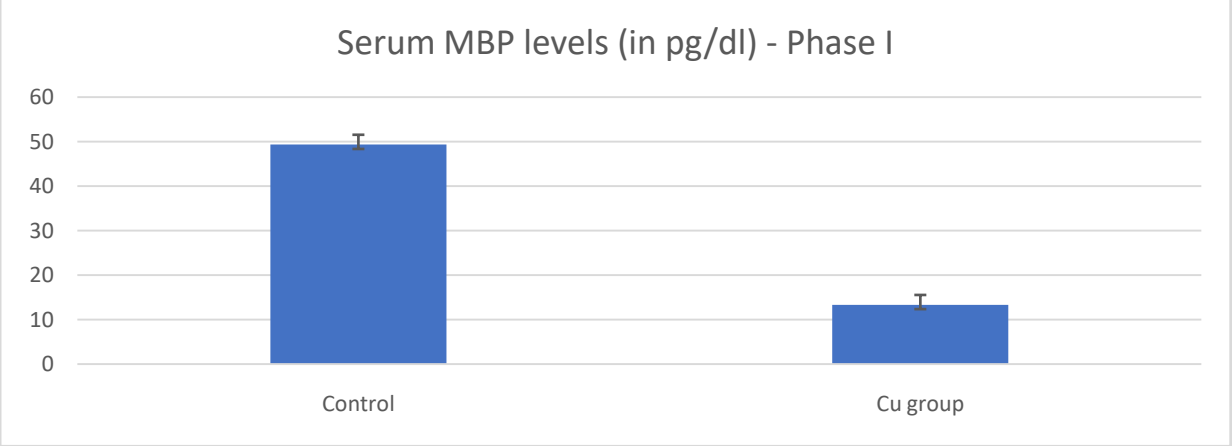

Fig 1: Showing serum MBP levels in Control and II (Phase I)

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Phase II - Sacrifice at the end of 5 weeks

Effects of Cuprizone on serum Copper level:

Cuprizone, 450 mg per Kg b.w was given in 1.5 ml of 1% of HPC solution through oral gavage for 5 weeks for Cu group and 1.5ml of 1% HPC solution was given for Control

Table III: Effects of cuprizone on serum copper levels expressed in mean±SE (N= 3)

Grouping	Serum Copper levels (in mg/dl)
Control	154.6±10.01
Cu group	65.58±4.65

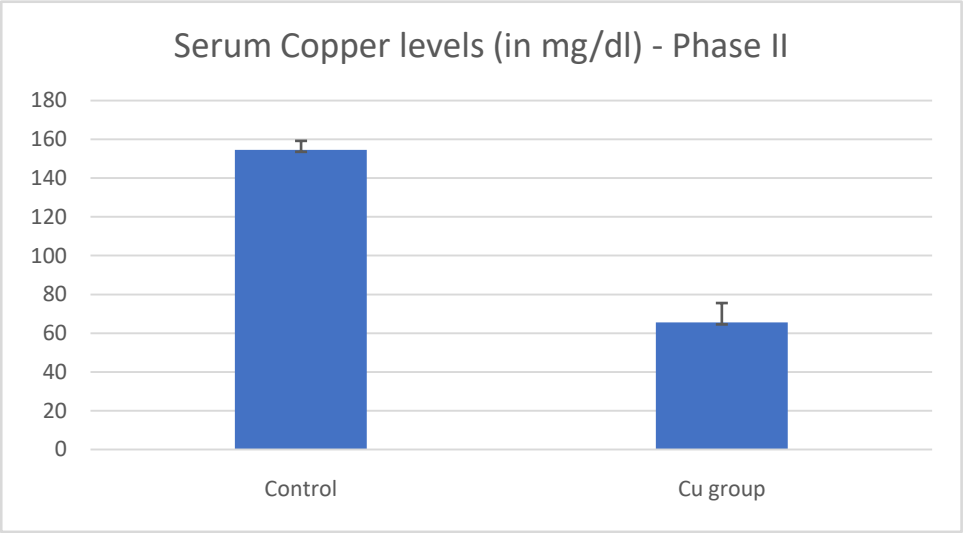

Fig 2: Showing serum copper levels in Control and II (Phase II)

Effect of Cuprizone on body and brain organ weight:

The animals were grouped after measuring the body weight, which was also measured constanty throughout the course of study. The brain weight was measured after both phase I and phase II sacrifice and the brain weight to the final body weight of the animal in both phases was calculated.

