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Influence of long-term cadmium ions exposure on mitotic and apoptotic activities and protein synthesis in mouse liver

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The aim of this study was to evaluate *in vivo* the effects of long-term exposure to cadmium ions (Cd^{2+}) on cell death, mitotic activity and translational machinery of mouse liver cells. It was determined that after intraperitoneal injections of cadmium chloride solution (0.16 mg Cd^{2+} per 1 kg of body mass) for six weeks, three times per week, the number of TUNEL positive cells containing fragmented nuclei was significantly higher than in control ($p < 0.0001$); the median score was 1. Cadmium-induced cell death was accompanied by activation of mitosis. These data indicate an activation of liver regeneration during sub-chronic exposure to Cd^{2+}. Examination of liver translation showed that Cd^{2+} reduced both the incorporation of [^{14}C]-labeled leucine into newly synthesized peptides and proteins and the acceptor activity of $tRNA^{Leu}$. In comparison with the control, liver exposure to Cd^{2+} caused activation of leucyl-tRNA synthetase.

Key words: cadmium, mitotic index, protein synthesis, tRNA, leucyl-tRNA synthetase, apoptosis

INTRODUCTION

Cadmium (Cd) has no known beneficial biological function and prolonged exposure to it has been linked to toxic effects in both humans and animals [1]. A diversity of toxic effects has been ascribed to Cd, including hepato-renal toxicity, carcinogenicity, teratogenicity and damage to endocrine and reproductive systems [2–5]. According to experimental and epidemiological data, mechanisms underlying Cd toxicity depend on the route of its entering into a mammalian organism, the dosage and chemical form of this metal, duration of exposure, the species and age of the experimental animal, etc. [6, 7]. The diversity of factors affecting the mechanism of Cd action makes experimental data difficult to compare, interpret and to develop a common model of this metal-induced injury to a mammalian organism. The molecular targets of Cd are well defined. These are thiol groups in proteins and peptides as well as nucleobases in nucleotides and nucleic acids [8, 9]. Cd can also mimic bivalent ions such as Ca^{2+} and Zn^{2+}, thereby interfering with signal transduction pathways, gene expression and bone formation [10, 11]. As Cd combines with biomolecules, it initiates a high variety of deleterious processes in cells. It has been shown that Cd inhibits mismatch repair of DNA, induces both the opening of membrane permeability pore of mitochondria and the production of reactive oxygen species [12–14].

Manifestations of these deleterious effects of Cd differ depending on the models of experimental intoxication, the doses and duration of Cd exposure as well as on

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chemical forms of this metal. It has been documented that liver can accumulate high amounts of Cd getting into the organism via lungs, digestive system or when injected to an experimental animal for a long period of time [3, 2]. As the liver retains considerable amounts of Cd, it becomes the primary target-organ for this metal. Under a long-term exposure, Cd-induced injury of other organs seems to be dependent on the Cd-detoxifying ability of the liver. The molecular mechanisms that underlie the resistance of an organism to Cd-induced injury of the whole organism and of the liver in particular are not yet completely understood. The present study was intended to investigate the effect of six weeks of exposure of mice to subtoxic doses of cadmium chloride ($CdCl_2$) on the viability of liver cells, an emphasis being placed on the efficiency of the machinery of translation. It was shown that even though in Cd-affected mouse liver the level of translation was reduced, it was efficient enough to maintain the level of regeneration activity (evaluated by the mitotic index) needed to replace apoptotic and necrotic cells.

MATERIALS AND METHODS

Experiments were carried out on 4–6-week-old outbred mice weighing 20–25 g. All the experiments were performed according to the rules for the protection of vertebrate animals used for experimental and other scientific purposes (License No 0028 of State Veterinary Service for working with laboratory animals). For six weeks three times per week, mice were intraperitoneally injected with $CdCl_2$ solution (0.16 mg Cd^{2+} per 1 kg of body mass, i. e. 0.05 LD_{50}). Control mice received injections of the same volume of saline solution according to the same scheme. Body weight, survival, and clinical signs were recorded daily throughout the experiment. After six weeks mice of control and experimental groups were terminated. An extensive necropsy was performed on all animals.

