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Full Length Research Paper

Assessment of the effect of *Echinacea purpurea* extract on the accumulation of cadmium in liver and kidney: Apoptotic - mitotic activity of liver cells

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Cadmium (Cd) is an important industrial pollutant, even though its mechanism of toxicity has not been completely clarified. Although Cd^{2+} is toxic to a wide range of organs and tissues, liver and kidneys are the primary target organs of cadmium toxicity. Cd^{2+} induces apoptosis, mitotic activity of cells and also causes necrotic cell death in certain pathophysiological situations. *Echinacea purpurea* (L.) Moench stimulates the production of immunoglobulins and/or interferons by stimulating one or another link of the immune system. The aim of our study was to investigate the effects of the liquid extract of *Echinacea purpurea* on the accumulation of Cd^{2+} in liver, kidney and on the mitotic and apoptotic activity of liver cells after the chronic intraperitoneal intoxication by Cd^{2+}. The experiments were performed on white laboratory mice using intraperitoneal injections of 0.05 LD_{50} of cadmium chloride solution. Two groups of mice were injected with *Echinacea purpurea* liquid extract of different concentrations - 0.05 LD_{50} and 0.1 LD_{50}. Mitotic and apoptotic activity of liver cells was expressed as an estimated number of mitotic and apoptotic liver cells in randomly selected reference areas in a histological slide. Cd^{2+} concentration in mice liver and kidney was detected using atomic absorption spectroscopy. Long-term injections of *Echinacea purpurea* extract combined with $CdCl_2$ lead to a significant increase in cadmium concentration in the liver and kidney of experimental mice. *Echinacea purpurea* decreased the cadmium-induced mitotic activity of liver cells, and increased the apoptotic activity of these cells. Long-term exposure to Cd^{2+} results in the formation of the foci of necrosis in liver, which may be reduced by the application of *Echinacea purpurea* extract.

Key words: cadmium, *Echinacea purpurea*, mice, mitotic and apoptotic liver cells, atomic absorption spectroscopy.

INTRODUCTION

Heavy metals affect almost every organ of the body. One such metal is cadmium (Cd), which is of concern because of its increasing prevalence as an environmental contaminant (Jarup et al., 1998). Prolonged exposure to Cd results in injury to liver, lung, kidneys, and testes. The redistribution of cadmium to the kidney with time is due to the longer time period required for the increase of metallothionein levels in the kidney (Kotsonis and Klaassen, 1977; Manca et al., 1991). The toxicity of cadmium is influenced by a number of factors such as the route of administration, the dosage, the chemical form of the metal, the duration of exposure and the age of

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experimental animals. Cd interferes with hepatic protein synthesis early after injection of a large dose, and that further degenerative changes occur later and possibly in response to protein inhibition (Dudley et al.,1984). It is concluded that the young might be at a special risk at the same level of environmental cadmium exposure because of the high oral cadmium toxicity at this age which is most probably due to a high cadmium retention in the gut (Casalino et al., 1997). The specific activities of D-3-hydroxybutyrate dehydrogenase (BDH) and glutamate dehydrogenase (GDH) are reduced in the liver and kidney of rats intoxicated with cadmium. In the same animals a great stimulation of antioxidant enzymes glutathione reductase and glutathione peroxidase occure. It has been observed that cadmium promotes a time-dependent iron release from biological membranes. The prooxidative effect of cadmium is an indirect one since it is mediated by iron (Casalino et al., 1997). Liver injury is manifesting by extensive necrosis, hepatocyte apoptosis, peliosis hepatis accompanied by a large increase in the plasma levels of alanine aminotransferase and other enzymes (Dudley et al., 1982; Skilleter et al., 1985).

