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Research Article

Copper and zinc dismetabolism in the mouse brain upon chronic cuprizone treatment

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Abstract. Recent reports describe successful treatment using copper chelation therapy in neurodegenerative animal models. However, the success claimed for chelation therapy in neurodegenerative diseases is still rather controversial. To acquire new information on copper metabolism/homeostasis, we utilized cuprizone, a very sensitive and selective copper-chelating agent with well-known neurotoxic properties, as a relevant chemical model in mice. Upon cuprizone treatment, mice developed a pronounced astrocytosis, with brain oedema and spongiosis characterised by vacuolisations of the neuropil predominantly in the white matter. In addition, cuprizone treatment severely altered copper and zinc homeostasis in the central nervous system (CNS) as well as in all other tissues examined, with increasing metal ion concentrations particularly in the CNS. Concomitant with this increase in the Cu and Zn concentration in the brain, metallothionein-I and -II were also highly immunoreactive in astrocyte, consistent with the astrocytosis and demyelination observed in our and other laboratories.

Key words. Cuprizone; metallothionein; copper; zinc; myelin; astrocyte; mouse brain; prion disease.

Copper is an essential element with an estimated safe and adequate intake between 1.5 and 3.0 mg/day [1]. The complexity of copper uptake in mammalian systems is further compounded by the need to absorb copper from the diet and distribute it throughout the tissues in the body [2]. Compartmental models of copper metabolism have been studied in humans and animals, demonstrating a very complex pathway for this metal ion [3, 4]. Incorporation of copper into ceruloplasmin, storage of the metal ion in metallothionein (MT), and transfer of copper into the cytochrome oxidase in the mitochondria with eventual excretion into the bile have been demonstsrated [5]. Non-ceruloplasmin-bound copper is linked to albumin, amino acids or small peptides that can cross the blood-brain barrier [6].

In higher organisms, cuproenzymes are thought to be important for the development and maintenance of many physiological systems: haemopoietic, cardiovascular, nervous, skeletal, reproductive and integumentary [7]. Some of the alterations in biological systems resulting from copper dishomeostasis have a firm biochemical basis [8]. The route of the metal ion administration does not apparently influence the dietary copper level and the liver is the major tissue involved in metabolism of copper, while plasma is the major fluid by which copper is transported [5]. Copper deficiency is rather rare and may

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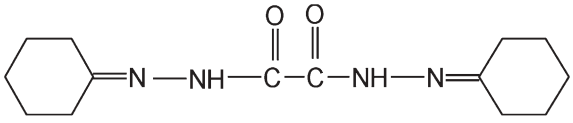
cause anaemia, changes in ossification and, possibly, elevated plasma cholesterol. In humans, despite widely different levels of dietary copper, it is possible to maintain relatively constant Cu-ceruloplasmin and copper plasma levels [5]. An exhaustive review of copper transport has been published recently [9].

Copper is known to be involved in some diseases with neurological implications such as Wilson's and Menkes' disease [8–10]. In addition, several authors have hypothesised that neuronal degeneration in a number of diseases could have copper deficiency as a relevant etiopathogenic factor [11]. Demyelisation has often been associated with copper deficiency, as in swayback disease in lambs, spongiosis in goats, mule deer [12, 13] and sika deer [14], and scrapie and Creutzfeldt-Jacob disease [15, 16]. However, copper analyses of the brain tissues for the purpose of ascertaining the alteration of copper concentration in these pathologies are either lacking or poorly reported [17].

In many organisms, a major role in metal homeostasis is played by MTs, a family of low-molecular-weight, cysteine-rich proteins with high binding capacity to Zn and/or Cu [18]. In mammals, four major isoforms (MT-I–MT-IV) are known, among which, MT-I and MT-II are expressed in most tissues including the brain. In normal brains, microglia and oligodendrocytes are apparently essentially devoid of MT-I/II whereas a significant MT-I/II immunoreactivity was observed in microglia of cerebral areas with marked damage of tissues with strong astrocytosis. MTs are generally referred to as a cytoplasmic component essential for Zn and Cu metabolism. In agreement, the promoter region of the multigene complex responsible for MT synthesis presents metal-responsive regulatory elements controlled by the metal regulatory transcription factor MTF1 [19]. Very detailed studies are available on the molecular physiology of MT, typically in liver, but an increasing interest is also focused on brain [10, 20]. Such studies are encouraged by evidence pointing to the importance of metal ion dismetabolism for the onset of neurodegenerative diseases, in particular ions such as Cu(I) that are typically bound to MTs. Thus, in addition to some encephalopathies that are relatively well-characterized from the metal physiopathological point of view – Wilson's, Menkes' and Alzheimer's diseases [10, 20, 21] – recent evidence shows that other diseases such as Binswanger's encephalopathy [22] involve alteration of brain MT levels. Evidence is increasing that these proteins in brain play a very important protective role. In particular, MT-I/II deficiency results in an increased incidence and worsening of clinical symptoms, including demyelination and axonal damage, during experimental autoimmune encephalomyelitis (EAE) in mice [23] and multiple sclerosis in patients and rats [24, 25]. Conversely, MT-II transcripts are the most significantly upregulated of 83 transcripts in the case of mouse focal cerebral ischaemia, while MT-I-overexpressing transgenic mice are protected against focal cerebral ischaemia and reperfusion [26, 27]. Furthermore, disturbances in brain copper homeostasis have been suggested, albeit controversially, in the case of prion disease [28, 29].

Treating animals with copper-chelating agents produces neuronal abnormalities including spongiform changes in white matter and reduced myelin development [30]. As far as we know, several compounds (e.g. porphyrin, polyanionic sulphated glycans, congo red, etc.) can interfere with metal ion metabolism apparently mimicking scrapie-like disease in mice [30, 31] with inhibition of the protease-resistant prion protein (PrP^{SC}) as observed in cell cultures [32].

