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Histopathological evaluation of IBA-1, GFAP activity in the brain cortex of rats administered cadmium chloride

ERTUGRUL GOK[1], ENGIN DEVECI[2], *

[1] *Department of Forensic Medicine, Faculty of Medicine, Dicle University, Diyarbakir, Turkey*
[2] *Department of Histology and Embryology, Faculty of Medicine, Dicle University, Diyarbakir, Turkey*
Ertugrul Gok, M.D., Assistant Professor; Engin Deveci, Ph.D, Professor

A B S T R A C T

Purpose: *This study aims to evaluate the changes in brain tissue and blood-brain barrier due to oxidative stress during cadmium (Cd) poisoning by biochemical, histopathological, and immunohistochemical methods.*
Methods: *170-190 g weighing eight-week-old female Wistar albino rats were divided into two groups (control and experimental), with 7 animals in each group. Experimental group rats were given 2 mg/kg/day powdered cadmium chloride dissolved in water intraperitoneally every day for two weeks. Biochemical, histopathological and immunohistochemical examination was performed.*
Results: *It was seen that brain malondialdehyde (MDA) levels increased significantly, and glutathione (GSH) and catalase (CAT) activity levels decreased. In addition to degeneration in some pyramidal cells and glial cells, deformity, and picnosis in the nucleus, dilation of the meninges and cortex vessels, and inflammation around the blood vessels were observed. An increase was found in ionized calcium binding adaptor molecule 1 (IBA-1) expression in microglia cells and degenerative endothelial cells, and increased glial fibrillary acidic protein (GFAP) expression was observed in astrocytes and degenerate neurons.*
Conclusions: *It has been shown that cadmium toxicity may cause microgliosis and astrogliogenesis by inducing cytokine production due to cell degeneration, vascularity, and inflammation in the brain cortex and by affecting microglia, astrocytes cells.*

Keywords
Cadmium • brain tissue • blood-brain barrier • oxidative stress • toxicity.

Introduction

Cadmium (Cd) is a toxic metal that can spread to the environment. It is used in the generation of other metals like zinc, lead, and copper in pigments, industrial products, pigments, and coatings[1]. Cadmium, a toxic heavy metal, is included in the priority list of hazardous substances reported by the Agency for Toxic Substances and Disease Registry in 2019. Cadmium can cause chronic and sometimes acute poisoning due to occupational and environmental reasons[2,3]. Accidental acute intake is rare, while the suicidal intake is much rarer[2]. It has been reported to cause significant damage to various organs such as the lung, brain, testis, kidney, liver, blood system, and bone[4-7]. Cadmium causes toxic effects on brain tissue and causes neurological changes in memory and learning center[8]. It has been reported that the deterioration of enzymatic effect in the brain and weakness in the defense system develop due to oxidative stress[9].

Cadmium can easily cross the blood-brain barrier (BBB) in infants and increase the permeability of young and adult animals and humans to improve neuronal degeneration and apoptosis by crossing the well-developed blood brain barrier[4]. Malondialdehyde, a toxic product of lipid peroxidation, has been reported to promote cross-

Corresponding author: Engin Deveci, Professor, Ph.D. Department of Histology and Embryology, Faculty of Medicine, Dicle University, Diyarbakir, Turkey. And, Editor-in-Chief of Analytical and Quantitative Cytopathology and Histopathology® (ISSN 0884-6812) - Email address: devecie32@hotmail.com

linking of nucleic acids, proteins, and phospholipids, which cause dysfunction of macromolecules, and MDA levels have been reported to function as a critical marker of lipid peroxidation[10].

Glutathione (GSH) acts as the primary scavenger of free radicals in the sequence of oxidative stress pathology. Cadmium-mediated free radical production in the brain depletes the cellular antioxidant defense mechanism and causes the GSH defense line to fall[11].

The ionized calcium-binding adapter-1 is a 17-kDa actin-binding protein, mostly expressed in microglia[12]. Anti IBA-1 is particularly reactive to microglia and macrophages. In brain tissue, GFAP antibody IBA-1 reacts with astrocytes to form a common association. GFAP, a brain-specific protein that represents the major fundamental component of the cell skeleton of astrocytes; following brain injury, GFAP releases the brain cells into the interstitial fluid in the environment and leads to deterioration in the blood-brain barrier[13].

