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Protective effect of zinc against cadmium toxicity on pregnant rats and their fetuses at morphological, physiological and molecular level

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Cadmium is a potent teratogen in laboratory animals, causing exencephaly when administered at early stages of development. Due to its heterogenicity with respect to molecular targets, the mechanisms behind cadmium toxicity are not well understood. In the present study, 40 pregnant rats (Sprague-Dawley) were divided into four groups (10 each); first group served as the control (G1), the second group (G2) received 61.3 mg/kg cadmium chloride daily from 7th to 16th day of gestation (organogenesis period) by oral tube. Group 3 (G3) was administrated a solution of 25 mg/kg zinc chloride orally from the 1st day to 20th day of pregnancy. Group 4 were administrated a solution of cadmium chloride (61.3 mg/kg) and zinc chloride (25 mg /kg) daily from the 7th to16th day of gestation. Maternal body weights were measured on gestational day 0, 6, 9, 12, 15 and 20. At the 20th day of gestation, blood samples were collected from the eye, using orbital sinus technique. Serum aspartate transaminase (AST) and alanine transaminase (ALT) were determined calorimetrically and serum, urea and creatinine were determined. All of the pregnant rats were sacrificed by ether anaesthesia at the 20th day of gestation and foetuses were removed from the uterus. The implantation sites, corpora lutea, living, dead and reabsorbed foetuses were counted and recorded. