Five Decades of Cuprizone, an Updated Model to Replicate Demyelinating Diseases

Jose M. Vega-Riquer\textsuperscript{1,2}, Gerardo Mendez-Victoriano\textsuperscript{1}, Raul A. Morales-Luckie\textsuperscript{3} and Oscar Gonzalez-Perez\textsuperscript{1,4,*}

\textsuperscript{1}Laboratory of Neuroscience, School of Psychology, University of Colima, Mexico 28040; \textsuperscript{2}Medical Sciences PhD Program, School of Medicine, University of Colima, Mexico 28040; \textsuperscript{3}Centro Conjunto de Investigación en Química Sustentable, Universidad Autónoma del Estado de Mexico, Mexico 50200; \textsuperscript{4}El Colegio de Colima, A.C. Colima, Col. 28000, Mexico

Abstract: Introduction: Demyelinating diseases of the central nervous system (CNS) comprise a group of neurological disorders characterized by progressive (and eventually irreversible) loss of oligodendrocytes and myelin sheaths in the white matter tracts. Some of myelin disorders include: Multiple sclerosis, Guillain-Barré syndrome, peripheral nerve polyneuropathy and others. To date, the etiology of these disorders is not well known and no effective treatments are currently available against them. Therefore, further research is needed to gain a better understanding and treat these patients. To accomplish this goal, it is necessary to have appropriate animal models that closely resemble the pathophysiology and clinical signs of these diseases. Herein, we describe the model of toxic demyelination induced by cuprizone (CPZ), a copper chelator that reduces the cytochrome and monoamine oxidase activity into the brain, produces mitochondrial stress and triggers the local immune response. These biochemical and cellular responses ultimately result in selective loss of oligodendrocytes and microglia accumulation, which conveys to extensive areas of demyelination and gliosis in corpus callosum, superior cerebellar peduncles and cerebral cortex. Remarkably, some aspects of the histological pattern induced by CPZ are similar to those found in multiple sclerosis. CPZ exposure provokes behavioral changes, impairs motor skills and affects mood as that observed in several demyelinating diseases. Upon CPZ removal, the pathological and histological changes gradually revert. Therefore, some authors have postulated that the CPZ model allows to partially mimic the disease relapses observed in some demyelinating diseases.

Conclusion: for five decades, the model of CPZ-induced demyelination is a good experimental approach to study demyelinating diseases that has maintained its validity, and is a suitable pharmacological model for reproducing some key features of demyelinating diseases, including multiple sclerosis.

Keywords: Cuprizone, myelin, oligodendrocyte, demyelination, remyelination, white matter, neuroinflammation, multiple sclerosis, demyelinating disease, microglia.

1. INTRODUCTION

Demyelinating diseases are neural disorders characterized by inflammation and damage or selective destruction of myelin associated with diffuse primary synaptic loss and progressive axonal degeneration [1-3]. Clinical deficits include ataxia, loss of dexterity, myoclony, spasticity, paraparesis or hemiparesis, vision loss and cognitive deficits. These alterations are due to disrupted electrical transmission along axons because of myelin loss. A crucial characteristic of demyelinating diseases is the limited ability to rapidly regenerate myelin and the extension of secondary damage of axons. There are several causes of myelin destruction, including immunological action, chemicals and infections [4]. Multiple sclerosis (MS) is associated with focal white matter plaques of demyelination, which are partially repaired during remyelination. Reappearance of oligodendrocytes within active lesions is frequently seen in patients at early stages of
MS. Some authors suggest that remyelination is a transient phenomenon and remyelinated shadow plaques may be affected by new bouts of demyelination, which ultimately lead to incomplete myelin repair [5, 6]. There are several animal models that resemble the clinical and pathophysiologic course of MS. To date, four experimental approaches to induce demyelination have been well-characterized and include: genetic myelin mutations, autoimmune inflammatory-induced demyelination (experimental autoimmune encephalomyelitis -EAE), virus-induced demyelination and toxic demyelination (cuprizone and lyssolecithin models). All these models partially mimic the MS pathology. Although EAE is a commonly used model that reproduces some of the autoimmune aspects of MS, the toxic demyelination with cuprizone can be still considered an appropriate model to study the remyelination process [7, 8].

Cuprizone (CPZ) is a copper chelator that targets many metalloenzymes as ceruloplasmin, impairs the activity of the copper dependent cytochrome oxidase, decreases oxidative phosphorylation and produces degenerative changes in oligodendrocytes (OLs). This cascade of events eventually ends in demyelination [9, 10]. In 2007, Zucconi, et al. found the copper deficiency induced by CPZ was related to microglial activation in cerebral cortex and thalamus [11]. This evidence suggests that CPZ may reproduce different pathological aspects of some neurodegenerative diseases [11]. The supplementation of animal diet with CPZ has been used as demyelinating model that mimics some histological hallmark of demyelinating diseases [12]. Cuprizone intoxication induces apoptosis of oligodendrocyte cell population that leads to extensive demyelination of white matter tracts in the corpus callosum, internal capsule, the thalamus, anterior commissure and cerebellar peduncles [13, 14]. Interestingly, when CPZ treatment is withdrawn, there is rapid remyelination and myelin proteins re-expression [15].