Cuprizone [oxalic acid bis(cyclohexylidene hydrazide)] (CZ) is a well-known neurotoxic agent acting as a selective and sensitive copper chelator. CZ forms a tight Cu-complex, in the pH range between 7 and 10, with a very strong absorption band at 595 nm [33].



Cuprizone: oxalic acid bis(cyclohexylidene hydrazide) $C_{14}H_{22}N_4O_2$ [MW: 278.36]

Several papers have reported that experimental animals treated with 0.5% CZ in the diet developed a spongiosis [34–41]. In addition, mice astrocytosis was observed in the deep cerebral cortex starting 8 days after CZ treatment in the solid diet, and after 17 days in the hippocampus and thalamus. Finally, CZ is reported to have a great effect on myelin metabolism, reducing nerve myelination dramatically and severely altering the immunological system [42]. From a clinical point of view, mice treated with CZ showed the hind-limb clasping that occurs in scrapie-affected mice [43], and which is considered characteristic of this disease [44]. Furthermore, growth retardation, posterior paresis and high mortality were also observed. Among the various animal species studied so far, the mouse appears to be the most sensitive to CZ treatment. Resistance to various effects produced by CZ treatment is highly dependent on the mouse strain utilised [38, 45, 46]. At the moment, the chemical form in which CZ interacts with brain cells is unknown. In this paper, unlike previous experimental approaches, CZ treatment in mice was carried out as a long-term treatment, administering CZ at low doses for several months. The presence of CZ in plasma and brain was demonstrated by mass spectrometry. As far as the biological effects are concerned, in addition to confirming the spongiosis with all other alterations previously described, the Cu, Zn and Fe concentrations in the brain and several other organs were found to be

markedly altered. The changes in the tissue metal patterns were correlated with the effects on the expression levels of MT, in particular the ubiquitous isoforms MT-I/II. The increase of MT-I/II expression levels were found to correlate positively with the increase in glial fibrillary acidic protein (GFAP), a characteristic of astrocytes [47], the expression of which is increased in the case of gliosis. The effects on myelination were studied using myelin basic protein (MBP) as marker.

Materials and methods

Animal treatment

CZ was purchased from Aldrich (Milan, Italy) and administered to a group of CD mice (Morini, Bologna, Italy) (male n = 30) per os with bidistilled drinking water (0.2%) for 9 months. Controls (male n = 10, bidistilled water) and CZ-treated animals were fed ad libitum with normal mouse food purchased from Morini (Bologna, Italy).

The animals were housed and fed under standard conditions, in accordance with the ECC Directive 86/609 and APA Ethical Principles for Animal Experimentation.

Metal ion determination

Tissue samples were taken immediately after animal decapitation following anaesthetisation with Zoletil 100 (Virbac srl, Milan, Italy). Samples were transferred in polystyrene test tubes pre-washed several times with HNO_3. A few milligrams of tissue were mineralised in 1 ml HNO_3 Suprapur (Merck, Milan, Italy) at 75 °C until the solution became clear. Analysis of total metal ion concentration (free + bound) was carried out using an atomic absorption spectrophotometer (Analyst 100; Perkin Elmer, Monza, Italy), comparatively with relevant standard solutions.

Spectroscopic measurements of the copper-CZ complex

The copper-CZ complex was prepared according to a procedure slightly modified with respect to that described by Nilsson [48]. The dark-blue complex was obtained both in solution and in the solid state. Visible absorption spectra of the copper-CZ complex were measured out with a Perkin-Elmer Lambda Bio 20 instrument at room temperature (25 °C).

Quantification of MTs by the silver saturation method

Total content measurement of MTs was carried out using the method described by Scheuhammer and Cherian [49].

Histology and immunohistochemistry

Animals (CZ treated and controls) were sacrificed by decapitation after anaesthetisation, and tissues were promptly removed and transferred into 4% buffered formalin. Immunohistochemical identification of MT-I/II was carried out as previously described [21]. Briefly, a mixture of mouse monoclonal antibodies directed against MT-I/II (anti-MTs) (DAKO-MT, E9; DakoCytomation, Milan, Italy) was utilised. Immunohistochemistry was carried out as follows. Microsections of 6 µm were incubated with 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min and, after washing, treated with normal goat serum for 30 min. Sections were then incubated overnight at 4 °C with anti-MTs at a dilution of 1:50. After washing twice with PBS, sections were reacted with biotinylated goat anti-mouse immunoglobulin G for 30 min, rinsed three times, and then processed with streptavidin-peroxidase complex (ABC complex; DakoCytomation). The sections were developed with 3,3′-diaminobenzidine and H_2O_2 in 50 mM Tris-HCl, pH 7.5, and then counterstained with haematoxylin.

All chemicals were of the purest commercial grade and were purchased from Sigma-Aldrich (Milan, Italy), unless specified otherwise. Reactive astrocytes were identified using either a polyclonal rabbit anti-GFAP antibody (Sigma-Aldrich) or polyclonal rabbit anti S-100 antibody (DakoCytomation). For the demonstration of MBP, especially in myelin sheaths of nerve fibres, polyclonal rabbit antibodies against human MBP were used (Dako Cytomation).

Mass spectrometry identification of CZ in plasma and brain samples

Plasma samples of 0.25 ml or lyophilised brain samples of 100 µg from CZ-treated animals were extracted with 1 ml of $CHCl_3$/CH_3CH_2OH (Merck) (1:1 v/v) and, after centrifugation at 2000 g for 15 min, the supernatant was evaporated to 100 µl under vacuum, and 1 µl was injected into a mass spectrometry system.