The aim of this study is to evaluate the changes in brain tissue and blood-brain barrier due to oxidative stress during cadmium poisoning through biochemical, histopathological, and immunohistochemical methods.

Methods

The research was carried out in line with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (2011). All procedures performed in this experiment were approved by the Ethics Committee for the Treatment of Experimental Animals (University of Dicle, Turkey, protocol number: 2021/02).

Animals and experimental design

170-190 g weighing eight-week-old female Wistar albino rats were divided into two groups (control and experimental), with 7 animals in each group and used in the trials. Animals had an acclimation period of 72 hours prior to use in the study. Control group rats were given 1 ml of 0.9% NaCl intraperitoneally every day for two weeks, while experimental group rats were given 2 mg/kg/day powdered cadmium chloride dissolved in water intraperitoneally every day for two weeks[14]. The animals were kept under standard laboratory conditions; 12/12 h light/dark period, with 50–70% humidity, at 23±2°C room temperature in standard steel cages at room temperature. Rats were fed as libitum with standard rat pellets and water. An intraperitoneal injection of 10 mg/kg xylazine HCl (Rompun; Bayer, Istanbul,Turkey) and 50 mg/kg ketamine HCl (Ketalar; Pfizer, Istanbul, Turkey) were used to anesthesize the rats and they were allowed to breathe spontaneously. The rats were euthanized by cardiac exsanguination. The frontal cortex was processed after the brains were dissected. Brain tissues were fixed in 10% formaldehyde solution, post-fixed in 70% alcohol, and embedded in paraffin wax for histological examination. Hematoxylin-Eosin was used to stain the sections. Malnutrition and decrease in food and water intake were investigated in groups.

Biochemical analyses

To determine MDA and GSH levels, homogenization of brain tissue samples was performed with cold 150 mM potassium chloride (KCl). MDA levels of lipid peroxidation products were analyzed. The results were expressed as nmol MDA / g tissue. The spectrophotometric method was used to explain GSH based on the use of Ellman's reagent. GSH was expressed as μmoL / g tissue[15]. A spectrophotometric method based on hydrogen peroxide's ability to form a stable stained complex with molybdenum salts was employed to determine CAT activity.

Immunohistochemistry technique

For advanced immunohistochemistry examination, formaldehyde-fixed tissues were embedded in paraffin wax. Deparaffinization of the sections was performed in absolute alcohol. Antigen retrieval process was carried out twice, first for 7 minutes and next for 5 minutes, in citrate buffer solution (pH:6.0), then they were boiled at 90°C×3 minutes in the microwave oven at 700 W. Next, they were cooled for 20 minutes at room temperature and following this, they were washed in distilled water for 6 minutes. Endogenous peroxidase activity was blocked in 0.1% hydrogen peroxide for 15 minutes. Before applying primary antibodies, IBA-1 antibody (dilution rate, 1/100), cluster of differentiation Glial fibrillary acidic protein (GFAP), antibody (dilution rate, 1/100) overnight, Ultra V block (Cat.No: 85-9043; Invitrogen, Carlsbad, CA, USA) was applied for 8 minutes. A secondary antibody was

applied for 20 minutes. Next, for 20 minutes, the slides were exposed to streptavidin-peroxidase. Chromogen diaminobenzidine (DAB; Invitrogen) was used. Control slides were prepared as mentioned above, with the exclusion of primary antibodies. Finally, the slides were mounted with entellan after counterstaining with hematoxylin, washing for 10 minutes in tap water, and holding in distilled water for 15 minutes. Total scores were calculated based as 0 for no degeneration, 1 for mild degeneration, 2 for moderate degeneration, 3 for intense degeneration, and 4 for the most intense degeneration for evaluating degeneration in nerve cells. Scores were calculated as 0 for no staining, 1 for faint staining, 2 for moderate staining, 3 for intense staining, and 4 for the most intense staining for evaluating IBA-1 expression. Scores were calculated as 0 for none, 1 for mild, 2 for moderate; 3 for intense; 4 for the most intense for evaluating vascular dilatation and congestion. Scores were calculated as 0 for none, 1 for mild, 2 for moderate; 3 for intense; 4 for the most intense for evaluating inflammation. Scores were calculated as 0 for no staining, 1 for faint staining, 2 for moderate staining, 3 for intense staining, and 4 for the most intense staining for evaluating GFAP expression.