The cuprizone-induced damage appears to be mediated by certain molecules, such as tumor necrosis factor alpha (TNFa), interleukin 1β (IL-1β) and interferon gamma (IFNy) that are secreted by microglia/macrophages in the brain. These events are associated with a decrease in mitochondrial activity in OLs, low energy production, and high activity of reactive oxygen species [16]. The administration of CPZ in vivo leads to fast proliferation of microglia/macrophages surrounding the lesion area. These immune cells are known to produce TNFα, which seems to exacerbates acute demyelination and remains undetectable on untreated mice, as reported by Arnett et al. [17]. However, some important features of MS, such as inflamed blood vessels and the presence of CD3+ T cells, have not been described in the CPZ model [18].

2. CUPRIZONE

CPZ, also referred as to cyclohexylidene hydrazide (Fig. 1), is a condensation product of oxalylhydrazide and cyclohexanone. CPZ is an effective and selective copper chelating agent. The first description of CPZ was made by Gustav Nilsson in the 1950s, he discovered that CPZ induced a subtle blue color reaction upon chelating copper (Cu2+) salts [19]. In 1966, Carlton observed that CPZ administration in mice produced low serum Cu2+ levels and demyelination [20]. Later, it was found that oral administration of CPZ also produced severe status spongiosus, hydrocephalus and hepatic lesions [21, 22].

3. COPPER METABOLISM

Copper is an essential element in all mammal’s nutrition. This element is a component of metalloenzymes in which it acts as an electron donor or acceptor [23]. Thus, copper is required for preserving tissue growth, cardiovascular integrity, neuroendocrine function, neovascularization, lung elasticity and iron metabolism. Copper acts as a coenzyme in aerobic metabolism for cytochrome c oxidase in the mitochondria, lysyl-oxidase in the connective tissue, dopamine monoxygenase in the brain, and ceruloplasmin in the liver. Copper is also a cofactor for apo-copper-zinc superoxide dismutase (ApoCuZnSOD) that protects against oxygen-free-radical damage to proteins, membrane lipids, and nucleic acids in several systems. Severe copper deficiencies are relatively rare in humans and may occur by gene mutations or low dietary copper intake. Copper deficit may produce mental retardation, anemia, hypothermia, neutropenia, diarrhea, cardiac hypertrophy, bone fragility, immune deficiencies, connective tissue weakened, brain function impaired, peripheral neuropathy, and accelerate hair loss [24]. Therefore, the copper metabolism is strongly regulated by a complex homeostatic process [25].

In mammals, absorption of copper primarily occurs in the small intestine. Copper absorption is performed by the brush border of the intestinal mucosa and transferred across the basolateral membrane into interstitial fluid and blood. Once copper is absorbed from the intestine, it binds to albumin and copper transport protein (transcuprein), and reaches serum levels of 40 mg Cu/L, approximately. Most of the serum copper is then deposited into the liver (Fig. 2). Nonetheless, only ~180 µg Cu2+ remains associated with albumin in human plasma. The rest of the copper (~1000 µg per liter plasma) is bound to ceruloplasmin (65%, approximately), transcuprein (12%), and other components of low molecular weight. Most of the incoming copper rapidly finds its way into the hepatic cells (Fig. 2), and minimal amounts of this metal enter the kidney. There are two phases of copper distribution into the blood serum. The first phase is mediated by transcuprein that facilitates copper intake into the liver. The second phase is mediated by ceruloplasmin that helps increase the copper levels into systemic organs (Fig. 2). The main role of copper is to function as a cofactor for specific enzymes and electron transport proteins involved in energetic or anti-oxidative metabolism [26].

![Molecular structure of N,N'-bis(cyclohexylideneamino) oxamide (cuprizone). Molecular formula: C₁₁H₁₂N₄O₂; molecular weight: 278.45006g/mol.](image-url)
4. CUPRIZONE AS DEMYELINATION MODEL

Since 1960s, several studies have been designed to determine the precise dose of CPZ to produce significant changes in the CNS. Significant myelin changes have been observed when 0.2%-0.6% CPZ is mixed with standard rodent chow [22]. The most common protocol consists in feeding 8-week-old mice with 0.2% CPZ (w/w) for 5-6 weeks (Fig. 3). Lindner et al. in 2008 demonstrated that high levels of demyelination can be achieved by increasing the dosage to 0.3% CPZ (w/w) [27]. However, as the CPZ concentration increases (from 0.2% to 0.3%) the mortality rate rises from <5% to more than 10% or 15% [28]. Thus, the 0.2% CPZ is the preferred concentration because it produces extensive demyelination with less side effects [29-34].

Oligodendrocyte apoptosis begins a few days after cuprizone administration. Recently, Hesse and colleagues demonstrated that oligodendroglial cell death and myelin gene downregulation start a few days after CPZ supplementation, but demyelination is only evident a few weeks after [35]. Recent evidence has shown that short-term exposure to CPZ (3 weeks) is enough to induce demyelination [29]. Astrogliosis and depletion of mature OLs is observed at 5th week. Similar histological changes can be observed with CPZ exposure for five weeks. Hence, it seems that once the mature OLs are perturbed the demyelinating activity progresses without needing additional exposure. Interestingly, early withdrawal of CPZ did not accelerate the recovery process, which suggests that a mild white matter insult triggers a cascade of demyelinating events in the following weeks (Fig. 4) [36]. Although matter degeneration in the CC is a key neuroanatomical sign of the CPZ model [33, 37, 38], several brain regions are also affected by CPZ (Table 1) [14, 32, 33, 37, 39-52]. The histopathological changes produced by CPZ include oligodendroglial cell death, microglia activation, astrocyte reactivity and grey matter demyelination [53].