Mass spectrometry (ESI-ITMS) analyses were performed using an LCQ_{Deca} ion trap mass spectrometer (Thermo, San Jose, Calif.) with electrospray interface and equipped with a 7725i Rheodyne injector. The following instrumental parameters were used for ESI-MS detection: capillary voltage 5.00 V, capillary temperature 250 °C, sheath gas flow 60 arb, ion spray voltage 5.0 kV.

Direct injection conditions: a standard solution of cuprizone was directly injected into the ESI-ITMS instrument by means of a syringe pump at a flow rate of 10 µl/min for optimising the ESI-ITMS conditions. Flow-injection (FI) conditions: the samples were injected through a Rheodyne injector (loop: 1 µl) into the ESI-ITMS instrument. Flow rate was achieved using a Phoenix 40 HPLC (Thermo, Milan, Italy) with 0.1% formic acid in 50% acetonitrile as eluent (flow rate 15µl/min).

The mass spectrometer was operated in positive mode from *m/z* 100 to 700 and 75 to 300 for full scan and MS/MS of molecular ion ($[M+H]^+$ = 279 *m/z*), respectively. For

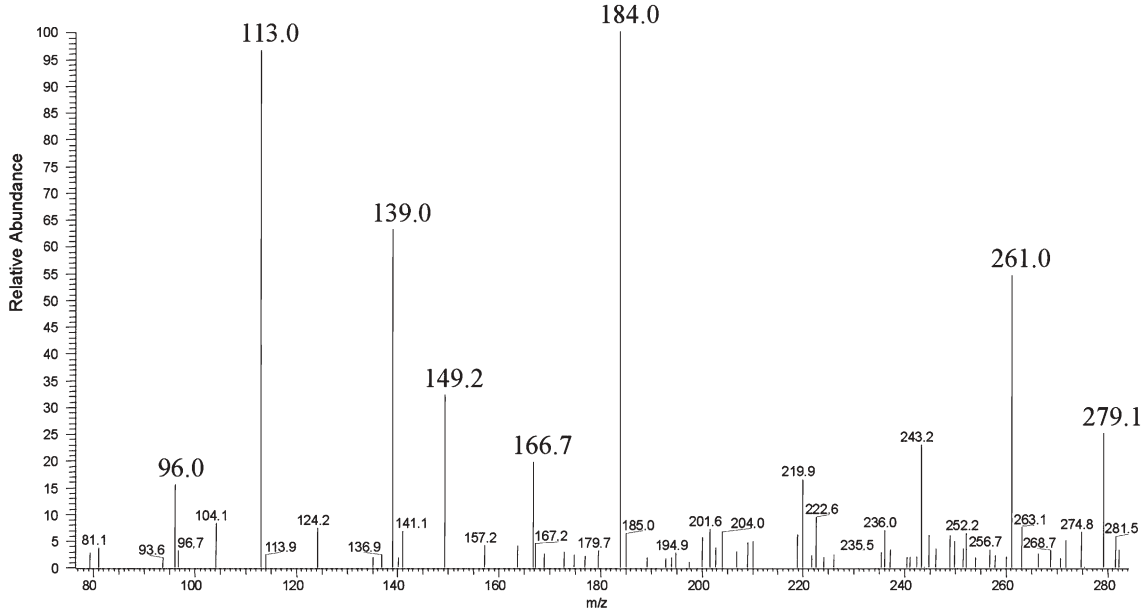


Figure 1. MS/MS fragmentation spectrum of CZ molecular ion ($[M+H]^+$ = 279 *m/z*) from a biological sample.

MS/MS analysis, the isolation width and normalised collision energy were 3.0 *m/z* and 35%, respectively.

Statistical analysis

Statistical analysis was performed using ANOVA test with Sheffe's post hoc analysis. Differences were considered significant at $p<0.05$.

Photomicrographs

The processed sections were photographed with a Nikon F-601 camera coupled to a Leitz Diaplan microscope, using Kodak Elit II (ISO 100/21°) colour slide film.

Results

Main chemical features of the copper-CZ complex

CZ is a copper-chelating agent extensively used for quantitative determinations [33] due to the intense absorption band (ε_{Max595} = 16,000) exhibited by its copper complex. CZ forms with Cu(II) a hydrophilic, intensely blue, complex the exact nature of which had never been clarified. We recorded the ESI MS spectra of this compound, which are dominated by an intense peak at 457 m/z (corresponding to CuL'_2 where L′ is the monohydrolysed derivative of CZ). This peak was not observed in the MS spectra of plasma from treated mice. However, we do not expect formation of significant quantities of such a Cu(II) complex in the plasma since CZ is not able to extract copper from copper(II) albumin (unpublished results from our laboratory). It can only be hypothesised that CZ in the plasma mainly exists as the free ligand and that in this form is able to cross membranes and enter cells. Thus, we expect that its reactivity with copper will mainly occur inside cells.

However, details of the solution behaviour and of the structure of the copper complex are not yet well known. This prompted us to investigate and characterise this species further, particularly as far as properties relevant for the interpretation of biological effects are concerned. An extensive chemical characterisation is in progress and will be the subject of a forthcoming publication.

In line with previous claims, we have observed that complete formation of the complex requires the presence of at least two CZ equivalents per copper ion. The complex is stable for several hours at physiological pH. The copper-CZ complex is highly hydrophilic; in fact, no colour transfer is observed when an aqueous solution is shaken with an immiscible organic phase (e.g. chloroform, n-butanol, CCl_4). Conversely, the free ligand is poorly soluble in water, moderately soluble in ethanol and very soluble in benzene and chloroform. The copper complex is hardly able to cross a semi-permeable membrane with a cut-off of 10,000 Da, strongly suggesting that it most likely exists in solution as an oligomeric species. This conclusion is further supported by preliminary electron paramagnetic resonance measurements that are suggestive of anti-ferromagnetic coupling among the copper centres (data not shown). Of note is that the tendency to form oligomeric species either in solution or in the solid state and the occurrence of strong anti-ferromagnetic coupling among copper centres had previously been described for related copper-acylhydrazone complexes [50].