Statistical analysis

All statistical analyses were performed using SPSS version 24.0 (IBM, USA) software. All data were evaluated statistically. A normality test using Shapiro-Wilk test was performed, and the data which were not normally distributed were subjected to the non-parametric Mann-Whitney-U test. The data obtained were expressed as median (interquartile range (IQR)). $p < 0.05$ was considered statistically significant in statistical analyses.

Results

Biochemical examination showed statistically difference, it was seen that brain MDA level increased significantly in the cadmium group when compared with the control group. When compared with the control group, GSH levels were found to decrease significantly in the cadmium group. When compared with the cadmium group, a statistically significant difference was found in the tissue CAT activities of the control group. The data showed that tissue CAT activity decreased after cadmium treatment (Table 1).

In the present study, the sections of the cadmium-treated group were compared with the sections of the control group. Histopathological examination showed that the pyramidal neurons had rich chromatin in the brain cortex, the diffuse glial cells were small rounded, the lumen of the capillary vessels in the cortex was regular, and the endothelial cells were flat (Figure 1a).

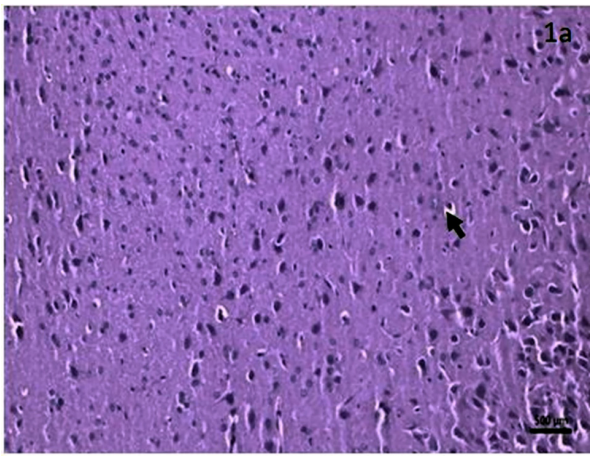

Fig. 1a - Pyramidal cells in the brain cortex and diffuse spread glia cells were rich in chromatin and were observed to be normal. The lumen of the capillary vessels was regular and flat endothelial cells were seen inside (arrow) with Hematoxylin-Eosin staining in the control group.

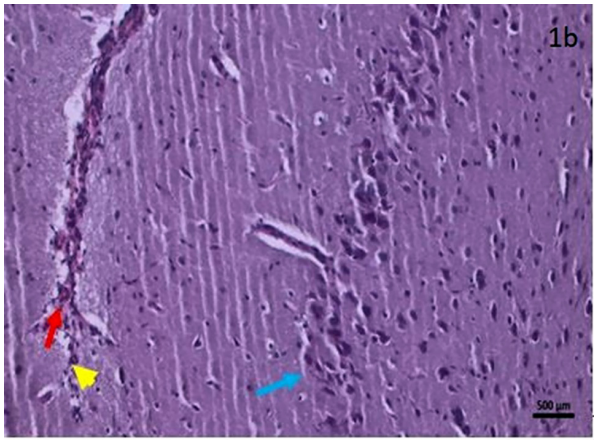

Fig. 1b - Apoptotic changes were observed in the area of the brain ventricles (yellow arrow), in the area of the meninges, and in the cortex, dilated blood vessels, marked congestion (red arrow), inflammation around of blood vessels, and degeneration of some pyramidal cells and glia cells (blue arrow). Hematoxylin-Eosin staining in the cadmium group.

As a result of cadmium application, the meninges and cortex vessels were dilated and markedly obstructed cortex vessels were dilated and markedly obstructed in the ventricles of the brain, and inflammation around blood vessels, degeneration in some pyramidal cells and glial cells, as well as a deformity in the cells and picnosis in the nucleus, were observed (Figure 1b).

When the IBA-1 reaction of the control group sections was examined, it was seen that IBA-1 expression was found in some pyramidal nerve cells and microglia cells, and small blood vessel endothelial cells (Figure 2a).

After cadmium administration, an increase in IBA-1 expression was seen in degenerative endothelial cells in dilated blood vessels and microglial cells around the blood vessel (Figure 2b).

When GFAP activity in the control group was examined, positive GFAP expression was found in astrocytic extensions and small glial cells around normal blood vessels, while negative GFAP expression was found in pyramidal and oval-shaped nerve cells (Figure 3a).

