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Research report

Behavioural changes observed in demyelination model shares similarities with white matter abnormalities in humans

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HIGHLIGHTS

- The demyelination induced by cuprizone in Lewis rats induces anxiety-like behaviour.
- The demyelination leads to cognitive impairment.
- Behavioural changes and neuroinflammation in Lewis rats share some similarities to those seen in human patients with white matter damage.

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ABSTRACT

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating disease of the central nervous system (CNS). Further to the symptoms resulting from demyelination, new studies point to the involvement of neuroinflammation and white matter abnormalities in psychiatric disorders and neurodegenerative diseases. Cuprizone, a model of MS, produces consistent demyelination and elicits behavioural, morphological and inflammatory changes in animals that share some similarities with those observed in humans. In this study, we used the cuprizone model in Lewis rats to evaluate clinical signs triggered by the demyelination process which could be comparable with the symptoms seen in white matter abnormalities in human beings. To induce the demyelination process, 0.6% cuprizone was added to the Lewis rats' diet for 4 weeks. We proceeded with behavioural, morphological and immunological analyses. Animals fed with cuprizone exhibited behavioural changes: higher scores in the neurotoxicity test, reduced exploratory and locomotion behaviour, and also an increase of permanency in the closed arm of the elevated plus maze test, were observed. In these analyses, the animals showed motor coordination impairment and anxiety-like behaviour. Demyelination also triggered changes in discrimination of objects identified by an increase in the time spent close to a familiar object. These behavioural alterations were associated with a significant increase in the levels of TNF-alpha and corticosterone, consistent with the activation of microglia and astrocytes. Taken together, the results of this work show the cuprizone/Lewis rat model demyelination as an attractive paradigm for studying the correlation between white matter abnormalities and behaviour.

1. Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating disease of the central nervous system (CNS), afflicting young adults during its productive phase, around 40 years old. It is believed the MS autoimmune attack is orchestrated against antigens of myelin and oligodendrocytes. During the autoimmune attack, the effector cells release cytokines leading to myelin destruction [1,2]. It has been shown that neuroinflammation and white matter abnormalities are related to a large number of

Abbreviations: MS, multiple sclerosis; CNS, central nervous system; PBS, phosphate buffered saline; LFB, luxol fast blue-staining; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein; OD, optical densities; TN, time spent with novel object; TF, time spent with familiar object; EPM, elevated plus maze; FOB, functional observational battery; TNF-alpha, tumour necrosis factor-alpha.

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diseases. However, besides the symptoms resulting from demyelination, new studies point to the involvement of neuroinflammation and white matter abnormalities in psychiatric disorders and neurodegenerative diseases [3].

It is believed that the increased expression of cytokines in the CNS could trigger behavioural changes, as seen in some patients who manifest symptoms of psychopathology before being diagnosed with the disease [4,5]. It is not clear if the behavioural changes observed in MS are correlated with the inflammatory process, white matter integrity and neurological deficit [6,7].

Traditionally used as a model of MS, cuprizone (bis(cyclohexanone)oxaldihydrazone) is able to produce consistent demyelination after 15 days of treatment [8]. The effects induced by cuprizone are not fully understood but have attracted much interest since white matter disorders are related to behavioural changes. Recent studies suggest that changes in communication between different regions of the brain are responsible for the clinical symptoms and cognitive dysfunction observed in patients with such neurological disorders, e.g. schizophrenia [9–11].

We hypothesized that the changes caused by demyelination in acute and chronic experimental models are comparable to the effects of demyelination observed in humans. Thus, the demyelination by cuprizone in Lewis rats could be used as an animal model for the study of inflammatory autoimmune demyelination and behavioural changes elicited by the white matter abnormalities.

Our results demonstrate that the behavioural changes and cognition impairment in animals treated with cuprizone are related to the occurrence of demyelination, specifically in the corpus callosum. These animals have elevated scores for neurotoxicity, gait and motor coordination, as well as having reduced exploratory activity, and signs of anxiety.

2. Materials and methods

2.1. Animals and treatment

Lewis rats, male, aged 7 weeks (CEMIB-UNICAMP) were housed in groups of 5 rats per cage at room temperature (24 ± 1 °C), and provided with food and water ad libitum. The animals were maintained in the animal care of Campus Baixada Santista – Universidade Federal de São Paulo, in a light/dark cycle (12/12 h). All procedures in this protocol were approved by the Animal Experimental Ethics Committee of the Universidade Federal de São Paulo (1254/10).

