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DISTRIBUTION OF GLIAL FIBRILLARY ACIDIC PROTEIN IN DIFFERENT PARTS OF THE RAT BRAIN UNDER CADMIUM EXPOSURE

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The chronic effects of low doses of cadmium on the distribution of soluble and filament forms of glial fibrillary acidic protein (GFAP) and their polypeptide fragments in different parts of the rat brain were investigated. Obtained results showed dose-dependent effect of cadmium on the soluble form of GFAP and more pronounced effect on the filament form and composition of the polypeptide fragments of the protein in the rat brain. Prolonged intoxication by cadmium ions in a dose of 1.0 μg/kg of body weight induced a significant decrease in soluble GFAP and an increase in the filament form in the rat brain, pointing to the development of reactive astrogliosis and the risk of neurodegeneration.

K e y w o r d s: glial fibrillary acid protein, rat brain, cadmium.

From all known metals, cadmium (Cd) has been identified to be one of the most harmful to mammals especially humans [1, 2]. Cells of various organs including the kidney, liver, gonads, bones, spleen, brain and red blood cells are susceptible to cadmium exposure. Cadmium is accumulated in tissues and exhibits carcinogenic effect [2]. Cadmium ions activate lipid peroxidation causing an increased loss of antioxidant capacity in response to the formation of free radicals. It is important that cadmium salts are classified as "thiol poisons" that block the sulfhydryl group of proteins including enzymes-antioxidants, inhibiting their antioxidant effects. Moreover, cadmium salts may also inhibit protein amine groups [3]. Effect of cadmium on the central nervous system (CNS) and its role in neurodegenerative diseases have been little studied. To date, though it has been shown that $CdCl_2$ can potentially be involved in the etiopathogenesis of neurodegenerative diseases [4] such as Alzheimer's disease [5]. Cadmium toxic effects, owing to its accumulation in various organs and tissues (including brain), depend on the concentration and exposure time. It was reported that the incubation of cultured spinal cord explants from human embryos with $CdCl_2$ for 24 h caused significant and dose-dependent alterations in the ratio of motor neuron/glial cells in the ventral horns of the human embryo's spinal cord [4]. These results indicate that $CdCl_2$ may significantly affect the ratio of neurons and glial cells during the development of the human spinal cord and therefore are potentially involved in the etiopathogenesis of neurodegenerative diseases.

Previous studies have shown that As, Pb and Cd induce apoptosis and morphological changes in rat brain cortical astrocytes [3]. Cd ions also disrupt the intracellular free calcium homeostasis, leading to apoptosis in various cells including primary rat neurons [6]. S. Hossain et al. showed that cadmium induces oligodendrocyte cell death mainly by apoptosis [7]. Considering the known damaging effects of cadmium ions on oligodendrocytes cells, it can be assumed that myelination process will be reduced under intoxication conditions.

Glial cells, including astrocytes, are very sensitive to any toxicity. Astrocytes outnumber neurons by over fivefold in the CNS. They perform the structural and nutritive functions as well as play an important role in neurotransmission and control interaction between the blood vessels and CNS cells [8]. A peculiarity of astrocytes is the ability to reactivate CNS after lesions of different nature [9]. Astrocyte activation known as reactive astrogliosis is an early signaling of cells' responses to damages [10]. Astrocytes express specific glial fibrillary acidic protein (GFAP), a component of astrocytes cytoskeleton

intermediate filaments (IF), which is a recognized histospecific marker of astrocytic damages [10]. This sensitive biomarker is a standard parameter of neurotoxicity [11, 12]. The total level of GFAP in different parts of the brain is uneven and depends on the number of astroglial cells. GFAP content is maximum in the medulla oblongata (complex inferior olive) and minimum in the cerebral cortex [13]. GFAP as a component of IF plays an important role in the astrocytes migration and supports the stable morphology of their outgrowths during the development of reactive astrocytosis [14]. It has been established that this protein is involved in the molecular mechanisms of neuron-astroglial interactions [15]. GFAP is released in the blood in case of damage of the blood brain barrier, particularly after a traumatic brain injury (that is, it may serve as a marker of the severity of the damage and prognosis of treatment) [16]. From the point of view of the marker concept, it is considered that the response of astrocytes to the effect of the stress-factors is nonspecific and the intensity of GFAP biosynthesis depends on the factors' dose and the exposure time rather than their nature. In this regard, comparative characteristics of negative factors of various origins, as well as their cumulative effect on GFAP metabolism are current points of interest.

