

Subacute effects of cadmium and zinc ions on protein synthesis and cell death in mouse liver

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Summary. *Objective.* The aim of this study was to evaluate *in vivo* the effects of cadmium and zinc ions on translational machinery and death of mouse liver cells.

Material and methods. Outbred mice received intraperitoneal injections of cadmium chloride solution (1.4 μmoles cadmium per 1 kg of body weight) and/or zinc sulfate solution (4.8 μmoles zinc per kg of body weight) three times per week for six weeks. Analogical volume of saline solution was injected to the control mice. Protein synthesis was evaluated by incorporation of [¹⁴C]-labeled leucine into peptides and proteins. Total tRNAs were isolated using deproteinized extract of liver tissue. Postmitochondrial supernatant was as a source of leucyl-tRNA synthetase. Activities of tRNA^{Leu} and leucyl-tRNA synthetase were measured by an aminoacylation reaction using [¹⁴C]-labeled leucine. Liver cell apoptosis was detected by TUNEL assay using *in situ* cell death detection kit.

Results. A decrease in incorporation of [¹⁴C]-labeled leucine into proteins was detected in liver, kidney, and heart as well as diminution of tRNA^{Leu} acceptor activity in cadmium-exposed liver. Cadmium caused activation of the leucyl-tRNA synthetase and induced liver cell apoptosis. Pretreatment of mice with zinc sulfate solution favored to protection of protein synthesis and acceptor activity of tRNA^{Leu} against cadmium-induced inhibition. Under co-exposure of mouse liver to cadmium and zinc, activity of the leucyl-tRNA synthetase was at the level of control. Zinc did not influence TUNEL-positive cell number in cadmium-exposed mouse liver.

Conclusions. Under subacute intoxication of mice by cadmium, zinc ions protect the translation machinery against inhibition, but do not decrease the number of apoptotic cells in the liver.

Introduction

Cadmium (Cd) is a toxic heavy metal that accumulates in the environment and is commonly found in cigarette smoke and industrial effluents. The data of experimental and epidemiological studies show that mechanisms of Cd toxicity depend on the route of its entering into an organism, the dosage, the chemical form of this metal, duration of exposure, species and age of experimental animal (1). Molecular targets of Cd depend on experimental model – *ex vivo* Cd combines with bases in nucleotides and nucleic acids (2), *in vivo* – with thiol-rich proteins and peptides (3). This is followed by a number of cellular responses including deterioration of cell-cell adhesion (4), changes in gene expression (5), abnormalities in cell signaling (6), changing in energy metabolism, and eventually occurrence of cell death (7). Diversity of factors effecting mechanism of Cd action makes experimental data difficult to compare, interpret, and develop com-

mon model of this metal-induced injury to mammalian organism.

One of Cd targets is the system of protein synthesis or translation. *In vivo*, effects of Cd ions on the protein synthesis depend on the intoxication duration and, probably, the dose of this ion (8). According to the data of *in vitro* study, Cd ions at low concentrations can activate both the rate and the level of translation but at high concentrations inhibit those parameters (8). As the protein synthesizing machinery is enormously complicated, sensitivity of its components to Cd ions may differ. *In vivo* and *in vitro* studies demonstrated a significant diminution of the tRNA and aminoacyl-tRNA synthetase activities under acute intoxication with relatively high concentrations of Cd ions (9).

Some authors have shown that Cd ions can induce apoptosis in mouse liver (10). This occurs via different mechanisms including oxidative stress (11), Bax-

and p53-dependent pathways (12). Under acute mouse intoxication, incidence of apoptosis depends on duration of liver exposure to Cd with maximum at the eight hour (7). In later periods of acute exposure, necrosis takes over apoptosis. Cell apoptosis is switched on and tightly regulated by proteins. Some of them are inducible, e.g. in response to injury and toxicants immune cells release cytokines, powerful inducers of apoptotic cell death. Heavy metals induce synthesis of metal-binding proteins, which inhibit apoptosis. However, almost nothing is known about relationship between protein synthesis and apoptosis in Cd-exposed liver.

Zinc (Zn) is a ubiquitous element essential for a number of cellular processes: DNA synthesis, transcription, and translation, but in excess it can be toxic (13). Suppression of both RNA-related and translation pathways is a mechanism underlying deleterious effects of high concentrations of Zn ions (14). On the other hand, Zn is essential for functioning of a number of enzymes including aminoacyl-tRNA synthetase (15). Complex effect of both Zn and Cd ions on the system of translation is little examined. *In vivo* study of acute exposure of mice to Cd (14 $\mu\text{mol/kg}$) has shown that Cd-inhibited protein synthesis can be restored by Zn but only at 2 h after Cd administration (16). So far, effects of prolonged exposure to low concentration of ions of these metals on the translation system have not been examined. Zn is known to be a suppressor of apoptosis (17). However, it is uncertain if Zn effect can compensate for pro-apoptotic action of Cd.

The present study was conducted to investigate the effect of 6-week exposures of mice to subtoxic doses of CdCl₂ and ZnSO₄ on protein synthesis, activities of tRNA^{Leu} and leucyl-tRNA synthetase, as well as apoptosis in the liver. We have found that Zn ions are capable to protect the translation machinery against Cd-induced inhibition, but do not effect liver cell death in mouse liver exposed to Cd ions.

Material and methods

Experiments were done on 4–6-week-old outbred mice weighing 20–25 g. All experiments were performed according to the Republic of Lithuania Law on the Care, Keeping and Use of Animals (License of State Veterinary Service for working with laboratory animals, No. 0135). Three times per week, mice of the first group received intraperitoneal (i.p.) injections of cadmium chloride (CdCl₂) solution (1.4 $\mu\text{moles Cd}$ per 1 kg of body weight) within 6 weeks. Mice of the second group were injected with zinc sulfate (ZnSO₄)

solution (4.8 $\mu\text{moles Zn}$ per 1 kg of body weight) according to aforementioned scheme. Mice of the third group were pretreated with of ZnSO₄ solution injections and after 20 min – with CdCl₂ solution according to the same scheme. Control mice (the fourth group) received injections of the same volume of 0.9% saline. Body weights, survival, and clinical signs were recorded daily throughout the experiment. After 6 weeks, mice of control and experimental groups were killed. An extensive necropsy was performed on all animals.

For the measurement of protein synthesis, mice received i.p. injection of [¹⁴C]-labeled leucine (7.4 MBq per kg of body weight) one hour before killing. 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 [¹⁴C]-labeled leucine into newly synthesized peptides and proteins (8). Protein amount in samples was determined by Warburg-Christian method. A procedure of isolation of total tRNAs and postmitochondrial supernatant (a source of leucyl-tRNA synthetase) from mouse liver and measurements of their activities were described earlier (15).

Apoptosis of liver cells was detected by immunohistochemical TUNEL assay using *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 re-hydrated 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 TUNEL technique as it is indicated in manufacturer instruction. After counterstaining with eosin, sections were analyzed under a light microscope (objective $\times 20$). The number of positively stained (TUNEL+) nuclei of liver cells was determined by counting in randomly selected 10 histological fields per section.

Results were expressed as the mean \pm standard error of mean. Nonparametric Kruskal-Wallis test was applied for evaluation of apoptosis of liver cells. Statistical significance was set at $P < 0.05$.

Results

The present study aimed to investigate subacute 6-week effects of low doses of i.p. injected CdCl₂ and/or ZnSO₄ solutions on translation machinery and the cell death in mouse liver. The overall survival rate was the same in all studied groups of mice suggesting that metals doses were well tolerated and did not cause in life-threatening intoxication. General toxic effect exerted by repeated exposures to solutions of salts of

these metals was assessed according to body weight gain and the relative weight indexes of organs (the ratio of an organ weight to body weight). Comparing to the control group, body weight gain exhibited two-phase response to Cd: after 2 weeks of exposure, body weight gain of experimental mice was by 7% lower than in control, but thereafter it started to increase and exceeded the control level by 13% after 6 weeks of Cd treatment (Fig. 1). Body weight gain of Zn-treated or Zn+Cd-treated mice was at the level of control group.