For histological examination, liver tissue specimens were fixed in 10% neutral buffered formalin for 48 h and then processed for routine paraffin embedding. Five-micron-thick sections were processed for routine staining with hematoxyline and eosin as indicated in [15]. Histological slides were examined with a light microscope (objective 40×). For each specimen, the number of mitotic cells was counted in 10 randomly selected reference areas (0.04 mm^2). An Olympus Digital Camera DP-11 was used for the histological imaging of slides. Apoptosis of liver cells was histochemically detected by the TUNEL assay using an *in situ* cell death detection kit (AP, Roche). Sections of formalin-fixed and paraffin-embedded liver tissue were de-waxed by washing in xylene and rehydrated through a gradual series of ethanol and distilled water. Proteinase K permeabilized sections were subjected to enzymatic *in situ* labeling of DNA strand breaks using the TUNEL technique as indicated in the manufacturer's instruction. After counterstaining with eosin, the sections were analyzed on a light microscope (objective 20×). The number of positively stained (TUNEL-positive) nuclei of liver cells was determined by counting in randomly selected 10 histological fields per section. The nonparametrical Kruskall–Wallis test was used for comparison between the groups. Statistical significance was set at $p < 0.05$.

For the measurement of protein synthesis, mice were injected i. p. with [^{14}C]-labelled leucine (7.4 MBq per kg of body weight) one hour before termination. Leucine was selected as a coding amino acid tag, because it is the most prevalent amino acid in the cellular proteins. Protein synthesis in mouse liver was evaluated by incorporation of [^{14}C]-labelled leucine into newly synthesized peptides and proteins as described in [16]. Protein amount in samples was determined by the Warburg–Christian method. The isolations of total tRNAs and post-mitochondrial supernatant (source of leucyl-tRNA synthetase) from mouse liver and measurements of their activities were described earlier [17].

Results were expressed as a mean ± standard error of the mean. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

The present study was aimed at investigating the subchronic (six-week-long) effects of low (0.05 LD_{50}) doses of intraperitoneally injected $CdCl_2$ on mice liver cell death and its regeneration activity with an emphasis on the hepatic machinery of translation *in vivo*. The general toxic effect exerted by repeated exposures to Cd was assessed according to mice body weight gain and the relative weight index of organs (the ratio of organ weight to body weight). In respect of the control group, the body weight gain exhibited a two-phase response to Cd: after two weeks of exposure to Cd, body weight gain in the experimental mice was by 7% lower than in control, but thereafter it started to increase and exceeded the control level by 13% after six weeks of Cd treatment (Fig. 1). The overall survival rate was the same in both the control and the Cd-exposed groups, suggesting that Cd doses were well tolerated and did not cause life-threatening intoxication. Gradual diminution of body weight gain in response to chronic oral and subcutaneous treatment of rodents with Cd salts has also been reported in other studies [2, 3, 18]. The reasons for the progressive increase in body weight gain observed beyond the 2nd week of Cd treatment are not completely clear. In our recent investigation we found a progressive diminution of body weight gain in juvenile mice during eight weeks of $CdCl_2$ administration *per os* [18]. In the present study, a systemic effect of Cd on the liver of experimental mice was evaluated according to the relative weight index, which is represented by the organ body weight ratio. In respect of the control, a statistically significant increment (by 10%) of the relative weight index was revealed in the group of Cd-treated mice (Fig. 2). Ultrastructural alterations, such as a thicker capsule and heaps of immune cells in liver, were also characteristic of Cd-exposed mice.