In cadmium administration, GFAP expression showed a positive reaction in degenerated astrocyte feet

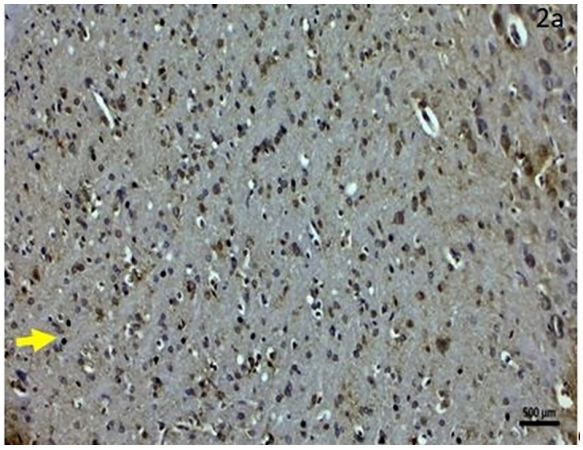

Fig. 2a - Some pyramidal nerve cells and microglia cells, and small blood vessel endothelial cells showed a positive reaction of IBA-1 expression. IBA-1 immune-staining in the control group.

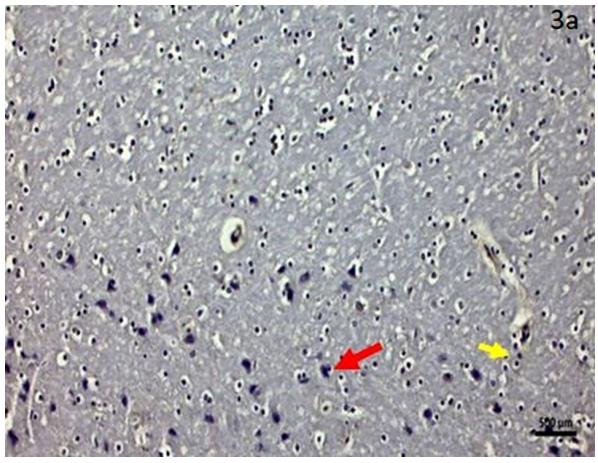

Fig. 3a - Positive GFAP expression was seen in astrocyte cell extensions and small glial cells around regular blood vessels (yellow arrow), while negative GFAP expression was observed in pyramidal and oval-shaped nerve cells (red arrow). GFAP immunostaining in the control group.

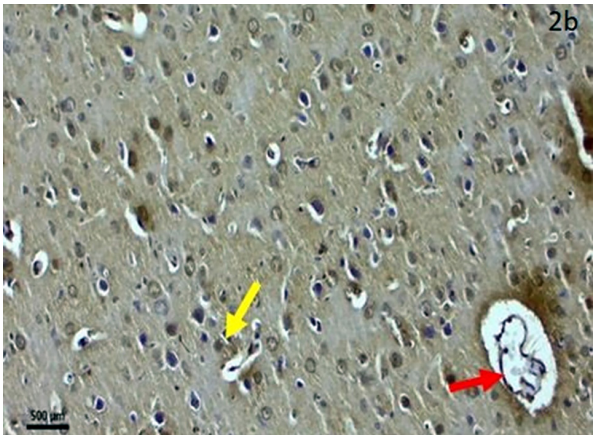

Fig. 2b - Degenerative endothelial cells in dilated blood vessels (red arrow) and microglia cells showing macrophage around the blood vessel showed an increase in IBA-1 expression (yellow arrow). IBA-1 immune-staining in the cadmium group.

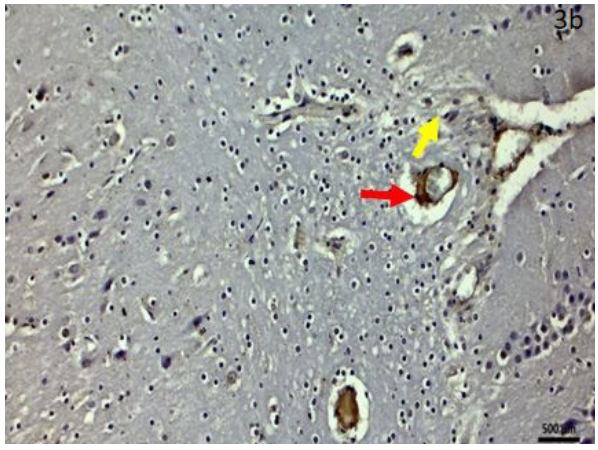

Fig. 3b - GFAP expression was positive in some degenerative neurons (yellow arrow) in astrocyte soles (red arrow) around dilated blood vessels. GFAP immunostaining in the cadmium group.

around dilated blood vessels and some degenerative neurons (Figure 3b).

