



## NOTE

Pathology

# Supplemental study on 2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase) activity in developing rat spinal cord lesions induced by hexachlorophene and cuprizone

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**ABSTRACT.** In a previous study, we showed that 2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase) expression is induced in different temporal patterns in the cerebrum, cerebellum and medulla oblongata of hexachlorophene (HCP) and cuprizone (CPZ) treated rats. Here, we additionally examined the histopathological changes and CNPase expression in the spinal cord to clarify the reproducibility of different temporal patterns of CNPase expression in the spinal cord showing low degree or lack of spongy changes. Spongy changes were observed in HCP-treated rats, but not in CPZ-treated rats. Immunohistochemistry showed that intense expression of CNPase was not induced following HCP or CPZ treatment. Our data reveal that expression intensity of CNPase may be dependent on the degree of HCP- and CPZ-induced damage of the myelin sheath.

**KEY WORDS:** 2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase, cuprizone, hexachlorophene, rat, spinal cord

*J. Vet. Med. Sci.*

81(9): 1368–1372, 2019

doi: 10.1292/jvms.19-0096

Received: 15 February 2019

Accepted: 10 July 2019

Advanced Epub: 23 August 2019

Several compounds have been reported to induce spongy changes in the central nervous system (CNS) [2, 3, 7, 12, 13, 15, 19]. The mechanisms underlying spongy changes induced by these compounds are varied. Hexachlorophene (HCP) and aniline induce spongy changes in white matter by increasing the cerebral water content by uncoupling mitochondrial oxidative phosphorylation, which interferes with ATP production [4, 10]. In contrast, cuprizone (CPZ) induces spongy changes in myelin via oligodendroglial apoptosis, causing oxidative and ER stress [8, 11]. 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), an enzyme exclusively expressed in oligodendroglia differentiation, localizes to immature and mature oligodendroglial cell membranes [8, 16]. CNPase is associated with myelinogenesis [6] and process outgrowth in oligodendroglia [9].

In our previous study, we demonstrated that cytotoxic insults to oligodendroglia and the myelin sheath in the cerebrum, cerebellum and medulla oblongata showing definite spongy changes induce different temporal CNPase expression patterns. CNPase expression appears to increase subsequent cytotoxic insults to oligodendroglia and the myelin sheath following HCP treatment and during demyelination due to damage to oligodendroglia following CPZ treatment [8]. In a CPZ-induced experimental demyelination model, Cu/Zn superoxide dismutase (Cu/Zn SOD) and Complex IV activity are decreased both in the corpus callosum with demyelination and intact spinal cord [1]. We showed that CNPase expression was increased in rat spinal cords with severe spongy changes following aniline treatment [7]. Here, we additionally examined the histopathological changes and immunohistochemical CNPase expression in the spinal cord to clarify the reproducibility of different temporal patterns of immunohistochemical CNPase expression in the spinal cord showing low degree or lack of spongy changes.

Materials were obtained from the same sources as those reported in our previous study [8]. Twelve female 19-day-old Crj: WI (Wistar) and 12 male 16-day-old Crj: (CD) SD rats, obtained from Charles River Laboratories Japan Inc. (Tsukuba, Japan), were used. The animals were cared for according to the Japanese Association for Laboratory Animal Science's and our institution's guidelines for the care and use animals. HCP and CPZ treatment regimens are shown in Table 1. Paraffin-embedded spinal cord sections were stained with hematoxylin and eosin. Immunohistochemical staining for mouse monoclonal antibody against human CNPase (1:400 in dilution, clone 11-5B, Millipore, Billerica, MA, U.S.A.) was performed using the streptavidin-biotin system (DAKO LSAB2 System-HRP, DAKO Japan Ltd., Kyoto, Japan). Antigen retrieval was performed by transferring slides to 0.5%

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Immunosaver (Nisshin EM Corp., Tokyo, Japan) and heating for 25 min at 95°C in a microwave.