The aim of the study was to investigate the chronic effects of low doses of cadmium on the distribution of soluble and filament forms of glial fibrillary acidic protein and its polypeptide fragments in different parts of the rat brain.

Materials and Methods

Wistar rats (20-26-weeks-old and weighing 190-200 g) used as experimental subjects were randomly divided into three groups *(n* = 6). These three groups were: 1 – control group of animals that were kept under standard conditions with a standard diet, 2 – animals that were given a diet with cadmium at a dose of 0.1 μg/kg body weight, 3 – animals that were given a diet with cadmium at a dose of 1.0 μg/kg body weight. The 18 animals from respective groups were used in the experiments. High-purified $CdCl_2x2.5H_2O$ (Sigma, USA) and drinking water for babies "Malyatko" (Econiya, Ukraine), that did not contain cadmium ions as a solvent were used for preparation of cadmium solution. Cadmium solution was introduced to the rats once a day before feeding the animals intragastrically through a sterile stainless steel probe integrated with adjustable dispenser. Control animals received the same volume of water. The animals were weighed prior to each introduction of cadmium solution. The volume of the solution was calculated on the basis of the studied dose. The rats were maintained under standard conditions with natural day/night cycle receiving standard diet. Water and food were freely available. The experiment lasted 37 days and was performed according to "Provisions for the Use of Animals in Biomedical Experiments" [17]. At the end of the experiment, the animals were decapitated under mild anesthesia (isofuran). Cerebellum and thalamus were isolated from the brain and further used to obtain protein fractions. For investigation of astocyte-specific proteins differing in localization, the fractions containing soluble and fibrillar cytoskeletal proteins were obtained by differential centrifugation and protein solubilization in the presence of urea (4.0 M) [18]. The initial buffer contained 0.25 mM Tris (pH 7.4), 1.0 mM EDTA, 2.0 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 3 mM sodium azide (NaN_3) (Sigma, USA).

The total protein concentration in the obtained fractions was determined by Bradford assay and measured in units of mg/100 mg of tissue [19].

The content of glial fibrillary acidic protein in the cerebellum and thalamus were determined by a competitive immunoenzyme solid-phase assay using monospecific polyclonal antibodies against GFAP (Santa Cruze Biotechnology Inc., USA), secondary antibodies against rabbit IgG conjugated with horseradish peroxidase (Sigma, USA) and pure GFAP (Boehringer Mannheim, Germany) as standard. The optical absorption was measured using ELISA reader Anthos 2010 (Finland) at 492 nm. Concentration of astrocyte intermediate filament protein was measured in ng/mg of tissue.

SDS-PAGE was performed in a gradient of polyacrylamide gel (7-18%) in the presence of 0.1% SDS as described in [20]. The content of an intact polypeptide 49 kDa and the composition of GFAP polypeptide fragments were determined by Western blot [21].

The assessment of the relative intensity of polypeptide bands was performed by processing of the scanned Western blot results using Image 2000 software (Bio-Techne Corp.). Relative quantity analysis of GFAP polypeptide fragments was performed by comparing the color intensity of the respective samples.

Statistical processing of obtained data was performed using software packages «Microsoft® Excel