Cadmium-induced liver injury did not involve inflammation at any time point. Apoptosis seems to be a major mechanism for the removal of damaged cells, and constitutes the major type of cell death in nonparenchymal liver cells. Apoptosis of nonparenchymal cells is the basis of the pathogenesis of peliosis hepatis (Tzirogiannis et al., 2003). Cd induces apoptosis by mitochondrial pathway which leads to the release of cytochrome c to the cytoplasm and the activation of procaspase-9 to caspase-9 (Kondoh et al., 2002). *Echinacea purpurea* (EP) has many beneficial features, especially by strengthening the immune system (Abdullah, 2000; Grimm and Muller, 1999). In animal studies, EP affects several aspects of the immune system: the biologic active components of EP increase the number of circulating white blood cells (Bauer et al., 1988), enhance phagocytosis (Roesler et al., 1991), stimulate cytokine production, and trigger the alternate complement pathway (Luettig et al., 1989). *In vitro*, EP displays direct bacteriostatic and antiviral activity, stimulates the production of cytokines (interferon, tumor necrosis factor, interleukin 1, and interleukin 6 (Braunig et al., 1992; Murray, 1995). Based on its stimulation of cytokine production, EP is being investigated as a possible antineoplastic agent in preliminary human trials (World Health Organization, 2003).Our study investigated the effects of EP liquid extract on the accumulation of Cd^{2+} in liver and kidney, as well as on the mitotic and apoptotic activity of liver cells after the chronic intraperitoneal intoxication with Cd^{2+}.

MATERIALS AND METHODS

Experiments were carried out on 4-6-week-old outbred white laboratory mice weighing 20-25 g. *I. p.* injections of $CdCl_2$ and EP extract solutions were performed as follows. Experimental mice (10 mice in each group) were periodically injected *i.p.* for 6 weeks (three times a week) with metal salts and EP extract solutions of two different concentrations dissolved in de-ionized water:

1) 0.05 LD_{50} Cd^{2+} (corresponding to Cd^{2+} 0.16 mg/1 kg body mass);
2) 0.05 LD_{50} EP (corresponding to EP extract 0.125 mg/1 kg body mass);
3) 0.1 LD_{50} EP (corresponding to EP extract 0.25 mg/1 kg body mass);
4) 0.05 LD_{50} Cd^{2+} and 0.05 LD_{50} EP;
5) 0.05 LD_{50} Cd^{2+} and 0.1 LD_{50} EP.

Mice of the control group were periodically injected *i.p.* with the same volume of saline. Mice were weighed weekly and decapitated after 6 weeks according to the rules defined by the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (License No.0028).

Detection of metals in organs

The concentration of Cd in tissue specimens taken from liver and kidney was determined using an electro-thermal graphite furnace atomic absorption spectrophotometer Perkin-Elmer Zeeman/3030. Tissue specimens were resolved with 0.125 M NaOH upon 90°C, and the digests were diluted up to the appropriate volume with twice-distilled water. The modified analysis method (Schlemmer, 1989) for the detection of heavy metal concentration in biological samples was used.

Histology

Samples from liver tissue were fixed in 10% neutral buffered formalin for 48 hours and were then processed for routine paraffin embedding. Five-micron-thick sections were routinely stained with hematoxylin and eosin. Histological slides were examined by light microscopy for necrosis and mitosis of liver cells. For each specimen, the number of mitotic cells was counted in 10 randomly selected reference areas (original magnification - x40). Their histological images were taken using DP-11 Olympus Digital Camera. The number of the foci of necrosis (the group of necrotic liver cells surrounded by inflammatory cells) was estimated in 10 randomly selected reference areas at the original magnification of x20.

Detection of apoptosis

Apoptosis of liver cells was histochemically detected by the TUNEL assay using alkaline phosphatase (AP) *in situ* cell death detection kit (Roche). Sections of formalin-fixed and paraffin embedded liver tissue were dewaxed by washing in xylene and rehydrated through a gradual series of the application of ethanol and distilled water. Proteinase K-permeated sections were subjected to enzymatic *in situ* labeling of DNA strand breaks using the TUNEL-technique. The DNA strand breaks were revealed by adding AP-converter with subsequent staining with NTB/BCIP solution employed as a chromogenic substrate. Following counterstaining with eosin, sections were analyzed under a light microscope (original magnification - x20). The number of apoptotic cells was estimated by counting TUNEL + positive cells with morphological signs of apoptosis in 10 randomly selected areas.

Preparation of the extract from the herb of Echinacea purpurea

Maceration and percolation were used to prepare an extract from

Table 1. Geometric mean and 25th–75th percentiles of Cd2+ concentration in liver and kidney of mice from the control group, EP 0.05 LD_{50} (EP 0.05) and EP 0.1 LD_{50} (EP 0.1).

In organ	Mice groups					
	Control group		EP 0.05		EP 0.1	
	Cd concentration (μg/g)	25-75 percentiles	Cd concentration (μg/g)	25-75 percentiles	Cd concentration (μg/g)	25-75 percentiles
Liver	0.020	0.013 – 0.029	0.021	0.018 – 0.022	0.024	0.020 – 0.031
Kidney	0.206	0.141 – 0.323	0.078	0.072 – 0.085	0.065	0.059 – 0.066

dried herb of EP.