Recent studies have demonstrated that juvenile mice are more vulnerable to CPZ. In fact, aged mice require high
Fig. (3). Cuprizone effects in the CD1 mouse brain. CD1 mice received 0.2% CPZ for 6 weeks. 30-µm-thick coronal sections immunostained with anti-myelin basic protein (MBP) and revealed with 3,3’-Diaminobenzidine. The control animal (A) shows a strong expression of MBP in the corpus callosum (CC), whereas the cuprizone-treated mouse (B) expresses low levels of MBP. CTX: cortex. Bar = 5µm.

Table 1. Cuprizone demyelination over susceptible regions.

| Brain Region                              | CPZ Concentration | Initial Demyelination (Week) | Complete Demyelination (Week) | Microgliosis Initiation (Week) | Astrogliosis Initiation (Week) | Refs.                  |
|-------------------------------------------|-------------------|------------------------------|-----------------------------|-------------------------------|-------------------------------|-------------------------|
| Corpus callosum                           | 0.1% – 0.5%       | 3                            | 4.5                         | 1                             | 3                             | [32, 33, 39]            |
| Anterior commissure                       | 0.2%, 0.25%       | 5                            | N/R                         | 5                             | N/R                           | [40-42]                 |
| Basal ganglia (caudoputamen, globus pallidus) | 0.2% – 0.4%       | 2                            | 5                           | 5                             | 2                             | [40-44]                 |
| Brainstem                                 | 0.2%              | N/R                          | 5                           | N/R                           | N/R                           | [41, 106]               |
| Cerebellum                                | 0.2%, 0.5%        | 2                            | 5                           | 2-3                           | 3-4                           | [20, 47, 48, 106-108]   |
| Cortex                                    | 0.2% – 0.5%       | 4                            | 5                           | 3                             | 3                             | [39-41, 49, 109-111]    |
| External Capsule                          | 0.2%              | N/R                          | 6                           | N/R                           | N/R                           | [110]                   |
| Fornix                                    | 0.2%, 0.25%       | 5                            | 12                          | 5                             | N/R                           | [40, 111]               |
| Hippocampus                               | 0.2% – 0.4%       | 3                            | 5                           | N/R                           | 5                             | [41, 51, 109-112]       |
| Hypothalamus                              | 0.2%              | 6                            | N/R                         | N/R                           | N/R                           | [110]                   |
| Internal capsule                          | 0.25%             | 5                            | N/R                         | 5                             | 5                             | [40]                    |
| Olfactory bulb-tract                      | 0.2%, 0.25%       | 5                            | 5                           | 5                             | N/R                           | [40, 41, 110, 111]      |
| Optic chiasm                              | 0.2%              | N/R                          | 5                           | N/R                           | N/R                           | [41]                    |
| Optic tract                               | 0.25%             | 5                            | N/R                         | 5                             | N/R                           | [40]                    |
| Septal nucleus                            | 0.25%             | 5                            | N/R                         | 5                             | N/R                           | [40]                    |
| Substantia innominata                     | 0.25%             | N/R                          | 5                           | 5                             | N/R                           | [40]                    |
| Thalamus                                  | 0.2%, 0.25%       | 6                            | 5-6                         | 5                             | 5                             | [40, 110]               |

Several brain regions have been reported to be susceptible to CPZ induced demyelination and show microgliosis and astrogliosis. N/R, Not reported.
doses of CPZ to achieve a similar degree of demyelination as compared to young mice, but the precise mechanism underlying this effect is still unknown. CPZ-induced demyelination in the corpus callosum of juvenile mice is more severe than that of middle-aged animals. Young mice (<57 days) exposed to CPZ show working memory deficit throughout both the CPZ intoxication period and the remyelination process (CPZ removal). In contrast, old mice (>57 days) only show working memory deficit immediately after CPZ exposure [54]. This age-related vulnerability also results in profound behavioral dysfunctions in young-adult mice, including anxiety, attention deficit hyperactivity disorder and schizophrenia-like phenotypes. [55, 56]. At week four after CPZ exposure, motor deficits are also observed as observed in the rotorod test and the open field maze [57-59]. The proclivity of young mice to demyelination may be explained by age-related changes in the expression of PDGFRA, Nkx-2.2 and Olig2 [60] that, in turn, modify the recruitment of oligodendrocyte progenitor cells and drive the cell fate of neural progenitors toward the astrocyte lineage [61-64]. Interestingly, short-term exposure to cuprizone do not produce evident demyelination, but induces astrocyte and microglia activation that have been associated with work memory impairment and social behavior disruption [65, 66]. Further evidence indicates some sex-related differences in CPZ-induced demyelination. In this regard, Ludwin reported that Swiss and SJL/J female mice did not show demyelination as compared to males. However, no significant intersex differences were observed in C57BL/6 mouse strain [67, 68]. C57BL/6 is the mouse strain most commonly used for the CPZ demyelination model [33, 69]; however, many other mouse strains and animals species have been used to study different aspects of the demyelinating effect of CPZ (Table 2).