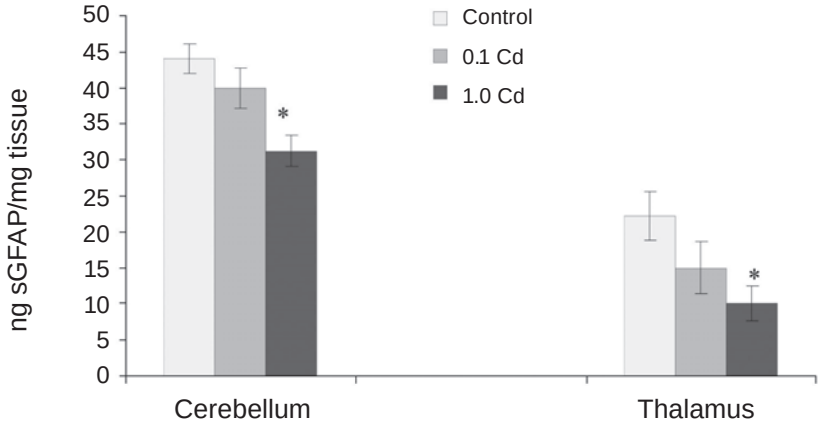

*Fig. 1. Content of the soluble form of glial fibrillary acidic protein in the rats' cerebellum and thalamus under Cd exposure in doses of 0.1 and 1.0 μg/kg; n = 6, * P < 0.05 with respect to control*

2000» (Microsoft®), «STATISTICA® for Windows 6.0» (StatSoft Inc.). Statistical analysis was carried out by Student *t*-test. Values with $P \leq 0.05$ were considered as reliable.

Results and Discussion

The results of the determination of GFAP soluble form content in the cerebellum and thalamus of control and experimental animals groups are shown in Fig. 1.

Coincidence of tendencies to the reduction of the concentration of the GFAP soluble form was observed in all studied brain parts under conditions of prolonged use of the standard diet (for 37 days) with addition of cadmium to drinking water (0.1 and 1.0 μg/kg of body weight). Increase of cadmium dose in drinking water to 1.0 μg/kg of body weight (over 37 days) induced a significant decrease in soluble GFAP level in all brain parts. Glial fibrillary acidic protein content in the cerebellum of the control animals was found to be 43.9 ± 0.02 ng/mg of tissue, while soluble GFAP levels in animals receiving cadmium at a dose of 1.0 μg/kg was 71% of control value and found to be 31.2 ± 2.1 ng sGFAP/mg of tissue. The highest decrease to 45.27% in sGFAP content was observed in the thalamus. sGFAP content in the thalamus of the control animals was found to be 22.2 ± 3.3 ng/mg of tissue, while soluble GFAP levels in animals receiving cadmium at a dose of 1.0 μg/kg of animal body weight were found to be 10.07 ± 2.3 ng sGFAP/mg of tissue. The obtained results indicate a pronounced dose-dependent effect of cadmium ions on astrocytes cytoskeleton.

It has been previously reported that the cumulative effect of As, Pb and Cd mixture present in the drinking water during rat postnatal period (60 days) led to a gradual inhibition of the expression of mRNA encoding GFAP isoforms in astrocytes. Several GFAP isoforms such as α, β, κ, δ and ε are expressed in the brain [22]. A decrease in soluble GFAP level in the mammalian brain during postnatal period was reported by A. Rai et al. [3]. The authors observed an increase in the apoptosis of astrocytes during the decrease in soluble GFAP by blocking the GFAP gene expression in the primary astrocyte culture. Thus, the accumulation of heavy metals in the brain can affect the changes in the composition of GFAP isoforms, whereas the inhibition of the expression of soluble GFAP may lead to stimulation of apoptosis in the mature rat astrocytes.

The obtained data indicate that the chronic effect of cadmium ions (for 37 days in drinking water in a dose of 1 μg/kg) induces a significant decrease in the levels of soluble GFAP that can be a result of decreasing the expression of this protein, as well as the destruction of intermediate filaments and rebuilding of astrocyte cytoskeleton.

The obtained Western blot data indicate no significant degradation of the soluble GFAP under cadmium ions exposure (Fig. 2).

The determination of total protein level in the cytosol fractions obtained from the tissues of the respective parts of the rat brain showed no notable changes in the total protein level under the chronic effect of cadmium ultralow dose of 0.1 μg/kg of body weight, although a tendency to increase was observed (Fig. 3).

Increasing the cadmium dose in drinking water to 1 μg/kg of body weight over 37 days resulted in a remarkable decrease in total protein in the cerebel-

Fig. 2 Western blot against GFAP of cytosol fraction from rat cerebellum and thalamus

lum, whereas a decrease in the total soluble protein in the thalamus was insignificant.