Harvesting of plant material

For optimum medicinal quality, EP plants were harvested in full bloom immediately after the dew was dried, but not in the heat of the day. Samples were harvested between 9-11 a.m. Great care must be taken to minimize the amount of soil that can splash up onto the leaves and stems (World Health Organization, 2003). Flowers, leaves and stems of EP were dried slowly in well-ventilated commercial dryers. Drying of the herb is easily accomplished; however, it should be noted that drying at a moderate temperature of 20 - 25 degrees Celsius works best (Schulthess et al., 1991). Dried herb (1 kg) of EP was ground into the particles sized 1 mm. The herb was evenly moistened with 1000 ml of 40% aqueous ethanol (menstruum) and then placed in a closed vessel for 24 hours. The moist herb was introduced into percolator. The percolator was placed in position, and a soak of 40% aqueous ethanol (1 liter) was poured on (to make 1:2 extract). Percolation was performed for 24 hours at the flow speed of extraction amounting to 0.2 ml/min. When the percolation was finished, the marc (the inert fibrous component) of EP was removed from the percolator, and was subsequently pressed. The expressed liquid was then mixed with the percolate. The 1:1 liquid extract was made using a concentration step. The resulting percolate was then filtered (British Herbal Medicine Association`s Scientific Committee, 2002). The extract (1 ml) included 0.089 g of dried herb of EP. During the experiment Cd^{2+} concentration in EP extract liquid was measured and was found to be 0.00362 μg Cd in 1 ml of the extract. Preparation of extract from roots of EP was made in the factory “Valentis” (Lithuania).

Statistical processing

The Student’s *t*-test with Bonferroni correction was applied for comparison of geometric means of Cd concentration. Nonparametric Kruskal-Wallis and Mann- Whitney tests (Kruskal and Mann, 1952) were applied for evaluation of difference among mitotic, apoptotic cells and focuses of necrosis in mice liver counts in different groups. Statistical significance was set at $p = 0.05$.

RESULTS

The concentration of Cd^{2+} in liver and kidney of mice was evaluated after periodical *i.p.* injections with $CdCl_2$ and two different EP concentrations for 6 weeks. The data on Cd^{2+} concentration in the liver and kidney of the control group and EP-exposed mice are presented in Table 1. There was no significant difference of Cd^{2+} concentration in the liver between the control and the EP group. The liver of mice injected with Cd+EP 0.05 LD_{50} and Cd+EP 0.1 LD_{50} contained, respectively, 1.78-fold and 2.11-fold higher Cd^{2+} concentration than it was estimated in mice injected only with $CdCl_2$ (Figure 1). Periodical injection of $CdCl_2$ together with EP during 6 weeks caused significant increase of Cd^{2+} concentration in kidney, as compared to Cd group. The metal concentration in kidney was 2.1-fold and 1.92-fold higher in Cd+EP (0.05) and Cd+EP(0.1) groups comparing to Cd group (Figure 2). Long-term intoxication with Cd resulted a significant increase in the mitotic activity of liver cells compared to the control group.

The administration of EP together with Cd decreased the number of mitotic cells, and the findings did not differ from those in the control (Figure 3A). Long-term intoxication with Cd induced apoptosis and necrosis in mice liver. It is of interest that the administration of high doses of EP (0.1 LD_{50}) induced apoptosis of liver cells as well. The maximal number of apoptotic liver cells was obtained after exposure to Cd and EP (0.1 LD_{50}) (Figure 3B). The number of apoptotic liver cells in mice injected with EP and $CdCl_2$ solutions was significantly higher ($p<0.05$) than it was in mice exposed only to Cd^{2+} or EP, or in control group. Long-term (6 weeks) intraperitoneal injection of $CdCl_2$ resulted the formation of focal necrosis in the hepatic tissue (Figure 4A, B) and the inflammatory reaction in the liver capsule (Figure 5A, B). The administration of EP solutions before intoxication by $CdCl_2$ significantly diminished the number of foci of liver necrosis (Figure 6).