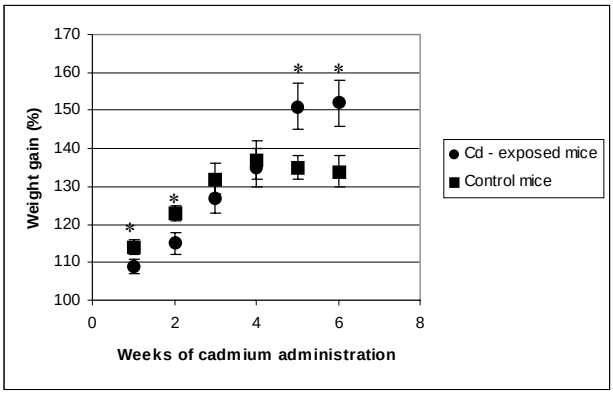

Fig. 1. Time-course of body weight gain of control group mice and mice treated with $CdCl_2$ (0.16 mg Cd^{2+} per 1 kg of body weight) for 6 weeks. The data are obtained by measuring the body mass of 45 mice in the control group and of 60 mice in the experimental group. Statistically significant differences ($p < 0.05$) between control and experimental groups are indicated by asterisks

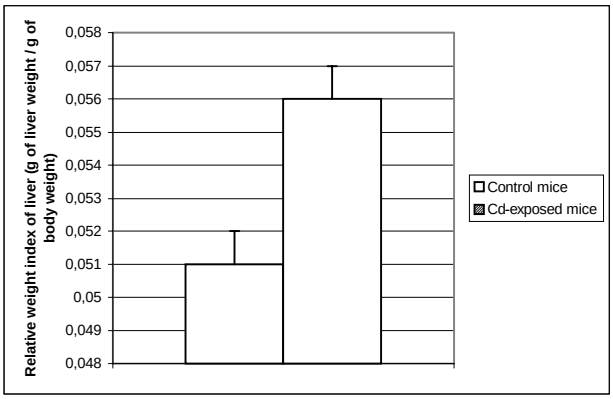

Fig. 2. Effect of repeated intraperitoneal injections of $CdCl_2$ solution on the relative weight index of mice liver. The control group included 45 and the experimental group 60 mice. The difference between control and experimental groups was statistically significant ($p < 0.05$)

As evidenced by our recent experiments, Cd concentration increased 655 and 112 times in mice liver and kidney, respectively, under subchronic treatment of mice with $CdCl_2$ [19]. These results are consistent with data reported by other authors [20, 21]. We determined that the absolute amounts of Cd retained in kidney did not reach the nephrotoxic levels indicated in other studies [22]. Accumulation of Cd is not necessarily related to tissue damage, since in liver and kidney cells Cd is sequestered to thiol-groups of proteins and polypeptides such as glutathione, hsp and metallothionein, a cysteine-rich protein [23]. The highest Cd-binding capacity is attributed to metallothioneins. So far, four isoforms of metallothioneins are known to exist: metallothioneins I and II are metal-ion inducible, and the others are constitutive [24]. Induction of metallothioneins was confirmed experimentally: subcutaneous injection of 0.5 mg Cd/kg in rats resulted in a progressive accumulation of Cd in liver and kidney for the first 10 weeks, which was related to induction of metallothioneins [20]. As Cd–metallothionein complexes are toxicologically inert, metallothionein levels correlate with resistance of an organism to Cd [24]. Although metallothionein is a powerful player in the protection of tissue from Cd, its effect is limited. Under subchronic exposure of mice to Cd, the primary pathological lesion is inflammation of liver parenchyma [21]. It is well documented that liver inflammation can progress into cell death such as apoptosis [26]. We quantified apoptosis immunohistochemically by determining the apoptotic cell number in formalin-fixed paraffin-embedded liver sections. In regard to the control, the incidence of apoptosis was higher in the liver of Cd-exposed mice (Fig. 3A). In the present study, regeneration activity of the liver was assessed according to the number of mitotic cells in the liver specimens. As Fig. 3B shows, the number of mitotic cells was higher in mice liver after subchronic exposure to Cd than in control. Thus, under repeated intraperitoneal injections of subtoxic doses of $CdCl_2$ for 6 weeks, the integrity of liver structure was maintained by eliminating critically damaged liver cells and their replacing them with new ones. The mechanisms of Cd-induced cell death differ in cases of acute and chronic exposure. Under acute exposure, Cd induces oxidative stress to liver cells, which progresses into apoptosis and then to necrosis [21]. Unlike acute effects, the chronic effects of Cd are related to a release of inflammatory pro-apoptotic cytokines, so that apoptosis occurs to be a predominant mode of damaged cell elimination [21]. Our present investigation confirms this mechanism, since only a tendency of marker enzyme release from the liver was observed in experiments (non-published data). Meanwhile, a number of studies indicate a significant release of those enzymes from the liver under acute exposure to Cd [26]. The molecular basis of Cd-induced mitosis is not clear. We can presume that TNF-α, a pro-apoptotic cytokine, is implicated in the process [27]. It is a well known fact that the integrity of any tissue is maintained when the degradation and synthesis of its components, e.g., proteins, is at least in balance. In this relation, we examined the effect of repeated intraperitoneal injections of $CdCl_2$ solution for 6 weeks on the intensity of protein synthesis in mouse liver *in vivo*. As the data presented in Fig. 4A show, the intensity of protein synthesis in Cd-treated mouse liver was significantly lower (by 21%) as compared with the control. A proteomic study showed that in yeast the expression of proteins unequivocally responded to Cd: 106 proteins were upregulated and 55 downregulated [28]. According to the data of the aforementioned study, the most prevalent upregulated proteins were those involved in translation. Limited cohesive information is available on the effects of Cd on protein expression in mammalian cells. It this relation, examination of *in vivo* effects of Cd on the machinery of translation in the liver was conducted in the present study. We compared the activities of $tRNA^{Leu}$ as a representative of all tRNA families isolated from the control mice liver and Cd-exposed mice liver. The effect of Cd ions on acceptor