Table 1. Concentration of total Cu (µg/g fresh weight) in different mice organs after 3, 6 and 9 months of CZ treatment. versus controls.

Organ	Control	3 months	6 months	9 months
Liver	7.69±2.52	8.46±1.6 p<0.1	11.03±1.79 p<0.2	20.6±4.52 p<0.05
Kidney	3.0±0.6	9.2±0.92 p<0.005	7.64±0.63 p<0.01	24.08±6.35 p<0.05
Heart	5.22±1.5	12.8±0.09 p<0.05	12.08±2.21 p<0.05	16.39±2.09 p<0.01
Stomach	11.73±0.95	13.3±2.74 p<0.2	13.9±1.76 p<0.1	46.35±1.62 p<0.005
Spleen	2.28±0.28	10.36±1.7 p<0.005	12.66±1.87 p<0.005	12.31±1.71 p<0.005
Large intestine	10±0.64	11.9±2.13 p<0.1	11.95±3.21 p<0.1	30.09±0.34 p<0.01
Small intestine	11.3±0.83	18.9±3.24 p<0.05	9.12±1.58 p<0.02	51.34±4.14 p<0.001

Values are the mean ± SE of ten determinations. Significance was evaluated by means of one-way ANOVA + Scheffe's post hoc test versus control.

Identification of CZ in the plasma of treated animals

Direct injection of a standard CZ solution into the ESI-ITMS instrument provided preliminary data to optimise the experimental conditions, which were then used in analysis using FI into the electrospray (FI/ESI-ITMS).

To confirm the identification of CZ, MS/MS experiments were performed. In particular, the main fragment ions were *m/z* 261, 184, 167, 139, 113 and 96. The analytical approach described above for studying the standard solution was then applied to the analysis of plasma and brain samples to determine the presence of CZ. Figure 1 reports an MS/MS fragmentation spectrum of ion *m/z* 279 in a mice plasma sample after 6 months of treatment. That the main fragment ions corresponded to those obtained for standard CZ: in particular 139+139, 113+167 and 184+94 are all related to the *m/z* 279 ion corresponding to free CZ. The Cu-CZ peak (see above) was not observed.

The same pattern was also obtained from lyophilised brain of CZ-treated animals, demonstrating the presence of the ligand in both compartments.

Metal ion distribution in various organs and tissues after CZ treatment

Table 1 reports the distribution of total copper (free and bound) in all organs and tissues, other than the brain, examined after 3, 6 and 9 months of CZ treatment, com-

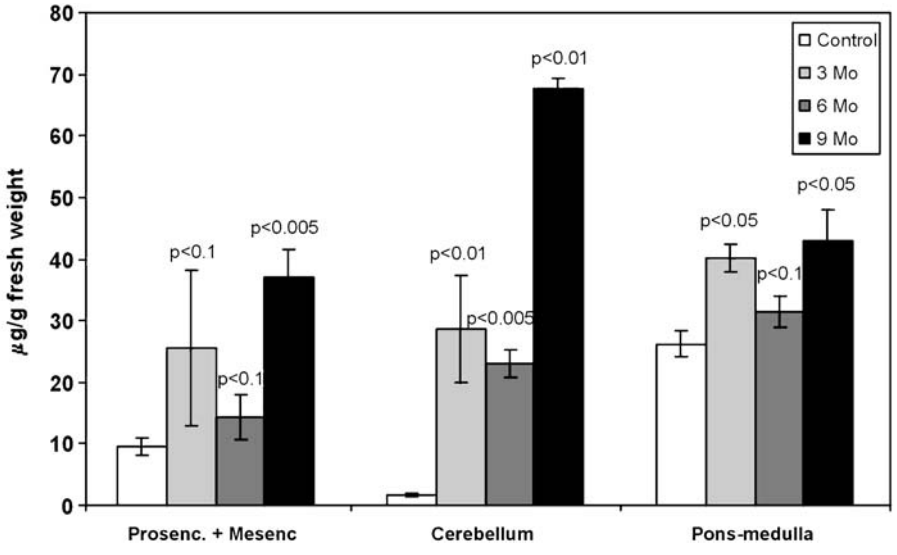


Figure 2. Total copper concentration in the prosencephalon-mesencephalon, cerebellum and pons-medulla of mice treated with CZ vs the controls.

Table 2. Concentration of total Zn (μg/g fresh weight) in CZ-treated animals and in the controls.

Organ	Controls	3 months	6 months	9 months
Liver	37.05±4.56	51.32±1.07 p<0.01	36.55±9.7 p<0.1	186.2±3.32 p<0.01
Kidney	19.9±2.7	43.22±0.81 p<0.0005	23.0±3.43 p<0.2	110.83±11.27 p<0.005
Heart	32.0±8.8	55.71±16.8 p<0.2	29.87±7.18 p<0.1	145.84±5.8 p<0.001
Lungs	31.0±1.48	40.0±11.7 p<0.1	35.38±5.08 p<0.1	247.54±2.46 p<0.01
Stomach	61.0±4.75	46.0±9.64 p<0.1	35.18±4.61 p<0.005	80.9±5.73 p<0.05
Spleen	44.71±8.12	56.28±11.36 p<0.1	35.77±8.48 p<0.1	424.3±2.51 p<0.01
Large intestine	34.23±1.1	97.49±5.1 p<0.0005	37.06±4.4 p<0.2	111.11±21.56 p<0.05
Small intestine	30.25±5.34	79.69±7.8 p<0.005	32.0±5.3 p<0.2	176.17±3.08 p<0.01

Values are the mean ± SE of ten determinations. Significance was evaluated by means of one way ANOVA + Scheffe's post hoc test versus control.

pared to the controls. Highly significant increases were observed for the various tissues. Evaluation of concentration together with the statistical significance of changes showed that there was a typical tissue-specific time dependence of the response. Copper levels in spleen and heart increased within the first 3 months, followed by a plateau. By contrast, small and large intestine and kidney showed a 3- to 6-month decrease period before exhibiting a further significant increase. Kidneys showed a typical biphasic behaviour including an initial moderate, yet significant, increase (3–6 months) followed by a more pronounced increase after 9 months.