DISCUSSION

Cadmium is an important industrial pollutant, although its mechanism of toxicity has not been completely clarified. The present study analysed the effects of EP in cases of long-term (6 weeks) intoxication induced by intraperitoneal injections of $CdCl_2$. Several parameters were chosen to investigate these effects: (1) measurement of cadmium concentration in liver specimens, (2) estimation of mitotic and apoptotic activity of liver cells; (3) evaluation of the formation of focal

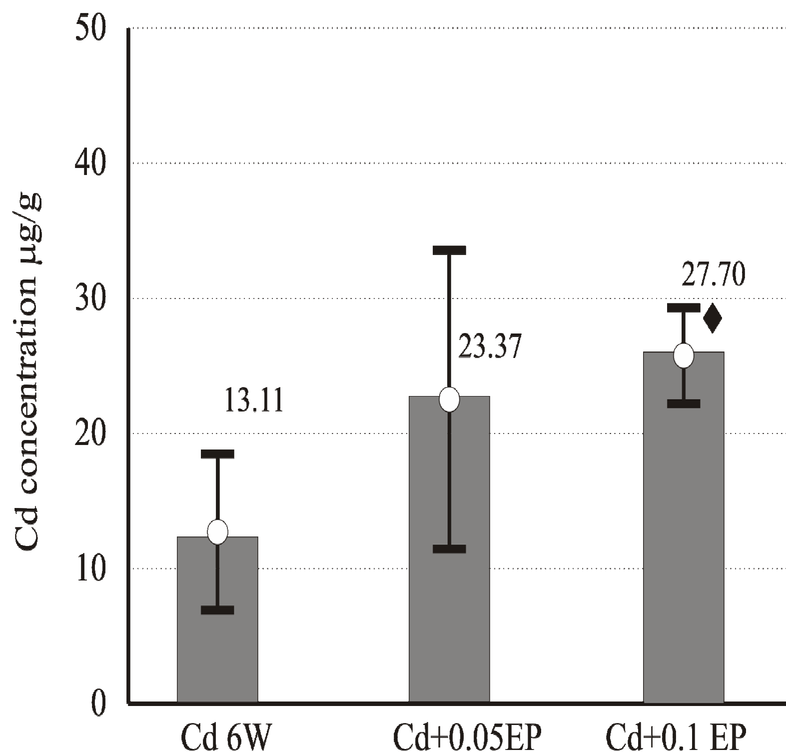

Figure 1. Cd^{2+} concentration in liver of mice after $CdCl_2$ (1.6 mg Cd^{2+}/1 kg body mass), after EP (0.125 mg EP extr./1 kg body mass); group Cd^{2+}+0.05 EP and after EP (0.25 mg EP extr./1 kg body mass); group Cd^{2+}+0.1 EP injections. ♦ - $p<0.05$ –compared to Cd^{2+} 6w group (administration of $CdCl_2$ for 6 weeks alone).

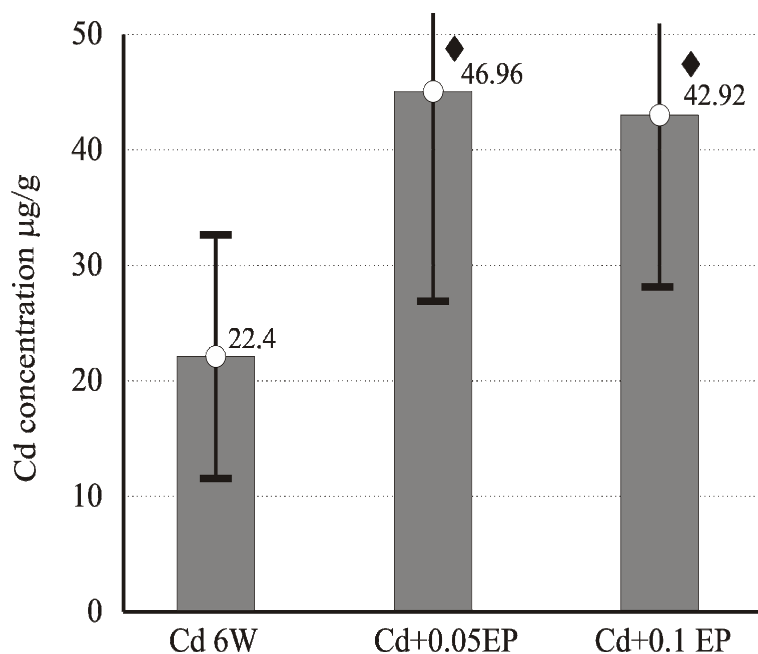

Figure 2. Cd^{2+} concentration in kidney of mice after Cd^{2+} (1.6 mg Cd^{2+}/1 kg body mass), and EP (0.125 EP extract/1 kg body mass) and EP (0.25 EP extract/1 kg body mass) injections. _- $P < 0.05$–compared to Cd^{2+} 6 w group (administration of CdCl2 for 6 weeks alone).