Comparing to the control group, a statistically significant increment (by 10%) of the relative weight index of liver was revealed in the group of Cd-treated mice (Fig. 2). The relative weight indexes of liver of both Zn- and Cd+Zn-treated mice were significantly lower than of control group mice.

In vivo effects of 6-week i.p. administration of Cd and Zn ions on the total protein synthesis in mouse

liver, kidney, heart, and skeletal muscle were evaluated by incorporation of [^{14}C]-labeled leucine into newly synthesized proteins and peptides. The intensity of total protein synthesis in organs of the control group mice was considered as high as 100%. The results showed (Fig. 3) that in response to Cd exposure for 6 weeks, reduction of protein synthesis intensity by 34%, 39%, and 43% was determined in mouse liver, kidney, and heart, respectively. In skeletal muscles of mice, however, the effect of Cd on the total protein synthesis was negligible. Repeated injections of solely ZnSO_4 solution did not induce appreciable effect on the intensity of protein synthesis in any of the tissues. However, repeated pretreatment of mice with ZnSO_4 solution favored to protection the intensity of protein synthesis in mouse liver, kidney, and heart from Cd-induced suppression (Fig. 3).

Sensitivities of protein synthesizing system components such as tRNA and aminoacyl-tRNA synthetases

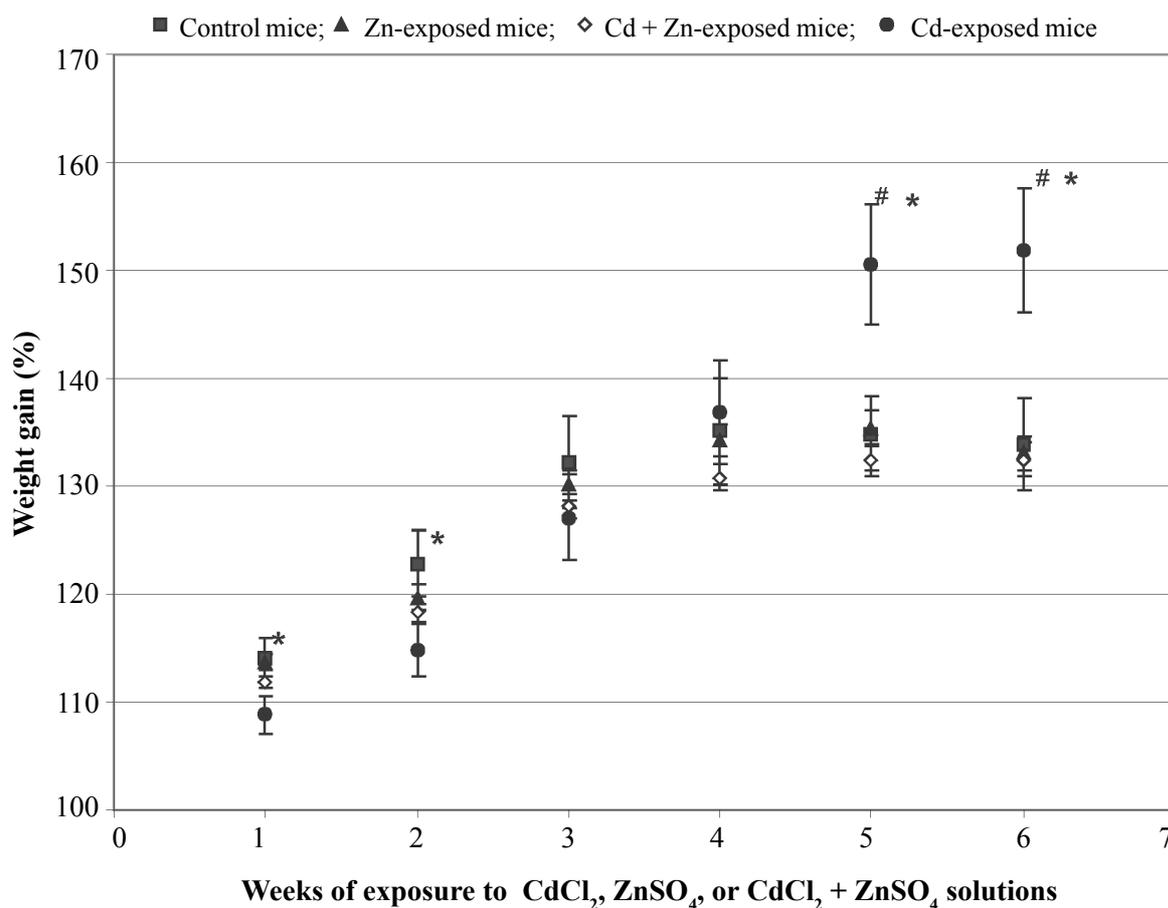


Fig 1. Time-course of body weight gain of the control group mice and the mice i.p. injected with CdCl_2 ($1.4 \mu\text{moles Cd per 1 kg of body weight}$) and / or ZnSO_4 ($4.8 \mu\text{moles Zn per 1 kg of body weight}$) for 6 weeks

The data are obtained by measuring the body weight of 45 mice in the control group, of 60 in Cd-treated mouse group, of 40 in Zn-treated mouse group, and of 45 in Zn+Cd-treated mouse group

* Differences are statistically significant in comparison to the control group mice.

Differences are statistically significant in comparison to the group of Cd-treated mice.

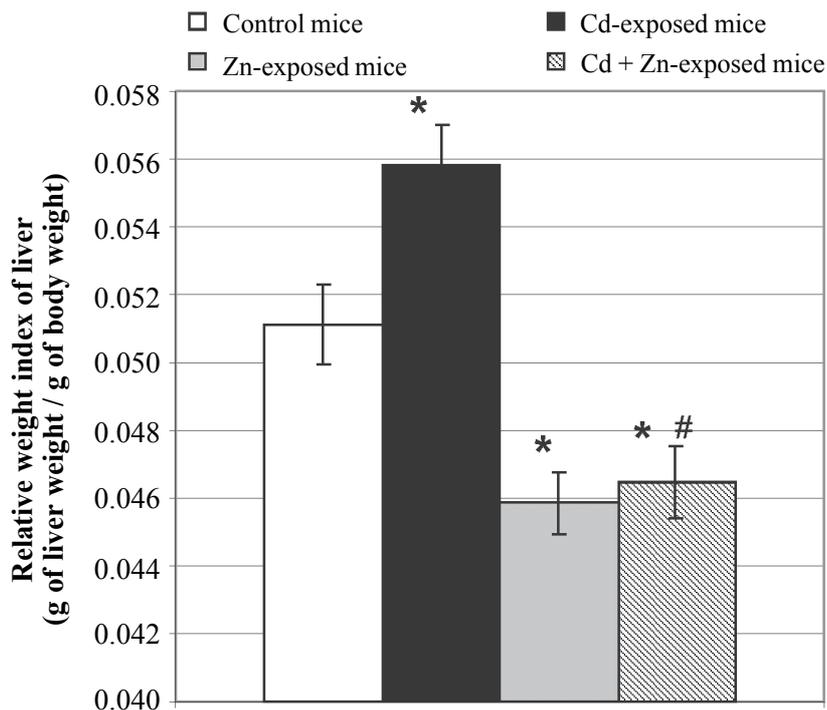


Fig. 2. Effect of repeated i.p. injections of CdCl₂ solution and / or ZnSO₄ solution on the relative weight index of mouse liver

Experimental conditions and statistics indicated in Fig. 1.

to Cd and Zn ions were examined in *ex vivo* studies. We compared acceptor activities of tRNA^{Leu} isolated from the control and the metal salts solutions-injected mouse livers. The observed effects are shown in Fig. 4. Subacute exposure of mouse liver to CdCl₂ resulted in 44% diminution of the acceptor activity of tRNA^{Leu}.

Under co-exposure of mice to Cd and Zn, the full restoration of the acceptor activity of tRNA^{Leu} was detected after the experimental period.