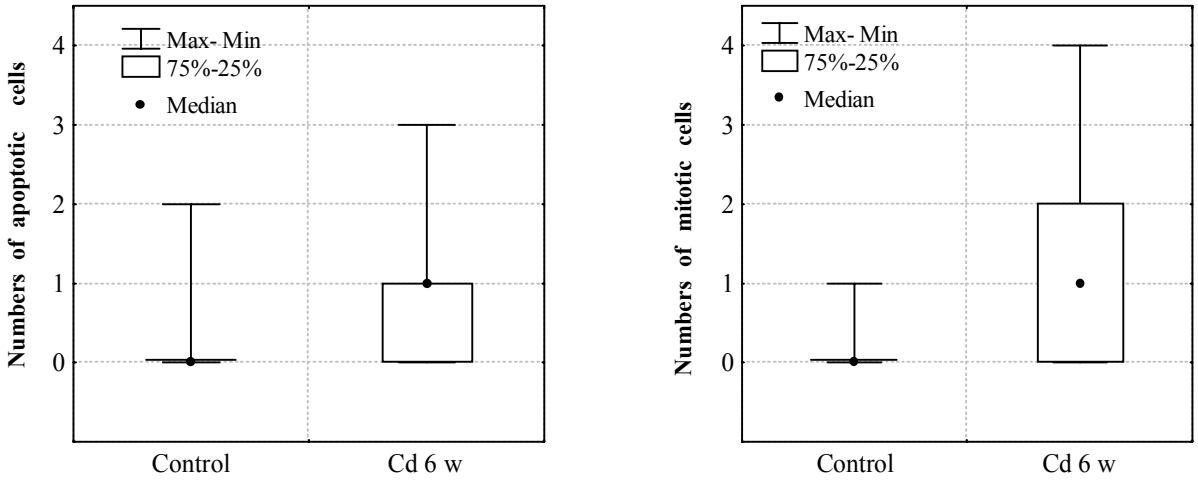

Fig. 3. Effects of six-week repeated intraperitoneal injections of $CdCl_2$ on the numbers of apoptotic (A) and mitotic (B) cells in mice liver specimens. The differences between control and experimental groups were statistically significant ($p < 0.05$). Data represent results of 3 separate experiments