The data obtained indicate a dose-dependent effect of cadmium on the content of the soluble form of glial fibrillary acidic protein and on the total level of cytosol proteins in the rat brain. Furthermore, the specificity of this effect is determined by the peculiarities of different parts of the brain that affect the functional properties of the respective brain parts and the central nervous system as a whole.

Unchanged characteristics under ultralow cadmium dose exposure were observed during the evaluation of the total protein level in filament fractions obtained from rat thalamus and cerebellum tissues (Fig. 4).

An increase in the cadmium dose in drinking water up to 1.0 μg/kg over 37 days led to a slight but important elevation in the total protein level. These results indicate that cadmium exhibits a dose-dependent effect on the content of the GFAP soluble form, total cytosol protein level and a strongly pronounced effect on the content of the fibrillated GFAP form and composition of the polypeptide fragments of this protein in the rat brain. Moreover, the specificity of the effect depends on the brain part.

The results of evaluation of the GFAP filament form showed almost no change in the cerebellum and thalamus at the exposure to chronic cadmium ions in dose 0.1 μg/kg. At the same time, the chronic exposure to cadmium at a dose of 1.0 μg/kg induced a significant increase in the expression of the cytoskeleton protein. Elevations in the GFAP filament form levels 2.23 and 1.75 times were observed in the cerebellum and thalamus, respectively (Fig. 5).

It should be noted that the filament form of GFAP in the rat brain is the primary form and significantly outweighs the soluble form [23]. Thus, an increase in the content of the filament form of GFAP indicates the intensity in the process of formation of intermediate filaments and the branching of astrocyte cytoskeletons in the rat brain of this studied group. Furthermore, Western blot results evidenced for the occurrence of low molecular mass polypeptide fragments of an intact GFAP 49 kDa (Fig. 6) under cadmium exposure at a dose of 1.0 μg/kg. Such fragments were not found in the rat cerebellum and thalamus from the control group and from the group that received cadmium dosage of 0.1 μg/kg.

Given the fact that overexpression of GFAP is a reliable marker of astrogliosis, a significant increase

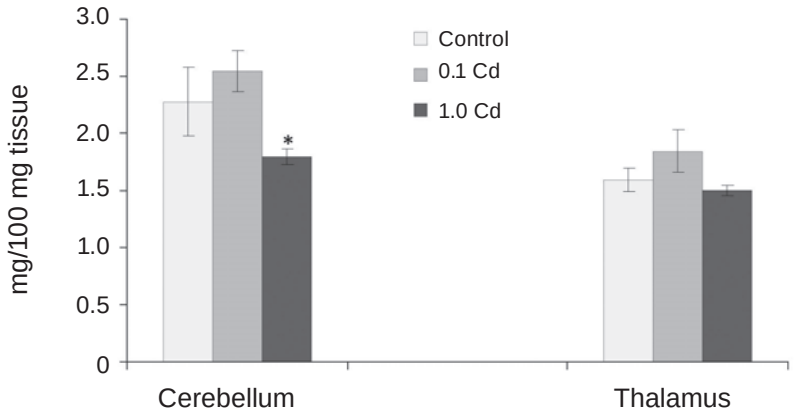

*Fig. 3. Total protein level in the cytosol fractions obtained from the different parts of the rat brain under Cd exposure in doses 0.1 and 1.0 μg/kg over 37 days; n = 6, * P < 0.05, with respect to control*

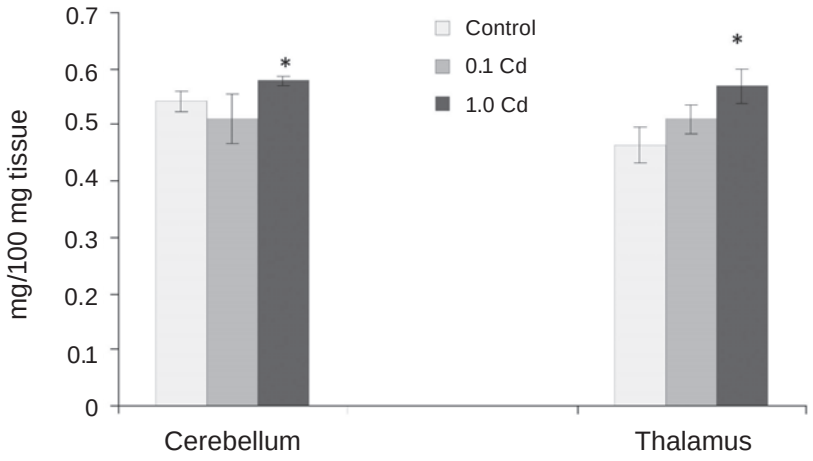

*Fig. 4. Total protein level in filament fractions obtained from rat cerebellum and thalamus under Cd exposure in doses 0.1 and 1.0 μg/kg over 37 days; n = 6, * P < 0.05 with respect to control*