The severity of spongy changes, oligodendroglia alteration (pyknotic nuclei for HCP or fragmented nuclei with condensed eosinophilic cytoplasm for CPZ) and anti-CNPase immunostaining was graded into – to ++. For spongy changes: –, not detectable; +, small vacuoles scattered in the white matter; ++, small or middle vacuoles diffusely present in the white matter. For oligodendroglia alteration: –, not detectable; +, slight; ++, mild. For CNPase staining: +, slightly positive in the submembranous region of oligodendroglia.

The severity of spongy changes determined by histopathological examination is summarized in Tables 2 and 3. In HCP-treated rats, spongy changes were observed in the white matter of spinal cords from day 2 to 12 (7 days after dosing ceased). Spongy changes were localized to the dorsal funiculi on day 2 but had spread throughout the white matter on day 5. On day 5, the size and density of vacuoles had also increased, and small or medium-sized vacuoles were diffusely present in the white matter (Fig. 1). As we partially

**Table 1.** Experimental design of hexachlorophene (HCP) and cuprizone (CPZ) treatment

Dosage level	Test article	Chemical information	Dosing route	Animal	Day of sacrifice
0 mg/kg/day	CMC-Na	carboxymethylcellulose-Na Shin-etsu Chemical Co., Ltd. (Tokyo, Japan)	Oral	Crlj: WI (Wistar) 19-day-old, female	Day 1, 2 and 5 Day 12 (7 days after dosing ceased)
35 mg/kg/day	HCP	Hexachlorophene C <sub>13</sub> H <sub>6</sub> Cl <sub>6</sub> O <sub>2</sub> , CAS No.70-30-4 Sigma-Aldrich Japan Co., Ltd. (Tokyo, Japan)			
0 w/w%	MF or CF-1	MF, CF-1 Oriental Yeast Co., Ltd. (Tokyo, Japan)	Powder chow	Crl: (CD) SD 16-day-old, male	Day 3, 6 and 8 Day 24 (16 days after dosing ceased)
1 w/w%	CPZ	biscyclohexanone oxaldihydrazone C <sub>14</sub> H <sub>22</sub> N <sub>4</sub> O <sub>2</sub> , CAS No.370-81-0 Sigma-Aldrich Japan Co., Ltd. (Tokyo, Japan)	Cotaining powder chow		

**Table 2.** Relationship between histological changes and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) positivity in the spinal cord treated with hexachlorophene (HCP)

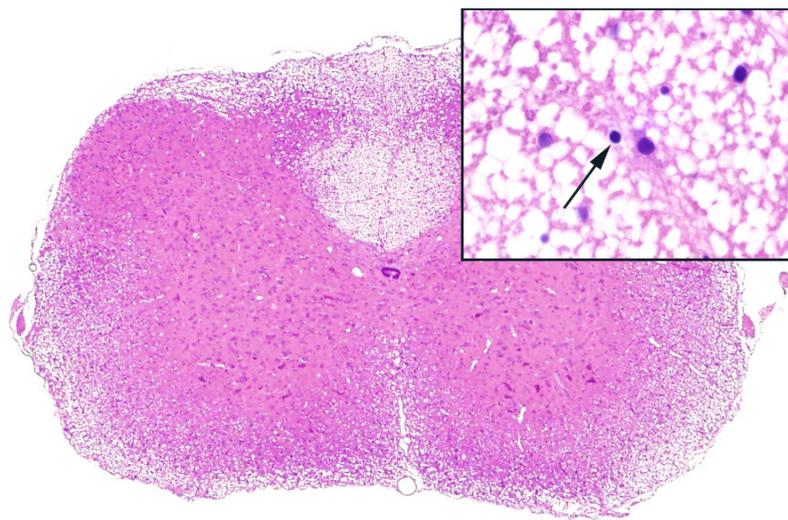
Day of necropsy	Treatment	1		2		5		12		
		Control	HCP	Control	HCP	Control	HCP	Control	HCP	
Number of rats		1	2	1	2	1	2	1	2	
Spongy change	- <sup>a)</sup>	1	2	1	0	1	0	1	0	
	+	0	0	0	2	0	1	0	2	
	++	0	0	0	0	0	1	0	0	
Degeneration, oligodendroglia	- <sup>b)</sup>	1	2	1	0	1	0	1	2	
	+	0	0	0	2	0	1	0	0	
	++	0	0	0	0	0	1	0	0	
CNPase positivity		+ <sup>c)</sup>	1	2	1	2	1	2	1	2