Statistical significance was found between the control group and the cadmium group in terms of all parameters (Table 1). Rats showed behavior changes after the cadmium administration for 4 weeks. Weight loss due to cadmium administration was significant ($p<0.001$).

Discussion

Cadmium is a metal capable of influencing the activation of various signaling pathways and producing reactive radicals that lead to oxidative stress, resulting in DNA damage and lipid and protein oxidation[6,16]. In a study, it has been reported that Cd causes polyneuropathy in the peripheral nervous system and increases cellular and fiber degeneration[17]. The application of high doses of Cd causes damage to the endothelial cells of the blood vessels of the central nervous system, causing obstruction of the lumen and edema. It has been reported to cause degeneration of nerve cells and fibers and alter the permeability of the blood-brain barrier[18]. In another study, cadmium has been found to affect the cell adhesion molecule, causing brain dysfunction, edema, neuron loss, and gliosis, causing destruction of the blood-brain barrier[19].

Elkhadragy et al. has shown cadmium to cause changes in neurons by depletion of the antioxidant mechanism that affects cellular redox after 5 days of cadmium (6.5mg/kg body weight) and causes oxidative stress[20]. After cadmium application, there were significant obstructions in the dilated blood vessels around the brain ventricles and in the meninges and in the cortex, inflammation around blood vessels, and apoptotic changes in some pyramidal cells and glial cells. It was found that cadmium application disrupted the vascular structure and caused cell loss in the cortex by increasing apoptotic cell development.

The increase in MDA level is an essential indicator of oxidative stress characterized by increased lipid peroxidation. Cadmium interacts with mitochondrial regions and has been reported to cause a decrease in mitochondrial potential leading to decreased glutathione levels[7]. In our study, malondialdehyde, a leading marker for lipid peroxidation, was found to be significantly increased in the brain tissue of rats injected with Cd compared to the control, GSH value decreased, and CAT value decreased. During cadmium poisoning, brain tissue becomes vulnerable to oxidative attacks. GSH and CAT antioxidant potential is inhibited.

GFAP, found in the skeleton of astroglia, is an intermediate filament protein. Data have shown increased local tissue GFAP immunoreactivity to be a sensitive indicator of neuronal damage, and its increase is considered to be a determinant of reactive astrocytosis. When cerebral tissue or spinal cord cells are damaged as a result of trauma or disease, the GFAP level in blood fluid increases[21,22]. The most important function of the astroglia cell

Tab. 1 - Effect of cadmium on brain tissue. Data represent the group median (25%-75%) values. Statistical significance is present between the control group and the cadmium group in terms of all parameters (*MDA:* Malondialdehyde, *GSH:* glutathione, *CAT:* Catalase, Nerve cell degeneration=Degeneration in nerve cells, *IBA-1:* Ionized calcium binding adapter-1, Vascular dilatation: Vascular dilatation and congestion, *GFAP:* Glial fibrillary acidic protein, *: Statistically significant).

Parameter	Control (n=7) (Median (25%-75%)	Cadmium (n=7) (Median (25%-75%)	p-value
MDA	34.03 (32.12-38.33)	48.42 (46.44-52.22)	<0.01*
GSH	1.09 (1.08-1.22)	0.77 (0.61-0.98)	<0.01*
CAT	6.44 (5.98-6.53)	3.82 (3.21-3.95)	<0.01*
Nerve cell degeneration	0.00 (0.00-0.00)	3.00 (3.00-4.00)	<0.01*
IBA-1 expression	2.00 (1.00-2.00)	3.00 (3.00-4.00)	<0.01*
Vascular dilatation	0.00 (0.00-1.00)	4.00 (3.00-4.00)	<0.01*
GFAP Expression	1.00 (1.00-2.00)	3.00 (3.00-4.00)	<0.01*
Inflammation	0.00 (0.00-1.00)	3.00 (3.00-4.00)	<0.01*

is reported as maintaining homeostasis for the proper functioning of neurons. Some neurotoxic agents, such as lead, cause an astrocyte response in which glial cells go through rapid changes, and the response of astrocytes is associated with reactive gliosis, morphological changes within the cell, and increased protein synthesis resulting in an increase in GFAP expression[23]. In our study, as a result of inducing astrocytic response to cadmium chloride, it caused cell morphology change and increased GFAP protein in the blood-brain barrier.