Next, we examined activity of leucyl-tRNA synthetase present in post-mitochondrial supernatant prepared from the control and experimental mouse

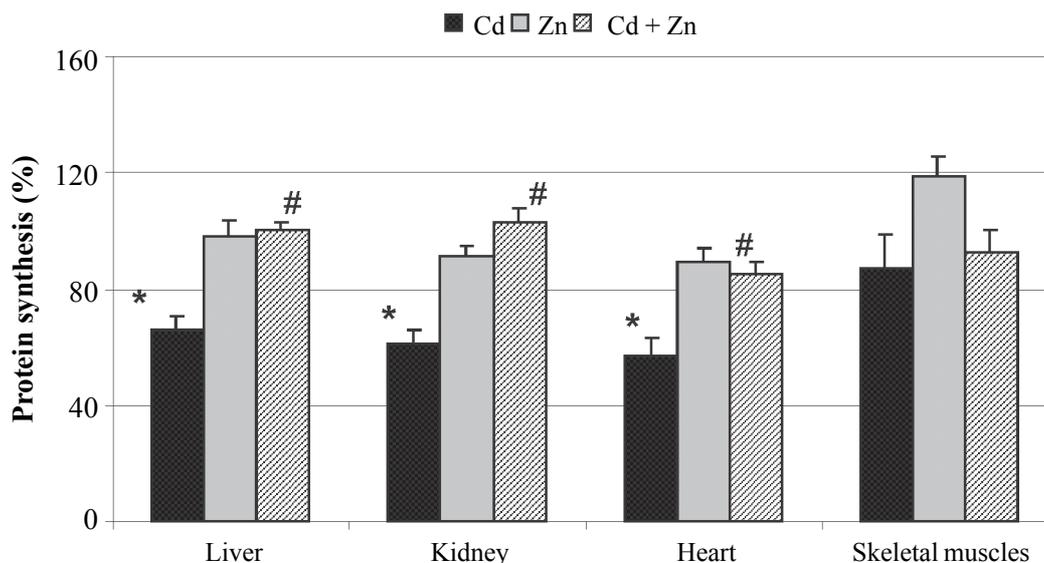


Fig. 3. Effects of repeated i.p. injections of CdCl₂ and / or ZnSO₄ for 6-week period on protein synthesis in mouse organs

In the organs of control group mice, protein synthesis was set at 100%. The data represent results of 8–21 separate experiments. Statistics are as in Fig. 1.

livers. The activity of leucyl-tRNA synthetase was increased by 59% of the control level following a 6-week exposure to Cd (Fig. 4). Exposure of mice to Zn for the same period did not cause appreciable activation leucyl-tRNA synthetase in supernatants from the liver. When injected prior to CdCl₂, ZnSO₄ solution caused partial return of leucyl-tRNA synthetase activity to the control level.

We also attempted to examine if there is a correlation between metal-induced changes of the components of translation *in vivo* and liver cell viability. Therefore, we assessed apoptotic cell number in formalin-fixed paraffin embedded sections from Cd-, Zn-, and Zn+Cd-exposed mouse liver. As Fig. 5 shows, the incidence of apoptosis was higher in the liver of Cd-exposed mice than in control. In solely Zn-exposed mouse liver, number of TUNEL-positive cells was similar to that one of the control group. However, pretreatment with ZnSO₄ did not reduce the number of TUNEL-positive cells in Cd-exposed mouse liver.

Discussion

The present study was conducted to evaluate the effects of common action of Cd and Zn on the translation system and cell viability in tissues of mice. General toxicological examination revealed two-week delay in mice body weight gain after repeated i.p. injections CdCl₂ solution (1.4 μmoles Cd per 1 kg of body weight) for six weeks: the body weight gain was by 7% lower than in the control and in other experimental groups. Three-weeks following Cd administration, body weight gain started to increase and exceeded the control by 13% at the fifth and sixth weeks. The data about effects of Cd on body weight gain are contradictory: from body weight gain retardation (18), no effect (19), to activation (20). Mechanisms of those discrepancies are still unclear. In the present study, Cd-induced activation of body weight gain correlated with 9% increase of the relative weight index of liver. Zn completely abolished these effects, thereby confirming antagonistic effect of these metals on living