2.2. Diet

The animals from the cuprizone group were fed with 0.6% cuprizone (bis(cyclohexanone)oxaldihydrazone – Sigma–Aldrich, St. Louis, MO, USA) mixed in a ground chow, for 5 weeks. The control group were fed with the same ground chow, without the cuprizone addition.

2.3. Histology and immunohistochemistry

Rats were perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. After dissection, tissue samples were fixed in 4% paraformaldehyde in PBS overnight at 4 °C, cryoprotected in 30% sucrose solution, embedded in O.C.T. compound (Tissue-Tek; Sakura, Alphen aan den Rijn, The Netherlands), frozen on dry ice, and stored at 80 °C. Sections of 12 μm were obtained by cryostat, mounted onto slides, and allowed to dry. The demyelination was detected by Luxol Fast Blue (LFB) staining: The sections were immersed in chloroform/ethanol (1:1 Merck – Darmstadt, Germany) to remove lipids, and afterwards were incubated in Luxol Fast Blue staining solution (1% in 95% ethanol with 0.5% acetic acid) at 56 °C overnight. Sections were then differentiated in 0.5% lithium carbonate solution for 30 s and counterstained with cresyl violet, then dehydrated in an ethanol series, cleared in xylene, and mounted in Entelan (Merck – Darmstadt, Germany). For the immunostaining, antigen retrieval was performed by incubating the slides in 10 mM sodium citrate, pH6, for 5 min at 95 °C and then for 30 min at room temperature. Slides were then incubated for 1 h at room temperature in blocking solution (5% normal donkey serum, 0.3% TritonX-100 in PBS). Primary antibody was added to the blocking solution which was then incubated overnight at 4 °C. Primary antibodies consisted of MBP (1:200 – Millipore, Watford, UK), GFAP (1:200), IBA-1 (1:200) and OLIG2 (1:300) (Abcam, Cambridge, UK). The slides were photographed in AxioVision (Carl Zeiss, Gottingen, Germany).

2.4. Cell quantification

The corpus callosum images were evaluated by placing random squares (10 mm^2) and measuring the optical densities (OD) of LFB stain for semi-quantification of the intensity of myelin. A higher OD indicates a higher intensity/transmittance and is represented by a lower LFB staining intensity [12]. The same principle was used to count the positive immunostained cells. We used ImageJ v.1.31 software (U.S. National Institutes of Health, Bethesda, Maryland, USA – available as freeware from http://rsbweb.nih.gov/ij/).

2.5. Behavioural assessment

2.5.1. Neurotoxicity test

In order to assess the evolution of behavioural changes, all animals were tested for behavioural and motor neurotoxicity (functional observation battery – FOB) on three occasions during the trial period: on the 1st day of treatment, on the 15th day and on the day before euthanasia. This test consists of neuromuscular, autonomic and sensorimotor analyses that allow the determination of activity and CNS excitability, which are the bases for scoring, as previously established [13].

2.5.2. Open field test

The Open Field test assessed the total distance crossed and the distance crossed in the central region for a period of 5 min. Testing was conducted by a trained, blind observer.

2.5.3. Novel object recognition

The test was made in the Open Field arena. Prior to testing, the animal was familiarized with the field for 30 min. At the start of the test, the animal was placed into the centre of the open-field and allowed to freely explore for 5 min. The objects to be discriminated had different shapes and could not be displaced by the animal. Then the animal was allowed to explore the new object, the familiar object and the environment for 5 min to assess cognition. The exploration was defined as the rat placing its nose within 2 cm of the object and any one of the following, which the observer determined to be exploration of the object: obvious movement of the vibrissae, sniffing, licking, or rearing onto the object. Some actions were not considered as exploration: sitting on the object or close contact in which the nose is not directed at the object. Tests were conducted during two periods: the 1st day of treatment and prior to euthanasia. The recognition index (RI) was calculated: RI = TN/(TN + TF) × 100, where TN = time spent with novel object and TF = time spent with familiar object [14,15].

2.5.4. Testing in elevated plus maze

The animals were individually tested on the elevated plus maze (EPM) for a 5-min period. An EPM consists of two open arms and two closed arms, and at the beginning of each test, the rat is placed