Recently, a modification in the CPZ model was described and consisted in the addition of 10 mg/kg i.p. rapamycin five times per week. This mTOR inhibitor produces extensive demyelination and prolongs the remyelination period, which help evaluate the effect of remyelinating treatments and downstream mechanisms involved in remyelination [70, 71]. Besides the CPZ model, other demyelinating models have been designed to reproduce the pathological course of demyelination and investigate possible treatments against MS (Table 3). Patients with MS may display three different clinical presentations: the relapsing-remitting form (RRMS) that is characterized by exacerbations of the symptoms (relapses), followed by periods of complete or partial remission; the primary progressive form (PPMS) that shows a continuous and irreversible evolution of the disease; and the secondary progressive form (SPMS) in which there is a progressive worsening of neurologic function over time, probably as a transition from RRMS [72]. To date, there are several experimental models that mimic some of the MS features and all of them have different characteristics. The autoimmune or allergic experimental encephalomyelitis (EAE) model in SJL/J mice is useful to study the relapsing forms. The MOG35-55 immunization of C57BL/6 mice is a good model to study a chronic-progressive disease [73] and it properly resembles the physiopathology of MS patter-II lesions [74]. The Theiler’s murine encephalomyelitis virus (TMEV) model, in which mice develop a chronic progressive demyelinating disease without remissions [75] and their histopathological patterns look alike the patterns III and IV of MS lesions [76]. The lyssolecithin injection model that produces focal and delimited demyelinating lesion with microglia/macrophage activation and recruitment, which provides a fast and well-defined remyelination pattern comparable to the patter III of MS lesions [38, 77, 78]. Finally, the CPZ model can be considered as a suitable model to reproduce extensive pattern-III lesions of MS [79], which are characterized by the presence of apoptotic OLs and early down regulation of myelin-associated glycoproteins (MAG) [80, 81]. The CPZ model also allows to the study of mechanism of spontaneous remyelination, because the mechanism of demyelination/remyelination occurs simultaneously and the white-matter damage reverts after CPZ withdrawal resembling the RMS [82]. In summary, the choice of the best model depend on the aim of study and the characteristics of the treatment to be evaluated (Table 3).

5. MECHANISM OF ACTION OF CPZ-INDUCED DEMYELINATION

5.1. Mitochondrial Response to CPZ Intoxication

The oral administration of CPZ via daily diet in adult mice produces a specific insult to mature oligodendrocytes by impairing their metabolic demands to support myelin production and by triggering oligodendroglia apoptosis [83]. These events are followed by microglia recruitment and myelin phagocytosis. Morphological and gene-expression studies indicate that during the CPZ administration some oligodendrocyte progenitor cells keep proliferating and invading demyelinated areas, but the magnitude of CPZ effect always ends in severe copper deficiency and secondary demyelination [14, 84-86]. Remarkably, demyelination and

| Mice Strain/ Species | Demyelination Severity | Refs. |
|----------------------|------------------------|-------|
| C57BL/6 mice         | Male ++ / Female ++    | [32-33, 39, 43] |
| ICI Mice             | Male ++                 | [14]  |
| Swiss-Webster Mice   | Male ++                 | [113] |
| Swiss Mice           | Male ++ / Female -     | [61]  |
| Albino Mice          | Male ++ / Female ++    | [114] |
| SJL Mice             | Male ++ / Female +     | [69]  |
| 129/SVJ Mice         | Male ++                 | [43]  |
| BALB/cf Mice         | Male +                  | [49]  |
| CD1 Mice             | Male ++                 | [115] |
| Cynomolgus Macaque   | Male - / Female -      | [116] |
| Wistar rat           | Male ++ / Female ++    | [117-120] |
| Guinea Pig           | Male ++                 | [119] |

Abbreviations: The CPZ demyelination pattern depends on the concentration, exposure time, sex, strain, and specie. (+ +) Acute; (+) Mild; (-) Not found.
oligodendrocyte damage produced by CPZ is not associated with injury of other neural cell types [85]. These findings contrast with those observed in liver, where the formation of megamitochondria (mitochondria enlargements or clusters) has been observed in hepatocytes. This alteration in liver mitochondria may be a consequence of deficient activity of cytochrome oxidase [16, 61, 85, 87, 88]. Interestingly, CPZ leads to a reduction in brain activity of cytochrome oxidase, monoamine oxidase (MAO) and inhibition of complexes I, II and III of the respiratory chain. After the CPZ intoxication, the formation of megamitochondria has been observed in liver, but not in neurons, astrocytes and other neural cells [16, 87, 89]. Remarkably, studies have confirmed that CPZ only affects mature oligodendrocytes, without modifying the absolute number of oligodendrocyte progenitor cells (OPCs) [85]. Consequently, CPZ does not affect de novo formation of oligodendrocytes.

Oxygen free radicals appear to be responsible for mitochondria enlargement. This process may be a protective reaction to reduce and suppress intracellular reactive oxygen species (ROS) levels, which restore normal cellular functions and organelle structure. However, if ROS levels dramatically increase into the oligodendrocytes and the resting membrane potential decreases with a concomitant caspase-3 activation and demyelination. During the first 3 weeks of CPZ intoxication the caspase-3 is strongly active. Next, the caspase-3 activity decreases, whereas the activity of poly ADP-ribose polymerase (PARP) increases and induces apoptosis via the apoptosis inducing factor (AIF) [29, 90, 91]. Hence, this evidence indicates that CPZ intoxication increases oxidative stress that, in turn, triggers apoptosis in mature OLs (Fig. 5) [83].