Percentage of body weight was measured using the formula:

Body weight % = final weight of the animal/initial weight of the animal X 100

Percentage of organ weight was measured using the formula:

Organ weight % = organ weight/ final weight of the animal X 100

PHASE I

Body weight %:

Table VI - showing the body weight % in Control and II

Phase I	Body weight %
Control	107.76
Cu group	86.81

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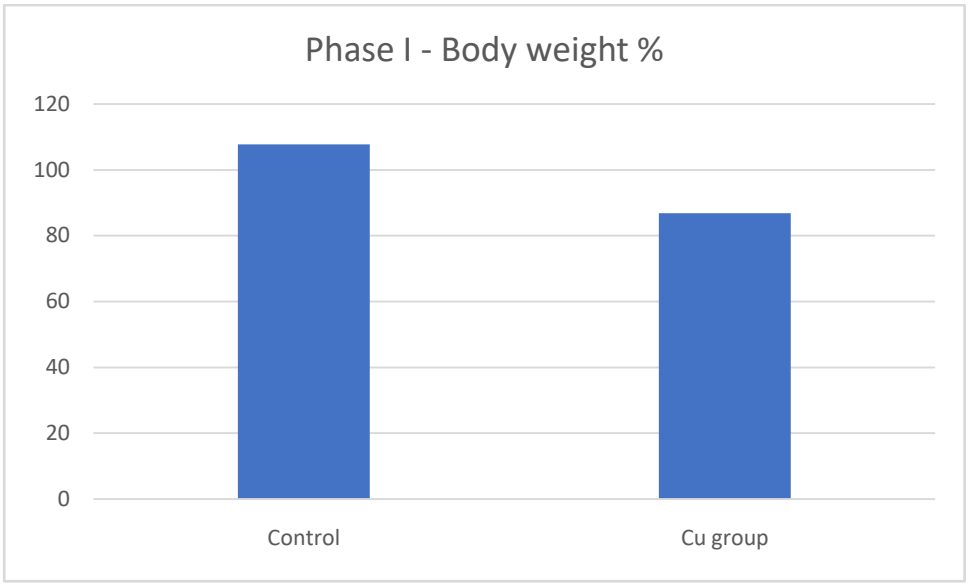

Fig 3: Showing % body weight in Control and II (Phase I)

Brain - Organ weight %:

Table VII - showing the brain organ weight % in Control and II

Phase I	Organ weight %
Control	1.009
Cu group	1.099

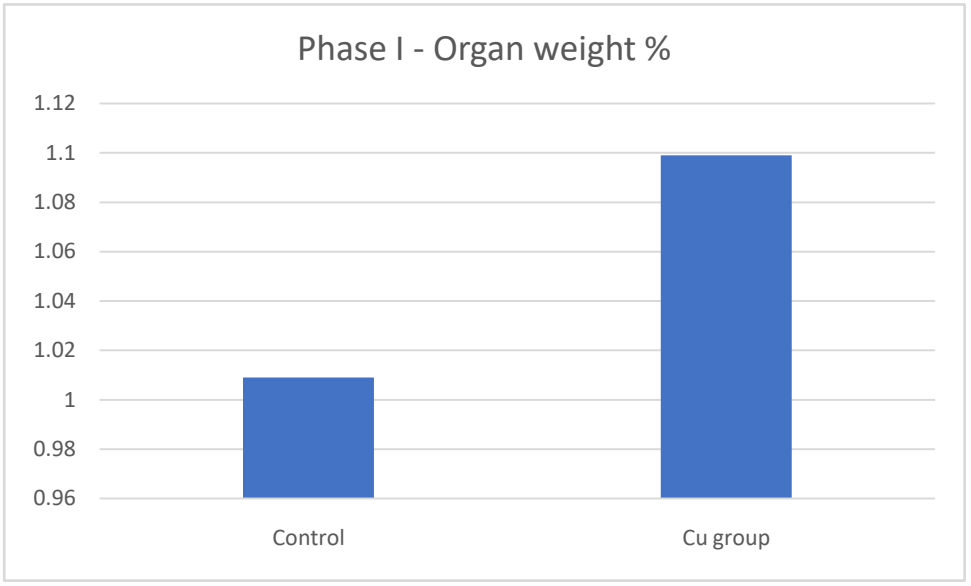

Fig 4: Showing % organ weight in Control and II (Phase I)

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PHASE II

Body weight %:

Table VIII - showing the body weight % in Control and II

Phase I	Body weight %
Control	132.07
Cu group	110.02

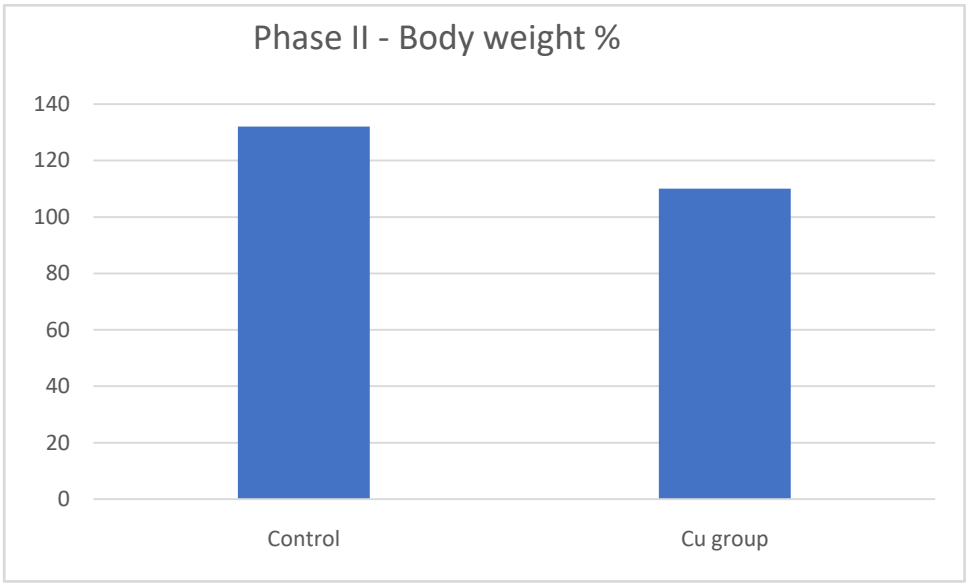

Fig 5: Showing % body weight in Control and II (Phase II)

Brain - Organ weight %:

Table IX - showing the brain organ weight % in Control and II

Phase I	Brain - Organ weight %
Control	0.89
Cu group	1.089

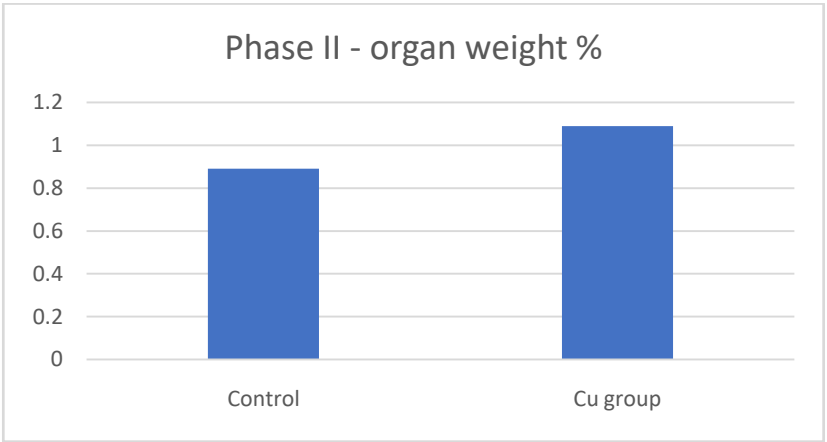

Fig 6: Showing % organ weight in Control and II (Phase II)

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Effect of cuprizone on GST (pi) Immunofluorescence (Phase I)

GST-Pi expression in the corpus callosum was analysed. Scale bar -50 μm. Immunofluorescenc was quantified using ImageJ software (NIH software). Number of positive cells per sq.mm was measured:

Phase I	Number of positive cells/sq.mm (mean)
Control	24
Cu group	110

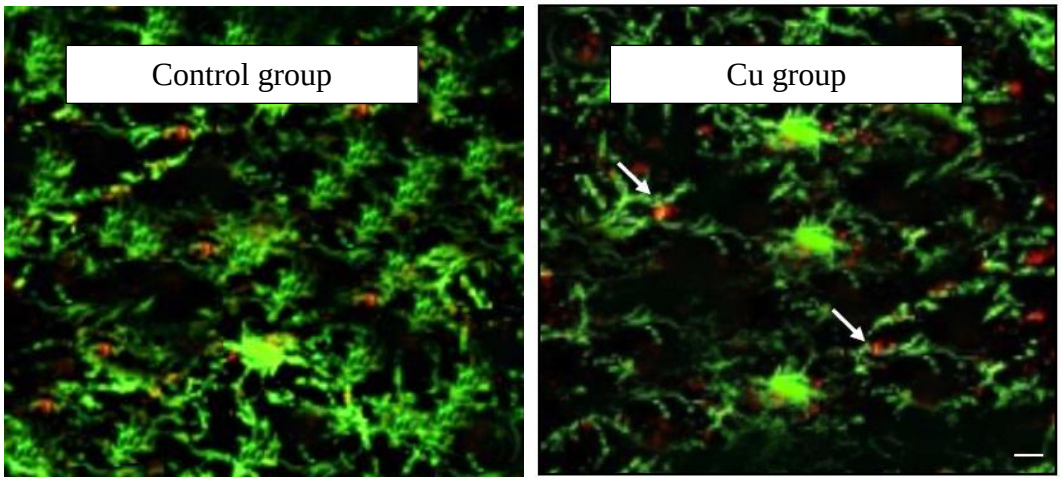

Fig 7: The figure in control group shows the green colour pointing the positive cells to GST [pi] and in Cu group shows the red colour pointing the positive cells to GST [pi].

Fig 8: showing the brain in Control (control) and Cu group (Cuprizone 450 mg/ Kg BW adminstered group)

5. Discussion:

Cuprizone - mode of administration:

Most of the previous studies have been conducted with mice as a model and including cuprizone in ground chow, which might not provide consistent dosage details. Other methods like mixing cuprizone mixed with drinking water [8] have also been in use. Recently, for precise dose measurement for the amount of cuprizone administration, oral gavage method has been in use.

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But, the reproducibility of these methods have not been well known. Cuprizone containing pellets have largely been able to create varying degrees of demyelination in mice [9], whereas in another study it was quoted that cuprizone in ground chow failed to provide consistent results in the form of demyelination[10]. Probably the reason for this can be explained by the fact that cuprizone is heat sensistive [11], which could partially deactivate its properties, but one more study has contradicted the same [12].

In a study done by Matsushima, G.K et al, 2001[13], they analysed the timeline of changes induced by cuprizone in corpus callosum and found that by oral adminstration of cuprizone by mixing it with rat chow, initiation of demyelination was found in 3 weeks and completion of demyelination was observed in 4.5 weeks. Spontaneous remyelination starts when cuprizone is stopped after 5 weeks. Some evidence of spontaneous remyelination has been reported even by 4 weeks [14]. If continued beyond 5 weeks, it will lead to chronic demyelination, which may not spontaneously recover, thus not mimicking relapsing remitting stage of Multiple Sclerosis.

Dose optimization:

In a study done by Zhen et al, 2017[15], different doses of cuprizone was given to eight- to 10-week-old male C57BL/6Bkl mice, and found consistent changes of demyelination seen in 400 mg/Kg B.W. They also found dosage of 200 and 300 mg/ Kg BW showed similar changes of demyelination and body weight and brain organ weight changes. The changes of demyelination and spontaneous relyelinationw were studied in forebrain and hindbrain regions.

In another study done by Abe et al, 2015 [16], cuprizone was administered at 120 and 600 mg/ Kg BW in male Sprague dawley rats. The highest safe dose was assigned at 600 mg/Kg BW and consistent myelin vacuolation and degradation in corpus callosum of the experimental rats was found at this dose. In the present study, a dose of 450 mg/ Kg BW was assigned and mixed with 1 % HPC and administered through a oral gavage, thereby leading to better optimization of the dosage and better reproducibility of results.