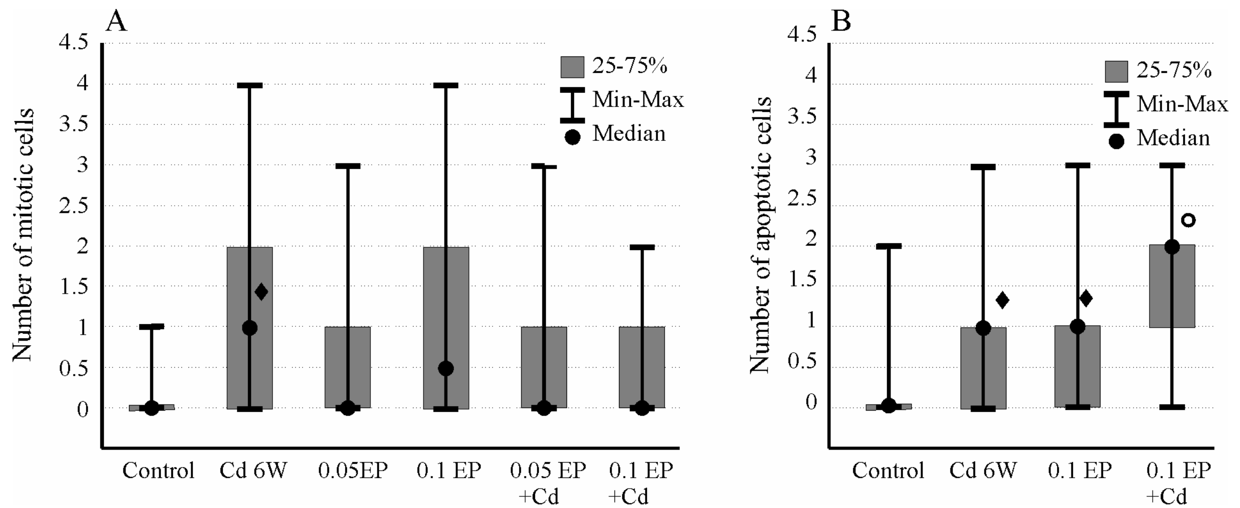

Figure 3. Number of mitotic (A) and apoptotic (B) of mice liver cells after $CdCl_2$ (1.6 mg Cd^{2+}/1 kg body mass), and EP (0.125mg EP extr./1 kg body mass) EP (0.25mg EP extr./1 kg body mass) injections. ♦ - $p<0.05$ – compared to control group. ○ - $p<0.05$ – compared to Cd 6w group (administration of $CdCl_2$ for 6 weeks alone).