a) -, no detectable; +, small vacuoles scattered in the white matter; ++, small or middle vacuoles diffusely present in the white matter, b) -, no detectable; +, slight; ++, mild, c) +, slightly positive in the submembranous region of oligodendroglia.

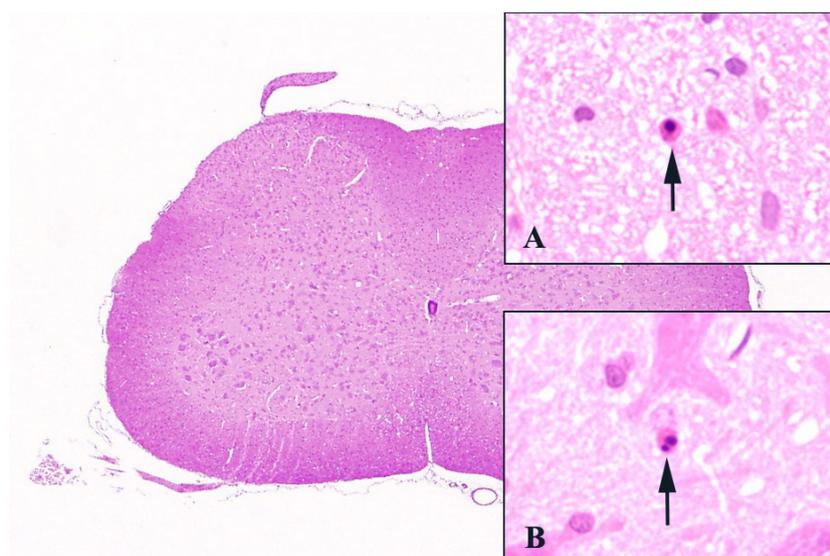
**Table 3.** Relationship between histological changes and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) positivity in the spinal cord treated with cuprizone (CPZ)

Day of necropsy	Treatment	3		6		8		24		
		Control	CPZ	Control	CPZ	Control	CPZ	Control	CPZ	
Number of rats		1	2	1	2	1	3	1	1	
Spongy change		- <sup>a)</sup>	1	2	1	2	1	3	1	1
Apoptosis, oligodendroglia		- <sup>b)</sup>	1	2	1	1	1	2	1	0
		+	0	0	0	1	0	1	0	1
CNPase positivity		+ <sup>c)</sup>	1	2	1	2	1	3	1	1

a) -, no detectable, b) -, no detectable; +, slight, c) +, slightly positive in the submembranous region of oligodendroglia.



**Fig. 1.** Transverse section of the lumbar level in the spinal cord from rats treated with 35 mg/kg of hexachlorophene (HCP). H&E staining. Mild spongy changes were observed in the white matter after HCP treatment on day 5. Inset, high magnification of the spongy changes area; small or medium-sized vacuoles and pyknotic nuclei of oligodendroglia (arrow) in the white matter.