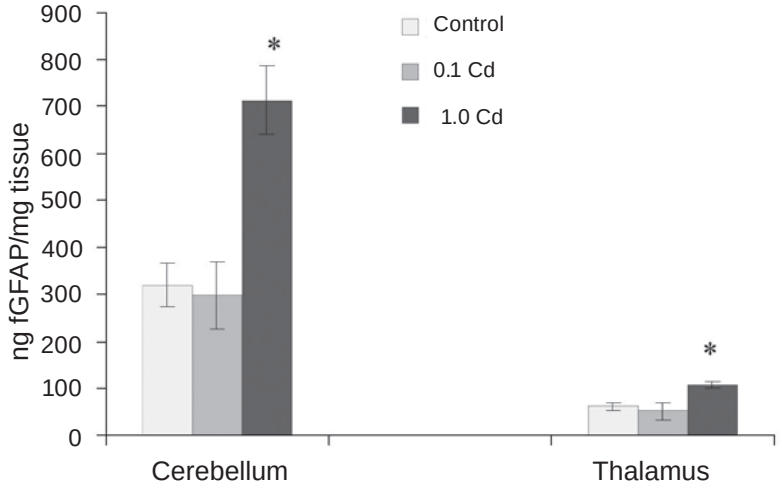

*Fig. 5. Content of the filament form of glial fibrillary acidic protein in the rats` cerebellum and thalamus under Cd exposure in doses of 0.1 and 1.0 μg/kg over 37 days; n = 6, * P < 0.05 with respect to control*

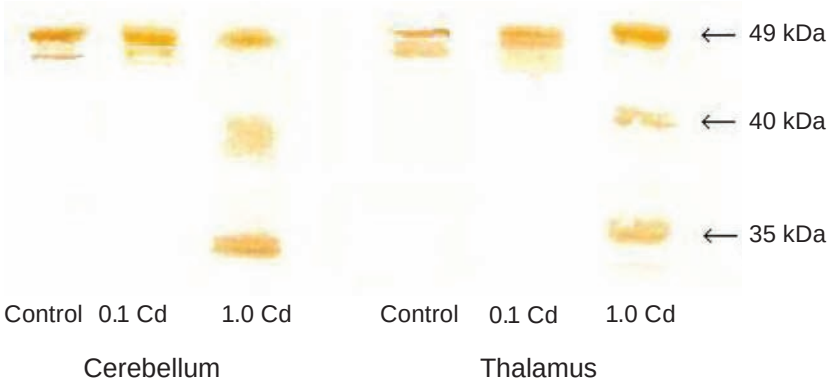

Fig. 6. Western blot against GFAP in filament fractions obtained from the rat cerebellum and thalamus

in the level of GFAP filament form observed in our study implies astrocytes reactivation in response to the damaging effect of cadmium ions.

Astrocytes play a vital role in the regulation of neurons' micro surrounding and the interaction between neurons and brain capillaries. Glial cells are more sensitive to neurotoxic insults than neurons [9]. Quick astrocytes reaction is an essential part of CNS cell response to various (in nature) damages [12]. This response is characterized by overexpression of GFAP during astrocytes proliferation and differentiation.

A strong correlation between the development of oxidative stress and the development of astrogliosis was observed on the various experimental models of damaged neural tissue cells [24]. Known and quite common effect of heavy metal ions exposure is a redox-balance disorder, increase free radical formation and development of oxidative stress. Thus, the increase of free radical formation caused by the chronic effect to cadmium ions exposure at a dose of 1.0 μg/kg can be one of the mechanisms of astrogliosis induction. The absence of such an effect under cadmium ions exposure at a dose of 0.1 mg/kg can be explained by the presence of the potent antioxidant protection system in glial cells that is capable of preventing the effect of ultra-low doses of metal ions on astrocytes. On the other hand, the effect of ultra-low concentrations of toxic xenobiotics is compensated by various mechanisms of detoxification and does not lead to the homeostasis disorders.