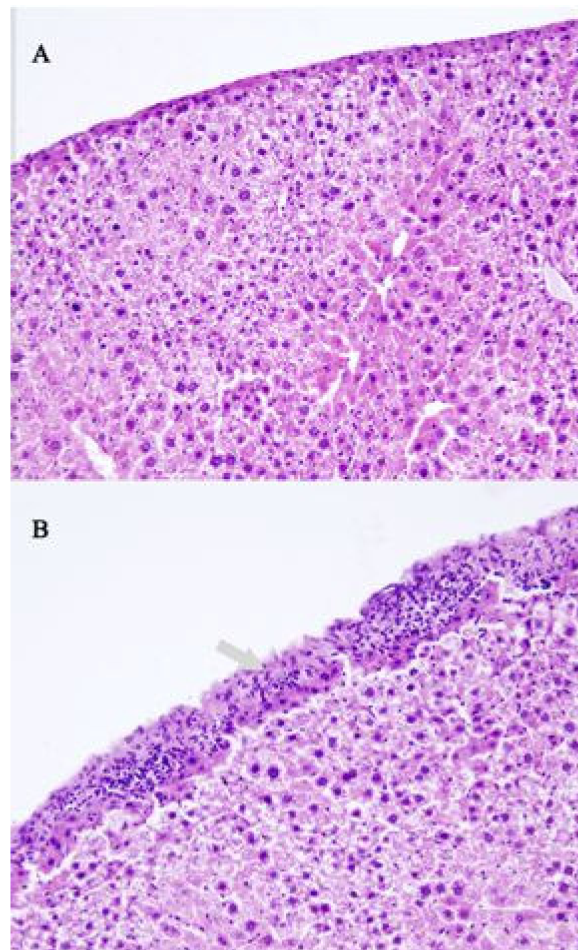

Figure 4. Mice liver from control group (A), and after 6 weeks $CdCl_2$ (1.6 mg Cd^{2+}/1 kg body mass) (B) multiinjections. Arrows indicates focal necrosis of liver. (Hematoxylin and eosin, original magnification X20).

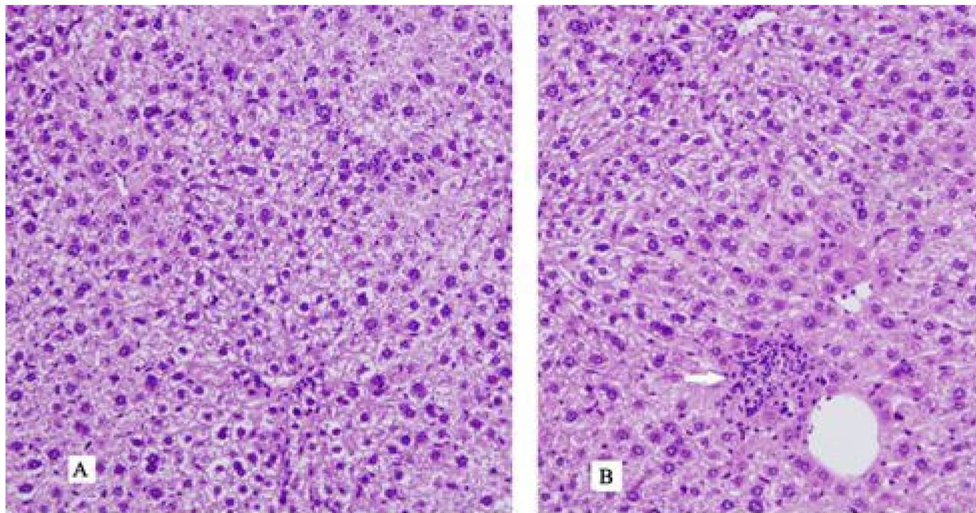

Figure 5. Mice liver from control group (A), and after 6 weeks $CdCl_2$ (1.6 mg Cd^{2+}/1 kg body mass) (B) multiinjections. Arrow indicates a thick liver capsule with inflammatory cells. (Hematoxylin and eosin, original magnification X 20).

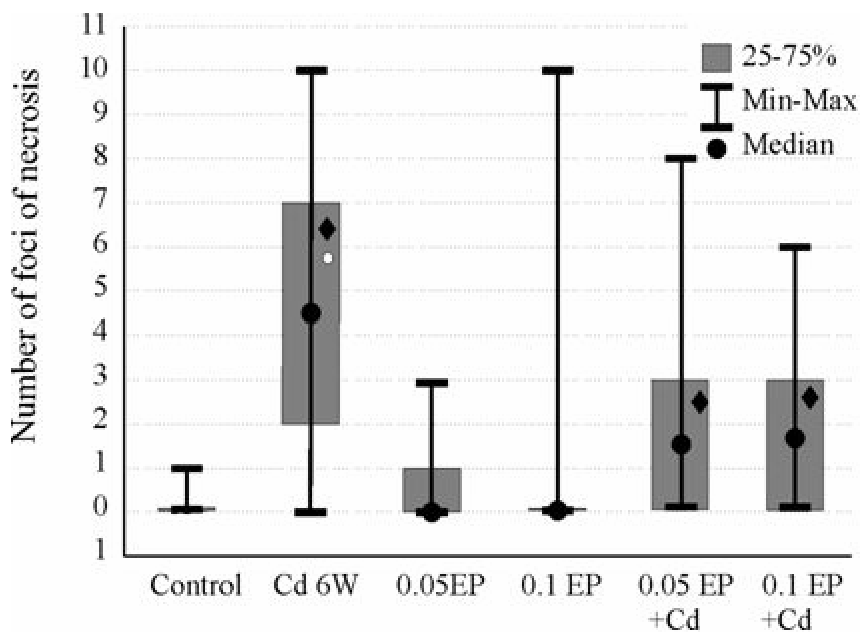

Figure 6. Number of focuses of necrosis in mice liver of control group, after $CdCl_2$ (1.6 mg Cd^{2+}/1 kg body mass), after EP (0.125 mg EP extr./1 kg body mass); group 0.05 EP and after EP (0.25 mg EP extr./1 kg body mass); group 0.1 EP injections. ♦ - $p<0.05$ – compared to control group. ○ - $p<0.05$ –compared to groups Cd^{2-}+0.05 EP and Cd^{2+}+0.1 EP.