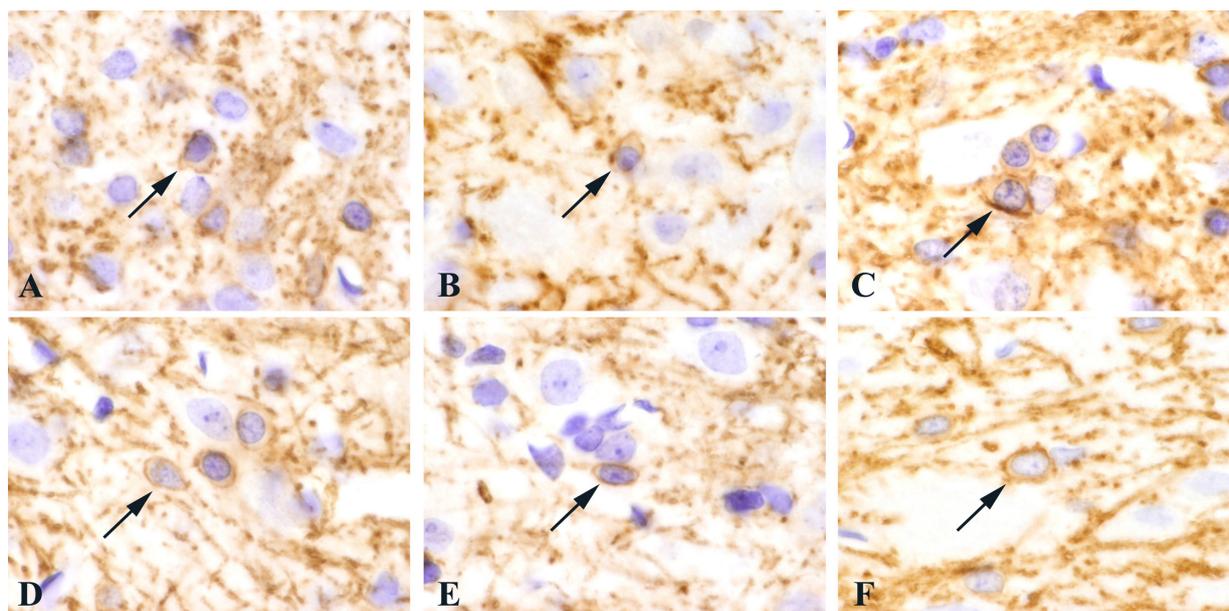


**Fig. 2.** Transverse section of the lumbar level in the spinal cord from rats fed with 1% cuprizone (CPZ) diet. H&E staining. No abnormality was found in the white and gray matter after CPZ treatment on day 8. Inset A, high magnification of the white matter; apoptotic morphology (arrow) such as fragmented nuclei and condensed eosinophilic cytoplasm is evident. Inset B, high magnification of the gray matter; apoptosis (arrow).

described in our previous report, pyknotic nuclei of oligodendroglia were present in the white matter with spongy change in the spinal cord on day 2 and 5 (Fig. 1, Inset). The nuclei were smaller than those in normal and showed dense and homogenous chromatin. These changes in the spinal cord were chronologically similar to those observed in the cerebrum, cerebellum and medulla oblongata. However, the severity of changes in the spinal cord was milder than those in the other CNS regions [8].

In CPZ-treated rats, no spongy changes were observed in the spinal cord at any time points examined (Fig. 2). From day 6 to 24 (16 days after dosing ceased), apoptotic morphology, such as fragmented nuclei and condensed eosinophilic cytoplasm, was detected in the white (Fig. 2, Inset A) and gray matter (Fig. 2, Inset B) of the spinal cord.

Immunohistochemical examination showed CNPase-positive staining in the submembranous region of normal oligodendroglia in controls (Fig. 3A). In HPC-treated rats, CNPase staining in the spinal gray matter was comparable to that of the controls from day 1 to 5 (Fig. 3B). Interestingly, on day 12 (7 days after dosing ceased), no strong CNPase staining was observed in the spinal gray matter (Fig. 3C). This is in contrast to our previous study, where although CNPase staining in the cerebral cortex was comparable



**Fig. 3.** Immunostaining of 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in the spinal cord in rats treated with hexachlorophene (HCP) (B and C) or cuprizone (CPZ) (D, E and F). CNPase reactivity is observed in the submembranous region of oligodendroglia in the controls (A, arrow), HCP-treated rats on day 5 (B, arrow) and 12 (C, arrow), and CPZ-treated rats on day 6 (D, arrow), 8 (E, arrow) and 24 (F, arrow).

to that of controls from day 1 to 5, expression increased on day 12 (7 days after dosing ceased). No strong CNPase expression was induced in the spinal cord at any time points examined.