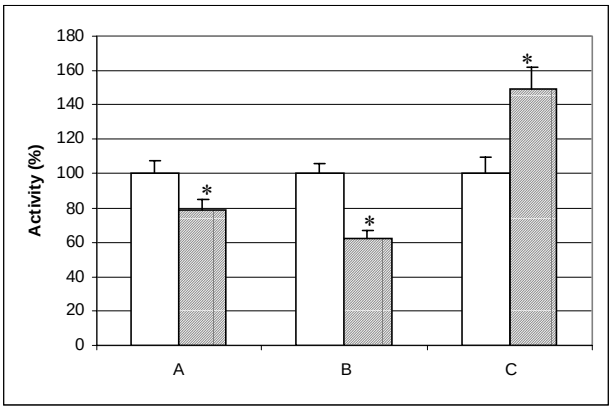

Fig. 4. Effects of six-week repeated intraperitoneal injections of $CdCl_2$ on mice liver protein synthesis (A), activity of $tRNA^{Leu}$ (B) and of leucyl-tRNA synthetase (C). Asterisks indicate statistically significant differences of values between experimental and control groups. Each column represents data obtained from 14–16 experimental animals

activities of $tRNA^{Leu}$ is shown in Fig. 4B. Subchronic exposure of mice to Cd resulted in a 38% decrease of the acceptor activity of $tRNA^{Leu}$ in respect of the control. As Cd^{2+} can combine with nuclei acids [9], it can result in both alteration of the structure and re-distribution of active and inactive forms of tRNA. The latter could be seen as diminution of acceptor activity of $tRNA^{Leu}$ observed in our experiments. Next, we examined the influence of Cd on the activity of one of the 20 aminoacyl-tRNA synthetases, namely leucyl-tRNA synthetase. The results indicated that the activity of leucyl-tRNA synthetase was increased by 49% versus the control level after six weeks of liver exposure to $CdCl_2$ (Fig. 4C). This effect can probably compensate the partial inactivation of $tRNA^{Leu}$ and thus maintain the total synthesis of proteins at a sufficiently high level (21% diminution of protein synthesis versus 38% diminution of acceptor activity of $tRNA^{Leu}$). The underlying mechanism of Cd-induced leucyl-tRNA synthetase activation is unknown. In mammals, leucyl-tRNA synthestase forms large supramolecular complexes with other aminoacyl-tRNA synthetases and non-catalytic proteins [29]. One may speculate that Cd-induced upregulation of proteins [28], e.g., leucyl-tRNA synthetase involved in protein biosynthesis, may result in increasing the contribution of this enzyme to the complexes. This mechanism could benefit the adaptation of mammalians to Cd-induced inhibition of vitally important molecules.

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LĖTINIS KADMIO JONŲ POVEIKIS MITOZINIAM IR APOPTOZINIAM AKTYVUMAMS BEI BALTYMŲ SINTEZEI PELIŲ KEPENYSE

S a n t r a u k a

Šių tyrimų tikslas – įvertinti šešių savaičių trukmės kadmio jonų (Cd^{2+}) poveikį *in vivo* pelių kepenų ląstelių žūčiai, mitoziniam aktyvumui ir baltymų sintezei. Nustatyta, kad pelių, kurioms tris kartus per savaitę į pilvo ertmę švirkštas kadmio chlorido tirpalas (0,16 mg Cd^{2+} vienam kg kūno svorio), kepenyse apoptozinių (TUNEL teigiamų) ląstelių buvo reikšmingai daugiau negu kontrolinės grupės pelių kepenyse ($p < 0,0001$), mediana 1. Kartu buvo stebima ir aktyvesnė negu kontrolės atveju kepenų ląstelių mitozė. Taigi šiomis eksperimentinėmis sąlygomis Cd^{2+} pažeistų kepenų audinys aktyviai atsinaujina. Tiriant Cd^{2+} poveikį baltymų sintezei kepenyse, nustatyta, kad šie jonai slopina tiek [^{14}C]-leucino įjungimą į naujai sintetinamus peptidus bei baltymus, tiek $tRNR^{Leu}$ akceptinį aktyvumą. Lyginant su kontroline grupe, kadmiu paveiktų pelių kepenų leucil-tRNR sintetazės aktyvumas buvo reikšmingai didesnis.

Raktažodžiai: kadmis, mitozė, baltymų sintezė, tRNR, leucil-tRNR sintetazė, apoptozė