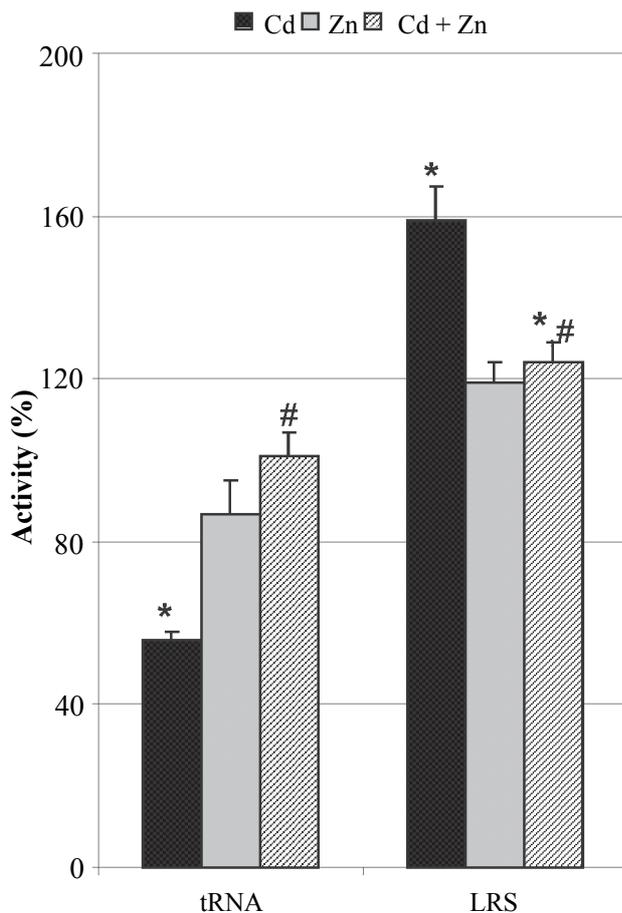


Fig. 4. Effects of repeated i.p. injections of CdCl₂ and / or ZnSO₄ for 6-week period on the activities of mouse liver tRNA^{Leu} and leucyl-tRNA synthetase
The data represent results of 9–21 separate experiments. Statistics are as in Fig. 1.

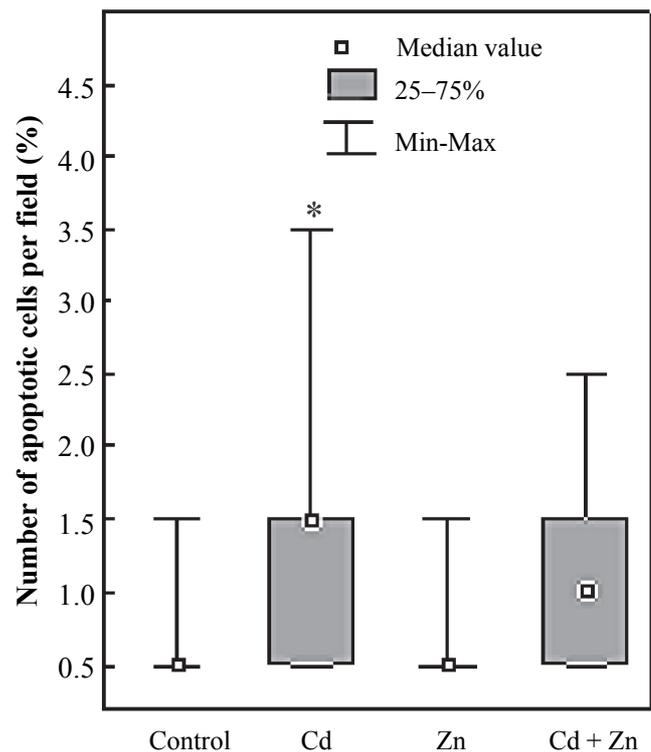


Fig. 5. Effects of repeated intraperitoneal injections of CdCl₂ and / or ZnSO₄ for 6 weeks period on the numbers of apoptotic cells in mouse liver specimens
The data represent results of three separate experiments. 25–75% – IQR, the interval encompasses numbers of cells in a half of all fields.
*Differences are statistically significant in comparison to the control group mice.