Body weight and organ weight %:

Body weight percentage was found to be reduced in Cu group as seen in study done by Abe et al and consistent increase of brain weight was also found similar to results given by Abe et al and Zhen et al, which could be owing to the inflammatory processes that are occusrring in concurrence with exposure to cuprizone.

Immunomarkers and Serum markers:

Carlton et al 1966 [17], found decreased levels of serum Copper on treatment with cuprizone and consistent demyelination. On further study, cuprizone was also found to cause severe staus spongiosus, hepatic lesions and and hydrocephalus[18]. In a review study done by Vega - Riquer et al [19], 2019, analysed the effects of cuprizone effects in the CD1 mouse brain. CD1 mice received 0.2% CPZ for 6 weeks and 30-μm-thick coronal sections immunostained with anti-myelin basic protein (MBP). it is found that the control group showed a strong expression of MBP in the corpus callosum wherase the cuprizone treated group expressed low levels of MBP expression.

The findings of the present study also co related with findings of Abe et al, 2015. They analysed the myelion vacuolation histopathologically, using MBP immunostained brain sections, where the results showed similar presentations as current study, with cuprizone treated group shwoing increased number of vacuolation in the cingulum, thalamus and medial cerebellar nuclei in the 600mg/ kg BW group compared to 120 mg/per kg of cuprizone treated group. mRNA expression

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levels of MBP in the hippocampus dentate gyrus was also evaluated which was also concurrent with 600 mg/kh BW group than 120 mg/Kg BW treated group.

GST[pi] Immunofluorescence:
GST [pi] is glutathione transferase [pi], which is a cytosolic isoenzyme which is used as a marker for mature oligodendrocyte in the mammalian brain. GST-pi is found in the cytoplasm of mature oligodendrocytes, which are immunopositive for 2=,3=-cyclic nucleotide 3=-phosphodiesterase (CNPase), in rodent cerebral cortex [21]. In a study done by Lou et al [22] showed Cu group showed a lower density of GST-pi positive profiles compared to CNT group, indicating oligodendrocytes loss occurred in cuprizone-treated mice as in the present study.

6. Conclusion:

Adminstration of Cuprizone to induce demyelination as a reliable method has been in use since long. But most studies have not properly validated the exact dosage where the adminstration has mostly been in ground or pellet form added to the feed of the animal. And most of the studies, have been conudcted with mice model. This study has used a standard dose of cuprizone, based on previous studied conducted and dosage has been administered in a calibrated format and the wistar rats has been been chosen as the experimental animal model, where many studies are not available. Consistent results of demyelination have been produced and further analysis has been made showing the difference of the extent of demyleination in 3 and 5 weeks of the study in 2 phases. This model can be very helpful in mimicking RRMS (relapsing remitting stage of multiple sclerosis) and thereby studies can be conducted on remyelination and for drug discovery and development.

Ethical clearance: Ethical clearance has been received from Institutional ethical clearance committee, Saveetha Medical College. [SU/CLAR/RD/010/12/2020].
Source of Support: Self funding
Conflicts of interests: None

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References

[1] Lassmann H, Brück W, Lucchinetti C (2007) The immunopathology of multiple sclerosis: an overview. Brain Pathol 17:210–218

[2] Lublin FD, Reingold SC (1996) Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. Neurology 46:907–911

[3] Scalfari A, Neuhaus A, Daumer M, Ebers GC, Muraro PA (2011) Age and disability accumulation in multiple sclerosis. Neurology 77:1246–1252

[4] Reich, D. S., Lucchinetti, C. F., & Calabresi, P. A. (2018). Multiple sclerosis. The New

England Journal of Medicine, 378, 169–180.

[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] LOVE S. Cuprizone neurotoxicity in the rat: morphologic observations. J Neurol Sci 1988; 84: 223-237.

[8] Zatta, P.; Raso, M.; Zambenedetti, P.; Wittkowski, W.; Messori, L.; Piccioli, F.; Mauri, P.L.; Beltramini, M. Copper and zinc dismetabolism in the mouse brain upon chronic cuprizone treatment. Cell. Mol. Life Sci.CMLS 2005, 62, 1502–1513.