### Table 3. Comparison of the most commonly used demyelination models.

| Model     | Specie      | Chemical Agent          | Demyelination                  | Administration Route | Spontaneous Remyelination | Complexity          | Disadvantages                                      | Refs.                  |
|-----------|-------------|-------------------------|--------------------------------|----------------------|---------------------------|---------------------|----------------------------------------------------|------------------------|
| Cuprizone | Rat, Mouse  | Cuprizone               | Initial: 3 weeks Complete: 4.5 weeks | Oral                 | Yes (completed at week 4) | Low [mixed with chow] | Not validated for functional readouts in some strains | [32, 39, 86]           |
| Lysolecithin | Rat, Mouse | Lysophosphatidylecholine | Initial: 2 days Complete: 1 week | Intracerebral injection | Yes (completed at week 4) | High [Stereotactic surgery] | High technical complexity                          | [121-123]              |
| EAE*      | Rat, Mouse, Guinea pigs | CFA+MOG/MBP/PLP peptides or rSCH | Initial: 11 days Complete: 2 weeks | Intraperitoneally      | No                         | Low [i.p. injection] | More related to ADEM than MS. Not for investigating remyelination process | [124-125]              |
| TMEV      | Mouse       | Theilovirus (cardiovirus/picornaviridae), TO and GDVII subtypes | Initial: 6 weeks Complete: 35 weeks | Oral, intranasal, footpad inoculation, intracerebral injection | No                         | High [Stereotactic surgery, virus culture] | The proportion of the disease caused by the virus activity or the inflammatory response is hard to determine | [8, 126-127]           |

**Abbreviations:** EAE: experimental autoimmune encephalomyelitis; TMEV: Theiler’s murine encephalomyelitis virus; CFA: complete Freund adjuvant; rSCH: rat spinal cord homogenate; MOG: myelin oligodendrocyte glycoprotein; MBP: myelin basic protein; PLP: proteolipid protein; W: weeks; ADEM: acute disseminated encephalomyelitis.  
*Demyelination pattern reported in the EAE model study was carried out on optic nerve of SJL/J mice, but demyelination pattern may variate between specie, strain and brain region.*

5.2. Lipid Metabolism Disturbance

Besides the inhibition of myelin protein synthesis, myelin lipid metabolism is also affected by cuprizone. Myelin sheet consist of 70% lipids, 40% phospholipids (mainly plasmalogens), and 30% proteins [92]. Membrane-bound plasmalogens can be hydrolyzed by plasmalogenase, leading to an increase of free plasmalogens. Phospholipase A2 (PLA2) can degrade both membrane-bound and free plasmalogens into arachidonic acid (AA), a key intermediate of pro-inflammatory signaling. The activity of both plasmalogenase and PLA2 is increased by CPZ intoxication, which contributes to myelin sheath degradation and elevated concentrations of AA [83]. In MS lesions, the AA cascade is activated by increasing activity of PLA2 and cyclooxygenase 1 or 2 (COX-1, COX-2). The produced AAs will be subsequently metabolized by COX-1 or COX-2 into prostaglandin H2 (PGH2) that strongly increase prostaglandin E2 (PGE2) related to OLs apoptosis via PGE2-E2 receptor, prostacyclin (PGI2), prostaglandin D2 (PGD2) and prostaglandin F2α (PGF2 α). Interestingly, this AA cascade is activated by cuprizone and is associated with a substantial involvement of sPLA2 isofrom [93].

Other organic compounds involved in the myelin sheet formation are cerebrosides (glycosphingolipids) and cholesterol [92]. These lipidic compounds are drastically reduced by increased activity of plasmalogenase during early stages of CPZ intoxication, and these findings are comparable to the increased phospholipase (PL) A2 and A3 activity found in MS and other neurodegenerative diseases [94-96]. These events produce myelin vacuolation and fluid accumulation between myelin lamellae with extensive space-occupying lesions, which cause axoplasmic displacement and axonal...
Fig. (4). **Time course of demyelination induced by 0.2% cuprizone feeding.** Oligodendrocyte death begins at day three to seven and this event is followed by myelin protein degradation that approximately occurs at week one to three (early demyelination). At 4th week, the severe demyelination period begins and culminates at 5th - 6th weeks, when a complete demyelination is observed in many brain regions. The recovery process (remyelination phase) is detectable in the first week after cuprizone withdrawal, but it is more significant between the weeks two and four.

Fig. (5). **Mitochondrial response to CPZ and lipid metabolism disturbance.** Cuprizone induces alterations in many Cu dependent enzymes, generate increased oxidative stress leading to apoptosis and impairing the synthesis of key compounds in the myelin formation. ($\Delta\psi_{m}$, mitochondrial membrane potential).
disruption [14, 97]. Although myelin vacuolation is reversible, demyelination becomes irreversible by cell-body degeneration of mature oligodendrocyte [67]. OLs degeneration and apoptosis precede the CPZ-induced demyelination. This sequence of events suggests that the initial deterioration of OLs is not linked to autoimmune response against myelin proteins. A few days after CPZ treatment, oxidative stress induces degeneration of oligodendrocyte and myelin sheath that progresses to OLs apoptosis. Once mature OLs are disrupted, a sequence of cellular and inflammatory processes drive caspase-3-triggered apoptosis and demyelination [36]. Caspase-3 is a crucial apoptotic protease [98] that is expressed at initial stages of cuprizone model in most of the injured oligodendrocytes. At later stages, the percentage of caspase-3-expressing oligodendrocytes decreases gradually until it is completely absent at 3rd week. This evidence suggests a biological switch in the cell-death mechanism triggered by CPZ, that begins with a caspase 3-dependent mechanism and progressively changes to a caspase 3-independent cell death [29] (Fig. 5).