organisms. Several mechanisms can underlie protective effect of Zn. One of them is competition of Zn and Cd for transport into cells. As both Zn and Cd share the same transfer mechanisms across cellular membranes, *in vitro* they compete for uptake into hepatocytes (21). This effect was disproved *in vivo* studies (22). In experimental conditions identical to those of the present study, Zn-pretreatment resulted in accumulation of Cd in mouse liver at the level as without pretreatment (23). Therefore, one may assume that *in vivo* Zn protects liver against Cd toxicity not by decreasing its uptake. The effect of Zn is presumably related to activation of synthesis of stress proteins such as metallothioneins and hsp, which decrease toxic potential of Cd (1). Our data about effects of Cd and Zn on the intensity of synthesis of proteins (Fig. 3) support this proposition: subacute co-exposure of liver, kidney, and heart tissues to Cd and Zn results in recovery of protein synthesis intensity in respect to Cd-induced suppression. Recently reported proteomic study (24) showed up-regulation of stress and rescue proteins in yeast exposed to Cd. Limited cohesive information is available about effects of Cd on protein expression in cells of mammals. Therefore, examination of *in vivo* effects of Cd on the machinery of translation in the liver was conducted in the present study. We showed that exposure of mice to Cd for six weeks resulted in a decrease of the acceptor activity of tRNA^{L^{eu}} and in an increase of leucyl-tRNA synthetase activity in the liver. Interestingly, diminution of tRNA^{L^{eu}} acceptor activity correlated with inhibition of the total protein synthesis. Although, Cd makes adducts with N-bases of nucleic acids *in vitro* (2), this scenario is rather unlikely *in vivo*, where Cd can indirectly affect nucleic acids either by induction of their oxidation (25) or by inhibition of repair mechanisms (5). Cd-induced activation of leucyl-tRNA synthetase can probably compensate partial inactivation of tRNA^{L^{eu}}, and it allows maintaining the total synthesis of proteins at a sufficiently high level. The underlying mechanism of Cd-induced leucyl-tRNA synthetase activation is unknown. Our recent study showed, that both Cd and Cd+Zn in a concentration range of 5–10 μ M caused a significant diminution of leucyl-tRNA synthetase activity *in vitro* (26). In this relation, Zn-caused return of Cd-activated leucyl-tRNA synthetase to its control level activity detected in the present study is presumably a consequence of indirect effect of Zn ions. As Cd and Zn up-regulate synthesis of proteins (24), the observed effects can be explained by changes in contribution of leucyl-tRNA synthetase to large supramolecular complexes, where it exists together with other aminoacyl-tRNA synthetases and non-cata-

lytic proteins (27). This mechanism could benefit to adaptation of mammals against Cd-induced inhibition of these vitally important molecules.

Cd-binding proteins such as metallothioneins decrease Cd toxicity; therefore, accumulation of Cd in organs is not necessarily related to tissue damage (3). Cd-metallothionein complexes are toxicologically inert; metallothionein levels correlate with resistance of an organism to Cd (3). Although a pivotal role of metallothioneins is to protect tissue against Cd, their effect is limited. Under subacute exposure of mice to Cd, the primary pathological lesion is inflammation of the liver parenchyma (7). It is well documented that liver inflammation can progress into cell death such as apoptosis (28). Thus, Cd-induced death of hepatocytes can occur because of integrated mechanisms coming from different types of cells in liver tissue. Some of those mechanisms, e.g. production of inflammatory cytokines, depend on active synthesis of macromolecules, such as RNA and proteins. In the present study, we found that the number of apoptotic cells is higher in the liver of mice subjected to subacute exposure to Cd than in the liver of control mice. It is known that mechanisms of Cd-induced cell death depend on duration of intoxication. Under acute exposure of liver to Cd, induced hepatocytes are subjected to oxidative stress, which results in apoptosis and then necrosis (7). Unlike acute effects, chronic effects of Cd are related to release of inflammatory proapoptotic cytokines so that apoptosis is likely to be a predominant mode of damaged cell elimination.