[9] Hochstrasser, T.; Exner, G.L.; Nyamoya, S.; Schmitz, C.; Kipp, M. Cuprizone-Containing Pellets Are Less Potent to Induce Consistent Demyelination in the Corpus Callosum of C57BL/6 Mice. J. Mol. Neurosci. 2017,61, 617–624.

[10] Hagemeyer, N.; Boretius, S.; Ott, C.; Von Streitberg, A.;Welpinghus, H.; Sperling, S.; Frahm, J.; Simons, M.; Ghezzi, P.; Ehrenreich, H. Erythropoietin attenuates neurological and histological consequences of toxic demyelination in mice. Mol. Med. 2012, 18, 628–635.

[11] Gudi, V.; Gingele, S.; Skripuletz, T.; Stangel, M. Glial response during cuprizone-induced de- and remyelination in the CNS: Lessons learned. Front. Cell. Neurosci. 2014, 8, 73.

[12] Heckers, S.; Held, N.; Kronenberg, J.; Skripuletz, T.; Bleich, A.; Gudi, V.; Stangel, M. Investigation of Cuprizone Inactivation by Temperature. Neurotox. Res. 2017.

[13] Matsushima, G.K.; Morell, P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. Brain Pathol., 2001, 11(1), 107-116.

[14] Hiremath, M.M.; Saito, Y.; Knapp, G.W.; Ting, J.P.Y.; Suzuki, K.; Matsushima, G.K. Microglial/macrophage accumulation during cuprizone-induced demyelination in C57BL/6 mice. J. Neuroimmunol., 1998, 92(1-2), 38-49

[15] Abe H, Tanaka T, Kimura M, Mizukami S, Saito F, Imatanaka N, Akahori Y, Yoshida T, Shibutani M. Cuprizone decreases intermediate and late-stage progenitor cells in hippocampal neurogenesis of rats in a framework of 28-day oral dose toxicity study. Toxicol Appl Pharmacol. 2015 Sep 15;287(3):210-21. doi: 10.1016/j.taap.2015.06.005. Epub 2015 Jun 7. PMID: 26057786.

[16] Zhen W, Liu A, Lu J, Zhang W, Tattersall D, Wang J. An Alternative Cuprizone-Induced Demyelination and Remyelination Mouse Model. ASN Neuro. 2017 Jul-Aug;9(4)

[17] Carlton, W.W. Response of mice to the chelating agents sodium diethyldithiocarbamate, alpha-benzoinoxime, and biscyclohexanone oxaldihydrazone. Toxicol. Appl. Pharmacol., 1966, 8(3), 512-521. [http://dx.doi.org/10.1016/0041-008X(66)90062-7] [PMID: 6006739]

[18] Benetti, F.; Ventura, M.; Salmini, B.; Ceola, S.; Carbonera, D.; Mammi, S.; Zitolo, A.;

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 12643 - 12653
Received 05 March 2021; Accepted 01 April 2021.

D'Angelo, P.; Urso, E.; Maffia, M.; Salvato, B.; Spisni, E. Cuprizone neurotoxicity, copper deficiency and neurodegeneration. Neurotoxicology, 2010, 31(5), 509-517. [http://dx.doi.org/10.1016/j.neuro.2010.05.008

[19] Vega-Riquer JM, Mendez-Victoriano G, Morales-Luckie RA, Gonzalez-Perez O. Five Decades of Cuprizone, an Updated Model to Replicate Demyelinating Diseases. Curr Neuropharmacol. 2019;17(2):129-141.

[20] Tanaka K, Nogawa S, Suzuki S, Dembo T, Kosakai A (2003) Upregulation of oligodendrocyte progenitor cells associated with restoration of mature oligodendrocytes and myelination in peri-infarct area in the rat brain. Brain Res 989:172–179.

[21] Cammer W, Zhang H (1992) Localization of Pi class glutathione-Stransferase in the forebrains of neonatal and young rats: evidence for separation of astrocytic and oligodendrocytic lineages. J Comp Neurol 321:40–45.

[22] Luo M, Deng M, Yu Z, Zhang Y, Xu S, Hu S and Xu H (2020) Differential Susceptibility and Vulnerability of Brain Cells in C57BL/6 Mouse to Mitochondrial Dysfunction Induced by Short-Term Cuprizone Exposure. Front. Neuroanat. 14:30. doi: 10.3389/fnana.2020.00030.