5.3. Immune System Influence in the CPZ Model

The classical hypothesis indicated that cuprizone intoxication produced a direct cell-autonomous toxicity in mature OLs with the concomitant demyelination. However, inflammatory mediators seem to be involved in the process of cuprizone-induced demyelination (Fig. 6). Some of this evidence includes: 1) Production of NO by upregulating inducible nitric oxide synthase (iNOS) and the neuronal isofrm (nNOS), which strongly determine the demyelinating process as demonstrated in eNOS^-/- mice [30, 31]. 2) Inflammatory cytokines produced by microglia and astrocytes that have a cytotoxic role and promote the overexpression of the endothelial nitric oxide synthase (eNOS) [16]. 3) Astrocyte production of interleukin-6 (IL-6) and interleukin-17 (IL-17) resulted in functional deficits associated with accelerated demyelination, reduced myelin synthesis and microglia activation [65, 99]. 4) Overexpression of CXCL10 produced by astrocytes that induces reactive microglia and, in turn, increases the levels of TNFα [13]. 5) IFN-γ that is crucial in the demyelinating process as demonstrated in transgenic mice that express low level of IFN-γ under the transcriptional control of MBP gene (MBP/IFN-γ mice) and display almost null evidence of injury in the white matter after CPZ administration [100-102]. 6) Interferon-beta (IFN-β) absence promotes remyelination by increasing the proliferation of oligodendroglial precursors cells [103]. 7) Activation of neutrophils that express the type 2 chemokine receptor (CXCRI2) and trigger demyelination, as observed in CXCRI2^-/- mice that are resistant to CPZ-induced demyelination [104]. Taken together, this evidence suggests that the immune system is a key component in the cascade of events triggered by cuprizone administration. Therefore, this model may represent an plausible tool for studying the role of immune system in the process of demyelination [30].
CONCLUSION

The CPZ model is a highly reproducible and easily implemented animal model that produces very consistent demyelinating lesions. The white matter damage, oligodendrocyte apoptosis and microglial activation induced by CPZ mimics some of the aspects of demyelinating diseases. Thus, CPZ model has some characteristics that help study the interactions between the immune system with the oligodendrocyte damage and myelin disruption, which can provide crucial information to test potential therapies against demyelinating diseases. Although it is generally accepted that CPZ model induces extended demyelination and important histological changes, the complete cascade of biochemical events triggered by CPZ remains unclear. In addition, more research needs to be done to establish these mechanisms and clarify the pathways on how CPZ can reach the brain.

LIST OF ABBREVIATIONS

AA = Arachidonic acid
CC = Corpus callosum
COX-1, COX-2 = Cyclooxygenase 1 or 2
CNS = Central nervous system
CPZ = Cuprizone
CXCR2 = Type 2 chemokine receptor
EAE = Experimental encephalomyelitis
eNOS = Endothelial nitric oxide synthase
IFNγ = Interferon gamma
iNOS = Inducible nitric oxide synthase
MBP = Myelin basic protein
MS = Multiple sclerosis
nNOS = Neuronal isoform
OLs = Oligodendrocytes
OPCs = Oligodendrocyte progenitor cells
PGI2 = Prostacyclin
PGH2, PGE2, PGD2, PGF2 = Prostaglandin H2, E2, D2 or F2
PLA2 = Phospholipase A2
ROS = reactive oxygen species
TMEV = Theiler’s murine encephalomyelitis virus
TNFα = Tumor necrosis factor alpha

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.
Copper and human health: biochemistry, toxicity, copper deficiency and neurodegeneration.

Neurotoxicology, 2010, 31(5), 509-517.

http://dx.doi.org/10.1016/j.neurotox.2010.05.008 [PMID: 20685220]

Carlton, W.W. Studies on the induction of hydroperoxide and spongy degeneration by cuprizone feeding and attempts to antidote the toxicity. Life Sci., 1967, 6(1), 11-19. [http://dx.doi.org/10.1016/0025-2230(67)90356-6] [PMID: 6030552]

Ransom Stern, B.; Solizio, M.; Krewski, D.; Aggett, P.; Aw, T.-C.; Baker, S.; Crump, K.; Dourson, M.; Haber, L.; Hertzberg, R.; Keen, C.; Meeck, B.; Rudenko, L.; Schoeny, R.; Slob, W.; Starr, T. Copper and human health: biochemistry, genetics, and strategies for modeling dose-response relationships. J. Toxicol. Environ. Health, 2007, 70(10), 157-222.

In Copper in Drinking Water; Washington, DC, 2000.

Scheibler, I.; Dringen, R.; Mercer, J.B.C. Copper: Effects of deficiency and overload. Interrelations between essential metal ions and human diseases; Sigel, A.; Sigel, H.; Sigel, K.O.R., Eds.; Springer Netherlands: Dordrecht, 2013, 359-387. [http://dx.doi.org/10.1007/978-94-007-7500-8_11] [PMID: 8615367]

Lindner, M.; Heine, S.; Haastert, K.; Garde, N.; Fokuhji, J.; Linsmeier, F.; Grothe, C.; Baumgartner, W.; Stangel, M. Sequential myelin protein expression during remyelination reveals fast and efficient repair after central nervous system demyelination. Neuropsychopharmacology, 2008, 33(1), 105-114. [http://dx.doi.org/10.1038/npp.2007.116] [PMID: 17961136]

Torkildsen, O.; Bruborg, L.A.; Myhr, K.M.; Be, L. The cuprizone model for demyelination. Acta Neurol. Scand. Suppl., 2008, 188, 72-76. [http://dx.doi.org/10.1111/j.1600-0404.2008.01036.x] [PMID: 18439226]

Hesse, A.; Wagner, M.; Held, J.; Brück, W.; Salinas-Riest, G.; Hao, Z.; Waisman, A.; Kuhlmann, T. In toxic demyelination oligodendroglial cell death occurs early and is FAS independent. Neurobiol. Dis., 2010, 37(2), 362-369. [http://dx.doi.org/10.1016/j.nbd.2009.10.016] [PMID: 19853662]