Zn ions are integral parts of many enzymes and transcription factors controlling cell proliferation, differentiation, and cell death. In response to Zn, cell line-dependent inhibition of Cd-induced apoptosis was reported (29). In the present study, the number of apoptotic cells in liver from Zn-exposed mice was the same as in control (Fig. 5). Furthermore, Zn did not decrease the number of apoptotic cells in Cd-exposed liver. As it was reported recently, Zn did not also affect mitotic index in mouse liver exposed to Cd for 6 weeks (23). Both facts can be explained by restricted distribution of Zn in organs. Recently several proteins implicated in zinc homeostasis have been described: plasma membrane zinc transporter and exporter proteins, metallothioneins, and transporters that mediate zinc sequestration into intracellular vesicles. As it was mentioned before, mouse liver can accumulate almost the same amounts of Zn under subacute exposure to both CdCl₂ and ZnSO₄+CdCl₂ (23). It may be assumed that intracellular pool of Zn limits the significance of this metal in protection of liver tissue against Cd-induced apoptosis. This, however, does not

make any impact on experimental mouse survival, which was of the same level in all our experimental groups.

Conclusions

1. Zinc pretreatment reverses inhibition of both the

total protein synthesis and the acceptor activity of tRNA^{Leu} induced by subacute exposure of the liver to cadmium chloride.

2. Zinc pretreatment does not decrease the number of apoptotic cells in the liver under subacute subtoxic exposure to cadmium.

Kadmio ir cinko jonų poveikis baltymų sintezei ir ląstelių žūčiai pelės kepenyse

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Raktažodžiai: kadmio, cinkas, baltymų sintezė, apoptozė, pelės kepenys.

Santrauka. Darbo tikslas. Nustatyti šešių savaičių trukmės kadmio ir cinko jonų poveikį baltymų sintezei ir ląstelių žūčiai pelių kepenyse *in vivo*.

Tyrimo medžiaga ir metodai. Eksperimento metu beveislėms pelėms tris kartus per savaitę, šešias savaites iš eilės į pilvo ertmę buvo švirkščiamas kadmio chlorido (1,4 μmol kadmio/1 kg kūno masės) ir (arba) cinko sulfato (4,8 μmol cinko/1 kg kūno masės) tirpalo. Kontrolinės grupės pelėms pagal tą pačią schemą buvo švirkščiamas atitinkamas tūris fiziologinio tirpalo. Baltymų sintezės intensyvumas nustatytas pagal [¹⁴C]-leucino įsijungimą į peptidus ir baltymus. Suminės tRNR preparatai buvo išskirti iš pelės kepenų ląstelių ekstrakto pašalinus baltymus fenoliu. Supernatante, gautame atskyrus mitochondrijas iš kepenų homogenato, buvo tiriamas leucil-tRNR sintetazės aktyvumas. Šio fermento ir tRNR preparatų aktyvumas buvo įvertintas pagal [¹⁴C]-leucino atsiradimą acilinimo reakcijos produkte leucil-tRNR. Kepenų ląstelių apoptozei įvertinti buvo atliekama TUNEL imunohistocheminė reakcija, kuriai naudotas ląstelių žūties *in situ* nustatymo rinkinys (Roche).

Rezultatai. Nustatėme, kad preparatuose, gautuose iš kadmio chlorido tirpalu paveiktų pelių kepenų, inkstų ir širdžių, sumažėjo [¹⁴C]-leucino įjungimas į naujai sintezuotus peptidus ir baltymus. Šių pelių kepenų preparatuose sumažėjo ir tRNR^{Leu} aktyvumas, tačiau leucil-tRNR sintetazės aktyvumas padidėjo. TUNEL teigiamų ląstelių, turinčių fragmentuotą branduolį, skaičius buvo žymiai didesnis kadmio paveiktų pelių kepenyse nei kontrolinių. Cinko sulfato tirpalas, sušvirkštas prieš kadmio chloridą, apsaugojo pelių kepenų sintezės sistemą ir tRNR^{Leu} nuo slopinamojo kadmio jonų poveikio. Cinkas taip pat sumažino kadmio sukeltą leucil-tRNR sintetazės aktyvinimą, tačiau nepakeitė TUNEL teigiamų kepenų ląstelių skaičiaus.

Išvados. Cinko jonai apsaugo kepenų baltymų sintezės sistemą nuo slopinamojo kadmio jonų poveikio, bet nekeičia apoptozės būdu žūvančių ląstelių skaičiaus.

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