Figure 2 reports the concentration of total copper in the mouse brain after CZ treatment with respect to the controls. Copper accumulated as a function of the time of treatment, the most significant accumulation being observed in the cerebellum. In the central nervous system (CNS) in particular, copper increased with respect to the controls by about 202% in the encephalon, 529% in the cerebellum and 255% in the mid-brain upon CZ treatment.

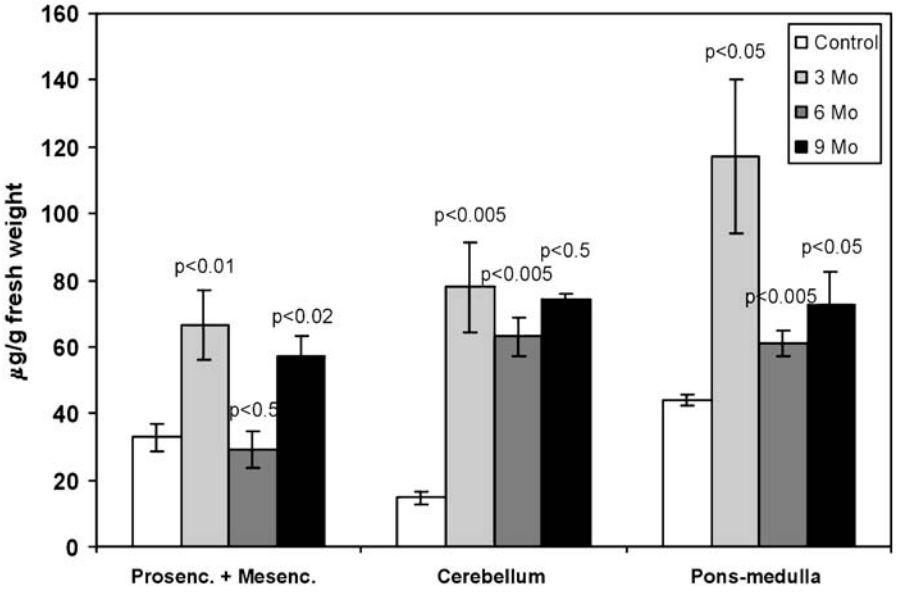


Figure 3. Total zinc concentration in prosencephalon-mesencephalon, cerebellum and pons-medulla of mice treated with CZ vs the controls.

Table 3. Concentration of total Fe (μg/g fresh weight) in CZ-treated animals and in the controls.

Organ	Control	6 months	9 months
Liver	135.29±33.1	163.7±27.84 p<0.05	48.67 ± 7.9 p<0.1
Kidney	93.21±5.38	83.37±13.85 p<0.1	45.67±0.39 p<0.005
Heart	259.17±13.55	141.76±12.94 p<0.001	49.97±11.53 p<0.001
Lungs	187.85±4.74	160.41±53.93 p<0.1	121.45±22.47 p<0.05
Stomach	78.39±5.12	45.5±22.58 p<0.1	85.1±4.61 p<0.01
Spleen	264.19±7.94	226.43±41.9 p<0.1	71.85±5.46 p<0.005
Large intestine	49.05±3.2	66.96±17.14 p<0.1	57.57±7.3 p<0.1
Small intestine	69.06±5.88	151.28±34.28 p<0.05	83.3±12.6 p<0.1

Values are the mean ± SE of ten determinations. Significance was evaluated by means of one-way ANOVA + Scheffe's post hoc test versus control.

Table 4. Excretion of Cu, Zn, Fe and MTs in 24 h with the urine of mice treated for 9 months with CZ, and in the controls.

	Cu (μg/g f. t.)	Zn (μg/g f. t.)	Fe (μg/g f. t.)	MT (μg/g f. t.)
Control	0.38±0.08	2.65±0.34	2.1±0.32	8.9±1.6
CZ treatment	0.70±0.01 p<0.05	4.05±0.42 p<0.01	2.6±1.41 p<0.1	15.5±1.4 p<0.01

Values are the mean ± SE of ten determinations. Significance was evaluated by means of one-way ANOVA + Scheffe's post hoc test versus control. (f. t., fresh tissue.)

In terms of accumulation kinetics, the different parts of the brain studied exhibited different behaviours: a long induction time was characteristic of the prosencephalon/mesencephalon before copper concentrations were found to increase significantly; the increase in the cerebellum was already manifest after the first 3 months of treatment, with a predominant accumulation at the 9th month; in the pons-medulla region, the increase was less marked, yet significant, since it was already manifest at the early stage of treatment and remained constant for longer periods.

Since Zn shares with copper a number of intracellular ligands, notably MT, the tissues of treated and control mice were also analysed for their Zn concentrations. Table 2 shows the concentration of total Zn in all tissues but the brain, after CZ treatment for 3, 6 and 9 months with respect to the controls. As expected, the Zn concentrations were higher than the Cu concentrations, yet significant increases were observed for almost all tissues. The stomach exhibited a lesser effect including an initial decrease; a 3- to 6-month induction period characterized the effects in the heart, lungs and spleen, whereas a rather prompt effect (within the first 3 months) was observed with the large intestine, small intestine and kidney.