Lìhores, D.; Taconis, M.; Maña, P.; Correa, M.; Fordham, S.; Staykova, M.; Willenberg, D.O. Neuronal nitric oxide synthase plays a key role in CNS demyelination. J. Neurosci., 2006, 26(49), 12629-12638. [http://dx.doi.org/10.1523/JNEUROSCI.0294-06.2006] [PMID: 17151270]

Arnett, H.A.; Hellendall, R.P.; Matsushima, G.K.; Suzuki, K.; Laubach, V.E.; Sherman, P.; Ting, J.P. The protective role of nitric oxide in a neurotoxicant-induced demyelinating model. J. Immunol., 2002, 169(1), 427-433. [http://dx.doi.org/10.4049/jimmunol.169.1.427] [PMID: 11751989]
Five Decades of Cuprizone, an Updated Model to Replicate Demyelinating Diseases

Zhang, Z.; Tan, Q.; Kong, J.; Huang, Q.; Li, X.

doi.org/10.1155/2011/826976

Page 139

Current Neuropharmacology, 2017, Vol. 17, No. 2

Acetylcholine is a neurotransmitter that is essential for normal brain function. Its importance is highlighted by the study of its metabolism in various conditions, such as schizophrenia and multiple sclerosis. This study examines the impact of acetate on acetylcholine levels and provides insights into the mechanisms underlying these conditions. It also discusses the potential therapeutic implications of acetate for the treatment of these diseases. The use of the cuprizone model is discussed in the context of these findings.
induced spongiform encephalopathy. I. Changes in the activities of myelin in the mammalian central nervous system. Biochem. Biophys. Res. Commun., 2014, 47, 485-505. [http://dx.doi.org/10.1016/j.убиовио.2014.10.004] [PMID: 25454182]

Carron, W.W. Studies on the induction of hydrocephalus and spongion degeneration by cuprizone feeding and attempts to antidote the toxicity. Life Sci., 1967, 6(11), 11-19. [http://dx.doi.org/10.1016/0025-2230(67)90356-6] [PMID: 6035552]

Bénardass, K.; Kotsiaris, A.; Skujie, J.; Koutoudaki, P.N.; Gudi, V.; Singh, V.; Vulinovic, F.; Sripurez, T.; Stangel, M. Cuprizone [bis-cyclohexylidenemethylazide]-induced is selectively toxic for mature oligodendrocytes. Neurotox. Res., 2013, 24(2), 244-250. [http://dx.doi.org/10.1007/s12046-013-9380-9] [PMID: 23392957]

Hiremam, M.M.; Saito, Y.; Knapp, G.W.; Ting, J.P.; Suzuki, K.; Matsuishama, G.K. Microglial/macrophage accumulation during cuprizone-induced demyelination in C57BL/6 mice. J. Neuroimmunol., 1998, 92(2-3), 38-49. [http://dx.doi.org/10.1016/S0165-5728(98)00168-4] [PMID: 9916678]

Kesterson, J.W.; Carlson, W.W. Monoamine oxidase inhibition and the activity of other oxidative enzymes in the brains of mice fed cuprizone. Toxicol. Appl. Pharmacol., 1971, 20(3), 386-395. [http://dx.doi.org/10.1016/0041-008X(71)90281-X] [PMID: 4399886]

Venturini, G. Enzymic activities and sodium, potassium and copper concentrations in mouse brain and liver after cuprizone treatment in vivo. J. Neurochem., 1973, 21(5), 1147-1151. [http://dx.doi.org/10.1111/j.1471-4159.1973.tb05769.x] [PMID: 4357499]

Suzuki, K. Giant hepatic mitochondria: production in mice fed with cuprizone. Science, 1969, 163(3862), 81-82. [http://dx.doi.org/10.1126/science.163.3862.81] [PMID: 5763494]

Wakabayashi, T. Megamitochondria formation - physiology and pathology. J. Cell. Mol. Med., 2002, 6(4), 497-538. [http://dx.doi.org/10.1111/j.1525-157X.2002.tb00452.x] [PMID: 12611638]

Veto, S.; Acs, P.; Bauer, J.; Lassmann, H.; Berente, Z.; Setalo, G.; Jr; Borgulya, G.; Sumbegi, B.; Komoly, S.; Gallyas, F.; Jr; Illies, Z. Inhibiting poly(ADP-ribose) polymerase: a potential therapy against oligodendrocyte death. Brain, 2010, 133(Pt 3), 822-834. [http://dx.doi.org/10.1093/brain/awp097] [PMID: 20157037]

Baumann, N.; Pham-Dinh, D. Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol. Rev., 2001, 81(2), 871-927. [http://dx.doi.org/10.1152/physrev.2001.81.2.871] [PMID: 11274346]

Palumbo, S.; Tiso, C.; Parente, L.; Weigert, R.; Bosetti, F. Time-dependent changes in the brain arachidonic acid cascade during cuprizone-induced demyelination and remyelination. Prostaglandins Leukot. Essent. Fatty Acids, 2011, 85(1), 29-35. [http://dx.doi.org/10.1016/j.pl Estado.2011.04.001] [PMID: 21530210]

Carey, E.M.; Freeman, N.M. Biochemical changes in Cuprizone-induced spongiform encephalopathy. I. Changes in the activities of 2',3'-cyclic nucleotide 3'-phosphohydrolyase, oligodendroglial cytidine galactosyl transferase, and the hydrolysis of the alkyl group of alkyl, acyl-glycerophospholipids by phospholipase A in different regions of the brain. Neurochem. Res., 1983, 8(8), 1029-1044. [http://dx.doi.org/10.1007/BF00965198] [PMID: 6512351]