Similarly in CPZ-treated rats, CNPase staining in the spinal gray matter was comparable to that of controls and no strong CNPase staining was observed in the spinal gray matter from day 3 to 8 (Fig. 3D and 3E). For CPZ-treated rats, no strong CNPase expression was induced in the spinal cord at any time points examined, including day 24 (16 days after dosing ceased) (Fig. 3F).

Using histopathology and immunohistochemistry, we showed that lesions in the myelin sheath and oligodendroglia in the spinal cord of HCP- and CPZ-treated rats were different from those in the brain, specifically, the cerebrum, cerebellum and medulla oblongata. Further, CNPase expression was not induced in the spinal gray matter following HCP and CPZ treatment.

The severity of spongy changes differed in the spinal cord compared to the brain. While a previous study showed that the spongy changes in the cerebrum, cerebellum and medulla oblongata occurred as severe lesions [8], we found that those in the spinal cord occurred as slight to mild lesions in this study. Further, CNPase expression is increased in the cerebrum during the recovery period following cytotoxic insults to the myelin sheath and oligodendroglia in HCP-treated rats [8]. However, intense expression of CNPase was not induced in the spinal cord during the treatment or recovery period. This difference in CNPase expression in the cerebrum and spinal cord may reflect the different degree of cytotoxic insult caused by HCP treatment.

Because HCP increases the cerebral water content, the spongy changes induced by HCP result from intramyelin accumulation of fluid [4, 15]. Therefore, the low degree of spongy changes observed in the spinal cord may reflect reduced water content, leading to no observable increase in CNPase expression upon restoration of the myelin sheath.

Although there were no spongy changes in the spinal cord of CPZ-treated rats, we did observe oligodendroglial apoptosis. Oligodendroglial apoptosis reportedly occurs before demyelination in the CPZ model [5, 8, 18]. Further, a previous study described demyelination in the corpus callosum but not in the spinal cord [1]. Therefore, our results are consistent with published data.

No CNPase-positive reaction was observed in the spinal cord at any time points examined in CPZ-treated rats, suggesting that oligodendroglial apoptosis does not induce intense expression of CNPase. Spongy changes were observed in the cerebrum, cerebellum and medulla oblongata from day 3, and increased CNPase expression accompanied these changes [8]. Therefore, increased CNPase expression may be induced by histopathological damage to the myelin sheath.

Acs *et al.* reported that copper-associated enzymes, copper-containing prosthetic groups, such as Cu/Zn SOD, and the respiratory chain protein Complex IV are decreased in both the corpus callosum with demyelination and intact spinal cord of CPZ model mice [1]. Although this observation suggests that there is a selective vulnerability of oligodendroglia to CPZ in CNS regions, the mechanism underlying this selective vulnerability is unknown. Acs *et al.* proposed [1] that differential expression of interacting intracellular molecules, different sources of oligodendroglial precursors [14, 17], or different cellular microenvironments may be associated with their selective vulnerability to CPZ. In our study, we observed differences in the distribution of spongy changes among CNS regions in CPZ-treated rats, which may reflect the differences in precursors or microenvironments suggested by Acs *et al.*

In summary, these data reveal different CNPase expression and severity of lesions in the spinal cord compared to the cerebrum,

cerebellum and medulla oblongata in HCP- and CPZ-treated rats. While the mechanisms underlying spongy changes differ following treatment with HCP and CPZ, expression intensity of CNPase may be dependent on the degree of HCP- and CPZ-induced damage of the myelin sheath.

**ACKNOWLEDGMENTS.** The authors thank Ms. Jun Sasaki for her assistance in the investigation, and Mr. Takayoshi Ito and Ms. Chie Shiroumaru for their excellent technical assistance. The authors also thank Ms. Kanae Tamatsukuri for help with language editing.

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