As far as the brain was concerned, figure 3 reports the concentration of total Zn in the prosencephalon-mesencephalon, pons-medulla and cerebellum of mice after 3, 6 and 9 months of treatment with CZ compared to the controls. In general, Zn increased in all three parts of the brain analysed, except in the prosencephalon/mesencephalon after 6 months treatment. However, the data were less homogeneous than those for copper, and higher accumulation was observed in the pons-medulla after 3 months of treatment.

Because iron uptake is known to be strongly affected by copper levels [51], the metal analysis was extended to iron. Table 3 reports the concentration of iron in all organs and tissues examined. Unlike copper and zinc, iron decreased, except in the stomach and small and large intestine. In the large intestine, the increase was not statistically significant due to the high standard deviation of the data. Within the tissues exhibiting significant decreases, the effects were more marked in the case of heart and spleen compared to liver, kidney and lungs.

Metal ion urine excretion

On the basis of the documented metal ion accumulation in tissues, including the brain, observed upon CZ treatment, we studied the possible effects on excretion of the metal through the urinary system. Typically, the concentration of Cu, Zn and Fe as well as MT was measured in the urine collected within 24 h from mice before and after treatment with CZ (table 4). Cu and Zn excretion was found to increase by a factor of two: from 0.38 ± 0.08 (controls) to 0.70 ± 0.01 [μg/g fresh tissue (f. t.) $p<0.05$] in the case of Cu, and from 2.65 ± 0.34 (controls) to 4.05± 0.42 (μg/g f. t., $p<0.01$) in the case of Zn. In agreement, the excretion of MT also increased in the same way: from 8.9 ± 1.6 to 15.5 ± 1.4 (μg/g f. t., $p<0.01$), since excretion of MT-I/II represents a physiological way to eliminate abnormally accumulated metal ions of note is that iron appeared to behave differently in that it remained unaltered after CZ treatment (2.1 ± 0.32 and 2.6 ± 1.41 μg/g f. t., before and after CZ treatment, respectively). Other excretion parameters are listed in table 5. Diuresis increased by a factor of about 10 ($p<0.05$); smaller, yet significant ($p<0.05$), increases were found for glucose, calcium and creatinine excretion. In contrast, protein excretion decreased by a factor of three.

Histology and immunohistochemistry

Brain sections clearly showed a reactive gliosis, especially of astrocytes, with 9 months of CZ treatment, and begin-

Table 5. Analyses of 24-h collected urine in CZ mice treated for 9 months with respect to the controls.

Analysis	Control	CZ treated
Diuresis (ml)	1.41 ± 0.11	11.63 ± 0.20
Glucose (mg)	1.64 ± 0.29	2.21 ± 0.29
Na^+ (mg)	6.57 ± 1.12	6.65 ± 0.36
K^+ (mg)	13.86 ± 1.41	18.42 ± 3.04
Cl^- (mg)	15.39 ± 1.79	18.43 ± 2.36
Ca^{2+} (mg)	0.16 ± 0.01	0.38 ± 0.71
Inorganic phosphates (mg)	4.77 ± 0.82	6.1 ± 1.12
Creatinine (mg)	0.72 ± 0.1	1.2 ± 0.11
Proteins (mg)	6.79 ± 2.19	2.03 ± 0.04

Values are the mean ± SE of ten determinations.

ning after 2–3 months of treatment. The astrocytosis was combined with a vacuolisation of the neuropil localised predominantly in the white matter (figs. 4, 5).

In brain sections of control animals there were only small regions which showed an immunopositivity for MT-I/II. As an example, figure 4 shows two typical fields observed in the control animals at the level of the dentate gyrus (fig. 4A) and of the deep cerebral cortex bordering the medulla (fig. 4C). Other areas showing low MT-I/II immunopositivity in the control animals were the cerebellar medulla, parts of the cerebellar cortex in the roof of the IV ventricle, the wall of lateral ventricles (ependymal and subependymal layers) and the fimbria hippocampi. The positive cells were moderate in number and showed the morphological characteristics of astrocytes. There were also MT-positive astrocytes in other areas, but very few. After CZ treatment, a moderate increase in the number and staining intensity of positive astrocytes was observed in similar CNS regions as those mentioned for the controls. An intense reaction was found in the gyrus dentatus with moderate extension to the CA1–4 areas of the hippocampal formation (fig. 4B). Other regions of experiment-enhanced expression were the corpus callosum, corpus striatum, the lamina quadrigemina, the capsula interna and parts of the cerebellar cortex/medulla (fig. 4D). In controls, GFAP-positive astrocytes were frequently found in the white matter of the brain, such as the cerebral and cerebellar medulla, the corpus callosum, capsula interna and marginal tracts of the pons and medulla oblongata. Few GFAP-positive astrocytes appeared in the grey matter with the exception of the hippocampal formation, which was characterised by an even distribution of cells in the different cortical layers.

Figure 5A and C show, as an example, the moderate GFAP positivity of control animals at the level of layer II/III (fig. 5A), and of the deep layer of the cerebral cortex bordering the white matter (fig. 5C). Beginning at the hippocampal sulcus towards the subiculum, the number of positive cells gradually decreased. In treated animals, a marked increase in the number and staining intensity of positive astrocytes was observed in similar regions as the controls. Additional regions showed the expression of GFAP, namely basal layers of the cerebral cortex and the molecular layer of the cerebellar cortex. Figure 5B and D show the same regions as figure 5A and C, but in animals after 9 months CZ treatment. Worth mentioning is the increase in reactivity in the corpus striatum.