Ohsawa et al.[24] stated that the IBA-1 protein, which is related to the calcium-retaining signaling pathway, is involved in cell migration and phagocytic activity of the microglia macrophage. It has been reported that the regulation of IBA-1 in active microglia contributes to cell migration and that the IBA-1 protein in brain macrophages is involved in phagocytic activity. Microglia are the glial cells that act as the first line of defense in the central nervous system. The effect of lead on microglia activation and its effect on the inflammatory response in the hippocampus of young mice has been reported. A significant increase was reported in the expression of IBA-1 in microglia cells in gyrus dentatus region of the hippocampus in lead metal rats[25]. Cadmium toxicity is thought to affect the neurogenesis in the brain, which may cause microgliosis and astrogliogenesis by inducing cytokine production due to cell degeneration, vascularity, and inflammation the brain cortex and by affecting microglia, astrocytes cells.

In our study, a significant increase in IBA-1 expression was found in the degenerative endothelial cells in dilated blood vessels and microglial cells around the blood vessel after cadmium administration.

In forensic medical examination, pathologies that occur in the acute or chronic period due to environmental and occupational exposure to cadmium and associated with cadmium should be considered. It has been reported that cadmium toxicity may cause end-stage renal disease, early-onset diabetes, renal complications, osteoporosis, impaired blood pressure regulation, and increased cancer risk[26-29]. In a recent study, cadmium has been reported to lead to subarachnoidal hemorrhage by causing vascular pathology[30]. It has also been reported in studies that cadmium may cause schizophrenia, impair cognitive functions, and affect the level of neuromodulatory biomolecules in the brain[31-34]. In one study, it was found that cadmium affected the structure and function of hippocampus neurons, leading to impairment of recognition memory[35]. In another study, cadmium was found to cause a loss in the structural density of cerebellum and motor activity[36].

Cadmium chloride has been conducted in specific experimental studies on brain tissue and its neurotoxicity. Branca et al.[37] reported that Cd barely reaches the brain in adults due to the presence of the blood-brain barrier; however, they stated that the change in the blood-brain barrier due to Cd contributes to the pathogenesis of neurodegeneration. However, it has been reported that the mechanism underlying the Cd-dependent BBB change remains unclear. Therefore, they investigated the signaling pathway of Cd-induced tight junction, F-actin and vimentin protein degradation in a rat brain endothelial cell line. Their results indicate that a reactive oxygen species-dependent endoplasmic reticulum stress-mediated signaling pathway involving caspase-3 activation and ATP release is behind the morphological changes of the blood-brain barrier induced by Cd. An experimental study investigating the neurotoxicity of low-dose cadmium chloride and chronic sodium dichromate administration has reported clearly showing that these two cations induce an oxidative stress resulting in tissue-damaging effects that may contribute to their toxicity and carcinogenicity[38]. In a study investigating the effect of metallothionein isolated from rat liver on rat cerebellum in culture, its relationship with Cd was investigated by comparison. Expressed that Cd significantly suppressed the overgrowth of nerve fibers, fibroblasts and glial cells compared to the control culture[39]. In parallel with our study, significant neural changes were detected in IBA-1 and GFAP activations at the level of neurons and microglia due to the effect of Cd.

In our country, cadmium is included in the list of occupational diseases caused by chemical substances. The decrease or deviation in the physical or mental functions of employees due to exposure to cadmium in the workplace can be evaluated by forensic medicine experts and a calculation of percent disability can be made.

Conclusion

As a result, the damage of cadmium, which is toxic to human health, on brain tissue, was shown by an experimental animal study. In poisoning, the degree of damage to the brain tissue will vary depending on the exposure time (acute or chronic). Thus, depending on the degree of damage in tissues, the degree to which physical and mental functions are affected in the clinic will also change. For this reason, it especially important to evaluate whether there is a causal link between the detected pathology in humans and cadmium by both forensic medicine experts and other health professionals.

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