Neu, I.; Woelck, H. Investigations of the lipid metabolism of the white matter in multiple sclerosis: changes in glycerophospholipids and lipid-splitting enzymes. Neurochem. Acta., 1982, 76, 727-735. [http://dx.doi.org/10.1007/BF00965525] [PMID: 7121719]

Balbo, M.A.; Varela-Nieto, I.; Killermann, Lucas, K.; Dennis, E.A. Expression and function of phospholipase A2 in brain. FEBS Lett., 2002, 531(1), 12-17. [http://dx.doi.org/10.1016/S0014-5793(02)03481-6] [PMID: 12401195]
Five Decades of Cuprizone, an Updated Model to Replicate Demyelinating Diseases

114. Elsworth, S.; Howell, J.M. Variation in the response of mice to cuprizone. *Res. Vet. Sci.*, 1973, 14(3), 385-387. [PMID: 4805158]

115. Bakker, D.A.; Ludwin, S.K. Blood-brain barrier permeability during Cuprizone-induced demyelination. Implications for the pathogenesis of immune-mediated demyelinating diseases. *J. Neurosci.*, 1987, 7(2), 125-137. [http://dx.doi.org/10.1016/0270-6474(87)90055-4] [PMID: 3553434]

116. Chen, Z.; Chen, J.T.; Johnson, M.; Gossman, Z.C.; Hendrickson, M.; Sakaie, K.; Martinez-Rubio, C.; Gale, J.T.; Trapp, B.D. Cuprizone does not induce CNS demyelination in nonhuman primates. *Ann. Clin. Transl. Neurol.*, 2015, 2(2), 208-213. [http://dx.doi.org/10.1002/acn3.159] [PMID: 25750925]

117. 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(1), 357-367. [http://dx.doi.org/10.1016/j.expneurol.2012.02.018] [PMID: 22421533]

118. Love, S. Cuprizone neurotoxicity in the rat: morphologic observations. *J. Neurol. Sci.*, 1988, 84(2-3), 223-237. [http://dx.doi.org/10.1016/0022-510X(88)90127-X] [PMID: 2837540]

119. Carlton, W.W. Spongiform encephalopathy induced in rats and guinea pigs by cuprizone. *Exp. Mol. Pathol.*, 1969, 10(3), 274-287. [http://dx.doi.org/10.1016/0014-4800(69)90057-4] [PMID: 5786627]

120. Valeiras, B.; Rosato Siri, M.V.; Codagnone, M.; Reînes, A.; Pasquini, I.M. Gender influence on schizophrenia-relevant abnormalities in a cuprizone demyelination model. *Glia*, 2014, 62(10), 1629-1644. [http://dx.doi.org/10.1002/glia.22704] [PMID: 24890315]

121. Hall, S.M. The effect of injections of lysophosphatidyl choline into white matter of the adult mouse spinal cord. *J. Cell Sci.*, 1972, 10(2), 535-546. [PMID: 5018033]

122. Jeffery, N.D.; Blakemore, W.F. Remyelination of mouse spinal cord axons demyelinated by local injection of lysolecithin. *J. Neurocytol.*, 1995, 24(10), 775-781. [http://dx.doi.org/10.1007/BF01191213] [PMID: 8586997]

123. Cerina, M.; Narayanan, V.; Göbel, K.; Bittner, S.; Ruck, T.; Meuth, P.; Herrmann, A.M.; Stangel, M.; Gudi, V.; Skripuletz, T.; Daldrup, T.; Wiendl, H.; Seidenbecher, T.; Ehling, P.; Kleinschnitz, C.; Pape, H-C.; Budde, T.; Meuth, S.G. The quality of cortical network function recovery depends on localization and degree of axonal demyelination. *Brain Behav. Immun.*, 2017, 59, 103-117. [http://dx.doi.org/10.1016/j.bbi.2016.08.014] [PMID: 27569659]

124. van der Star, J. In vitro and in vivo models of multiple sclerosis. *CNS & Neurological Disorders-Drug Targets*, 2012, 11(5), 570-588.

125. Shindler, K.S.; Ventura, E.; Dutt, M.; Rostami, A. Inflammatory demyelination induces axonal injury and retinal ganglion cell apoptosis in experimental optic neuritis. *Exp. Eye Res.*, 2008, 87(3), 208-213. [http://dx.doi.org/10.1016/j.exer.2008.05.017] [PMID: 18653182]

126. Ulrich, R.; Baumgärtner, W.; Gerhauser, I.; Seeliger, F.; Haist, V.; Deschl, U.; Alldinger, S. MMP-12, MMP-3, and TIMP-1 are markedly upregulated in chronic demyelinating theiler murine encephalomyelitis. *J. Neuropathol. Exp. Neurol.*, 2006, 65(8), 783-793. [http://dx.doi.org/10.1097/01.jn.0000229990.32795.0d] [PMID: 16896122]

127. Gudi, V.; Gai, L.; Herder, V.; Jejedor, L.S.; Kipp, M.; Amor, S.; Sühs, K.W.; Hansmann, F.; Beineke, A.; Baumgärtner, W.; Stangl, M.; Skripuletz, T. Synaptophysin is a reliable marker for axonal damage. *J. Neuropathol. Exp. Neurol.*, 2017. [Epub ahead of print] [http://dx.doi.org/10.1093/jnen/nlx114] [PMID: 28177496]