Immunostaining with antibodies against MBP showed the typical pattern of myelinated nerve fibres in most brain areas of control as well as experimental groups. There were two areas with pronounced differences between animal groups: the cerebral cortex (fig. 6A, B) and the hippocampal formation (fig. 6C, D).

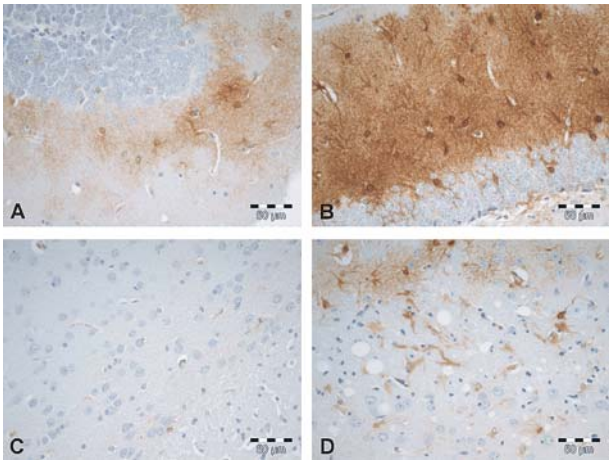


Figure 4. Immunostaining reactivity to metallothionein-I/II in the different brain areas. MT-positive astrocytes in the dentate gyrus of controls (*A*), and after CZ treatment (*B*), and in the deep cerebral cortex bordering the medulla of controls (*C*), and after CZ treatment (*D*). Note the numerous vacuoles distributed in the neuropil (*D*).

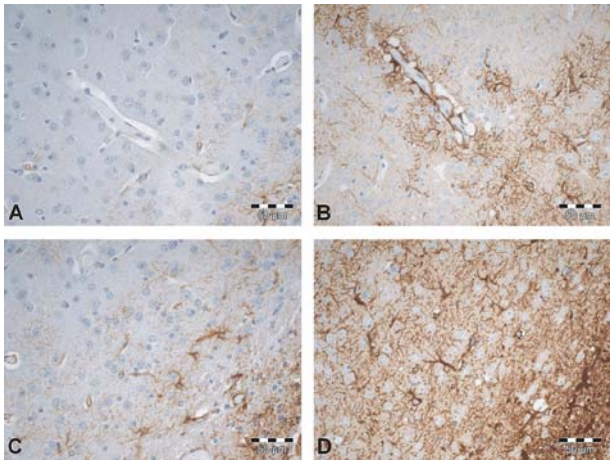


Figure 5. Immunostaining reactivity to GFAP in different layers of the cerebral cortex. GFAP-positive astrocytes in layer II/III of controls (*A*), and after treatment with CZ (*B*) and in the deep layer of the cerebral cortex bordering white matter in controls (*C*), and after treatment with CZ (*D*).

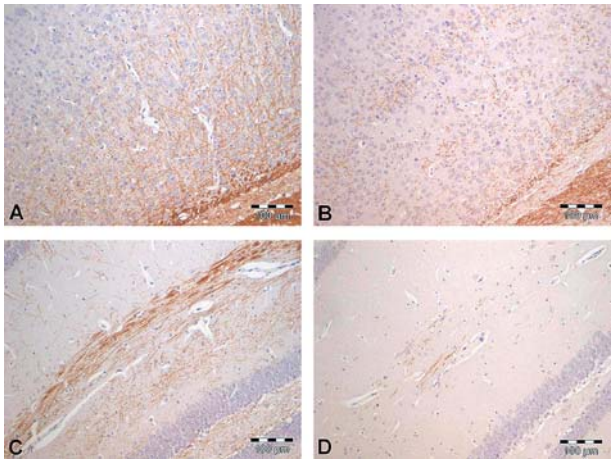


Figure 6. Immunostaining for MBP in cerebral cortex and hippocampus. Myelinated nerve fibres in deep layers of the cerebral cortex of controls (*A*) and after treatment with CZ (*B*); the border of white matter is present in the right lower corner. Myelinated nerve fibres in the hippocampal formation of controls (*C*) and after treatment with CZ (*D*); the dentate gyrus is observable in the right lower corner of both photographs.

Compared to controls, in CZ-treated animals there were clear signs of myelin loss. Demyelination took place in deeper layers of the cortex and showed the pattern of rounded spots with variable diameter. In the hippocampal formation, there was a nearly total disappearance of myelinated fibres in the entorhinal cortex as well as afferent and efferent fibre tracts. Immunostaining for both MT and GFAP showed a massive increase in the activation of astrocytes induced by the CZ treatment, with a more pronounced reaction in GFAP samples. As regards positive areas of the brain, there was a high degree of coincidence. The number of positive astrocytes and the pattern of distribution, however, seemed to be greatly different. Some of the reactive astrocytes showed only GFAP expression, others only MT, while a third set co-expressed both. Besides the astroglial reaction, the circumscribed decrease in myelinated fibres indicated that oligodendroglia were also involved in CZ-induced alterations.