The obtained results showed a dose-dependent effect to cadmium ions exposure on the astrocytes cytoskeleton and their involvement in reactive gliosis in the rat CNS. Differences in alterations of levels of soluble and filament forms of the astrocyte intermediate filament protein under cadmium exposure at a dose of 1.0 μg/kg indicate the toxicity of such dose on the CNS cells of animals. A significant increase in the expression of the filament GFAP under cadmium exposure at a dose of 1.0 μg/kg indicates active involvement of the astrocytes in the protective reactions under cadmium ions intoxication.

The obtained data indicate the participation of intermediate filaments of astrocyte cytoskeletons in processes that determine the peculiarities of the neurotoxic effects of cadmium. The results of evaluation of the content and composition of polypeptide fragments of GFAP indicate a dose-dependent effect of chronic cadmium ions. Exposure to cadmium ions over 37 days at a dose of 0.1 μg/kg did not affect the total protein pool and the metabolism of glial fibrillary acidic protein in the rat brain. Intoxication by cadmium ions at a dose of 1.0 μg/kg led to a significant decrease in the soluble form of GFAP and an increase in the filament form of GFAP in the studied parts of the rat brain, thus indicating the development of reactive astrogliosis and the risk of neurodegenerative disorders.

РОЗПОДІЛ ГЛІАЛЬНОГО ФІБРИЛЯРНОГО КИСЛОГО ПРОТЕЇНУ В РІЗНИХ ВІДДІЛАХ ГОЛОВНОГО МОЗКУ ЩУРІВ ЗА ВПЛИВУ КАДМІЮ

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В експерименті на лабораторних щурах досліджено хронічний вплив малих доз кадмію на розподіл розчинної та філаментної форм гліального фібрилярного кислого протеїну (ГФКП) та вміст їхніх поліпептидних фрагментів у різних відділах головного мозку. Одержані результати показали, що вплив кадмію виявляє дозозалежний ефект не тільки на вміст розчинної форми ГФКП, загальний рівень цитозольних протеїнів, але і на вміст фібрилізованої форми ГФКП і склад поліпептидних фрагментів цього протеїну в мозку щурів. Тривала токсикація іонами кадмію в дозі 1,0 мкг/кг призводить до вірогідного зменшення розчинної форми ГФКП та значного збільшення його філаментної форми в дослідних відділах мозку щурів, що вказує на розвиток реактивного астрогліозу та ризик розвитку нейродегенерації.

Ключові слова: гліальний фібрилярний кислий протеїн, мозок щурів, кадмій.

РАСПРЕДЕЛЕНИЕ ГЛИАЛЬНОГО ФИБРИЛЛЯРНОГО КИСЛОГО ПРОТЕИНА В РАЗЛИЧНЫХ ОТДЕЛАХ ГОЛОВНОГО МОЗГА КРЫС ПОД ВЛИЯНИЕМ КАДМИЯ

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В эксперименте на лабораторных крысах исследовано хроническое воздействие малых доз кадмия на распределение растворимой и филаментной форм глиального фибриллярного кислого протеина (ГФКП) и содержание его полипептидных фрагментов в различных отделах головного мозга. Полученные результаты показали, что влияние кадмия имеет дозозависимый эффект не только на содержание растворимой формы ГФКП, общий уровень цитозольных протеинов, но и на содержание филаментной формы ГФКП и состав полипептидных фрагментов этого протеина в мозгу крыс. Длительная токсикация ионами кадмия в дозе 1,0 мкг/кг приводит к достоверному уменьшению растворимой формы ГФКП и значительному увеличению его филаментной формы в исследуемых отделах мозга крыс, что свидетельствует о развитии реактивного астроглиоза и о риске развития нейродегенерации.

Ключевые слова: глиальный фибриллярный кислый протеин, мозг крыс, кадмий.

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