necrosis in liver tissue. The results of the study showed that periodic intraperitoneal administration of $CdCl_2$ for 6 weeks in mice leads to an increased Cd^{2+} concentration in liver and in kidney as compared to control. It is well known that the clearance of cadmium from the circulation and its deposition into tissues is rapid. Over 50% of the body burden of cadmium is localized in liver and kidneys (Bernard and Lauwerys, 1986). The accumulation may be due to the ability to produce large amounts of metallothioneins. It is of interest that administration of EP significantly increased the concentration of Cd in liver tissue as compared to the administration of Cd alone.

One can be speculated that the administration of EP leads to the increased amounts of metallothioneins and chelators in liver.

Cadmium bound to metallothionein can leak into plasma, leave storage sites in the liver, and be taken up by the kidney. The cadmium-metallothionein complex is dissolved and free cadmium is released in the kidney and reabsorbed in the proximal tubules. These free cadmium ions can again be bound by newly synthesized metallothionein. If production of kidney metallothionein and non-metallothionein defense and detoxification systems (glutathione) are not sufficient, free cadmium can damage cellular membranes in the renal tubules. The presence of metallothioneins within cells markedly decreases cadmium toxicity (Klaassen et al., 1999). It is possible that the EP herb has some metal binding substances. Among these could be cichoric acid derivatives, polysaccharides (arabinogalactan), glycoproteins, flavonoids, as well as others. Such compounds that exhibit high chelating properties may also constitute an antidote to heavy metals *in vivo* (Clifford, 2000; Soczynska-Kordala et al., 2001). This hypothesis can be justified by the decreased toxic effects observed due to Cd during its administration with EP including the diminished number of Cd-induced focal necrosis. Long term intoxication by Cd^{2+} leaded to the significant increase of mitotic activity of liver cells as compared to the control group. Administration of EP together with Cd^{2+} decreased number of mitotic cells and it did not differ from the control. Our results showed that ES normalizes increased mitotic index of liver cells and protects liver protein synthesizing systems from cadmium toxicity and DNA mutations. According to our data, the number of apoptotic cells increased during the administration of Cd and EP.

It is indicated that Cd induces apoptosis via the mitochondrial pathway (Kondoh et al., 2002). In spite of this finding, the causes that switch on Cd-induced apoptosis remain obscure. It is suggested that oxidative stress is a most important factor involved in the toxicity of cadmium ions. This suggests that reactive oxygen species can provoke various outcomes, such as increased lipid peroxidation, DNA damage, membrane damage, altered gene expression, and apoptosis. EP appears to have strong anti-inflammatory activity, it is an antioxidant, has woundhealing actions, stimulates the immune system, and may be effective against some viral and bacterial infections (Bauer et al., 1990; Burger et al., 1997; Schulthess et al., 1991). EP increases the number of cytotoxic T cells and suppressor T cells. The mechanism likely involves an increase in IFN-γ levels. EP decreases IgG and IgM production by increasing the production of IFN-γ associated with secondary T lymphocyte stimulation by macrophages. It also activates macrophages and T cells and stimulates cytokine production (e.g., IFN-γ) (Mishima et al., 2004). Macrophages primed with IFN-γ elicites induction of cell apoptosis and complete suppression of mitosis (Duffield et al., 2000). The explanation for these phenomena will require a more detailed investigation to reveal which mechanisms are responsible.

Conclusion

Long-term injections of an extract of *Echinacea purpurea* combined with $CdCl_2$ leads to a significant increase of cadmium concentration in the liver and kidney of experimental mice as compared to the intoxication by cadmium ions only. *Echinacea purpurea* decreases cadmium-induced liver necrosis and mitotic activity of hepatic cells and increases the apoptotic activity of liver cells.

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