Discussion

The capacity of CZ to form stable copper complexes with the associated development of an intense blue colour has long been known. The reaction has been extensively employed for analytical purposes [33]. However, no detailed description of the structure and the stability of the copper CZ complex has been reported so far. In this paper, we reported the preparation and a preliminary characterisation of the main chemical features of the copper-CZ species. The poor solubility of the ligand in water together with the high water solubility of the Cu-CZ complex point to a rather complicated pattern of CZ absorption through the absorbent epithelia of the intestinal tract. This scenario is further complicated by the apparent oligomeric character of the complex, which does not favour absorption through membranes. For this reason, we preferred CZ administration through the drinking water to prevent the formation of Cu-CZ complexes by reaction of the free ligand with the metal present in the foodstuff. The chronic effects of CZ were studied via a steady-state administration of low doses for a rather long period of time (up to 9 months). For this reason, the use of drinking water is a more preferable route than intraperitoneal or subcutaneous injection. Great interest in the utilisation of CZ as a chemical and biological model arose in the 1960s when Carlton (see the introduction) described a spongiform encephalopathy in rats, mice and guinea pigs upon CZ treatment, with impressive similarity to the brain lesions observed in scrapie [38, 39]. Young animals appeared to be more susceptible to CZ toxicity that older animals [45] but, most interestingly, clinical as well as histological recovery of the brain lesions is possible upon cessation of CZ treatment [52] which excludes any progressive infective status. The mechanism of CZ action has not yet been clarified and supplementation of copper failed to reduce [35] or prevent CZ spongiosis as well as other observed biochemical effects [53]. In his rather controversial paper Venturini [54] proposed that CZ toxicity could be due to inhibition of the active transport of some electrolytes and to the inhibition of the activity of some key enzymes such as monoamine oxidase, succinate dehydrogenase and cytochrome oxidase. The decrease in copper-related enzymatic activities could be interpreted as a consequence of a copper deprivation depending on the copper-chelating capacity of CZ. The possible oligomeric structure(s) of the Cu-CZ complex would explain the observation that addition of the complex, rather than of the free ligand, does not induce any pathological manifestation in experimental animals. However, the results obtained in the current work demonstrate that all tissues respond to the CZ treatment by increasing their copper concentrations, yet they exhibit different time dependencies in their biological responses, which point to the existence of tissue-specific toxicity patterns. The coupling of copper metabolism with that of Zn is also fully confirmed by our data, although, again, tissue-specific behaviours are manifested as far as the time dependence of the Zn increase and its correlation with the time dependence of Cu increase are concerned. This observation suggests that the increase in metal content is, at least in part, correlated with an increase in MT levels via activation of the nuclear metal-responsive elements. In agreement, the twofold increased Cu and Zn urinary excretion correlates with a similar increase in MT excretion in treated animals.

The decrease in the copper-dependent enzymatic activities in CZ-treated animals reported by some studies, although not depending on intracellular metal deprivation, can

explain the decrease in intracellular iron levels observed in this study. Since the iron metabolism requires the ferroxidase activity of the serum copper oxidase ceruloplasmin, a decrease in the redox activity would negatively affect the iron balance. This issue will be specifically addressed in further work. Worth noting is that the copper-chelating properties of CZ may still represent an important aspect to be considered to explain its biological effects. Since CZ appears in the blood, it might also, at least in some tissues, be absorbed by the cells where it could impair the physiological balance and distribution of metal ions within different molecular targets. This hypothesis has been validated by MS/MS analysis showing the occurrence of CZ both in brain and serum. Hence, despite an increase in metal levels, the bioavailability could be reduced by the presence of CZ. However, study of an impairment of enzymatic activities due to a direct effect of CZ on cuproenzymes was beyond the aims of this work.

The morphological observations described in our experimental approach coincide with other data reported by other laboratories demonstrating that the model for CZ biological effects is reliable and reproducible. The novelty of our data is the fact that CZ causes a dramatic and unexpected increase in Cu and Zn concentration in the brain, stimulating the immunopositive reaction of MT-I/II. This is concordant with the increase of Cu and Zn in other tissues, and confirmed by the astrocytosis as observed by our and other laboratories.

The dramatic decrease in myelinated nerve fibres as seen in circumscribed regions of the brain is a further characteristic of the biological effects of CZ. CZ produces alterations that are very similar to those exhibited by animal models with an experimental autoimmune encephalomyelitis mimicking the demyelinating human disease multiple sclerosis. With this animal model, a protective role of MT-I/II could be demonstrated, since exogenous administration of the protein significantly prevented demyelination and axonal damage while such effects were significantly increased in MT-I/II-deficient mice [23, 24]. Thus, the increase in MT-I/II levels in CZ-treated mice can be related to the defence mechanism against CZ toxicity. Another important issue resulting from our work is that the pattern of distribution of GFAP and MT expression strongly suggests the existence of different subtypes of astrocytes defined by the characteristics of their response to CZ treatment, astrocytes responding with GFAP expression or with MT expression or with the co-expression of both proteins.

Taken together, the results of our work suggest the hypothesis that the chelating properties of CZ induce an alteration in metal homeostasis with concomitant induction of the metal-binding protein that has a major role in cellular metal partition, namely MT, resulting in the same resistance pathway typical of pathological conditions or animal models. With respect to this last hypothesis, a different susceptibility to CZ effects is expected from MT-deficient or MT-overexpressing animals.

Although the mechanism of CZ action is not yet fully understood, we demonstrated that CZ is effectively absorbed in mice and CZ treatment produces remarkable effects on the CNS and other tissues. As far as the CNS is concerned, from a morphological viewpoint, we confirm that CZ administration in drinking water produces the same effects observed by administering CZ together with a solid diet, but over a longer period of treatment, because the quantity of CZ administered in drinking water is lower with respect to the 0.5% CZ utilized in the solid-diet treatment. Besides the temporal correlation with the method of treatment, gliosis with hypertrophy of astrocytes and vacuolisation of the neuropil was seen using standard haematoxylin-eosin staining.

We have demonstrated that the subcellular effects of CZ are not based on its ability to produce copper deficiency by chelation of copper; rather, the Cu and Zn levels are increased by the treatment. CZ administration can be considered a good protocol to induce the alteration of myelin synthesis [41, 55, 56], as well as for an overaccumulation of copper and zinc in the brain and, eventually, a good approach for a better understanding of the mechanisms of spongiform encephalopathy from a biochemical, biophysical as well as a molecular point of view.

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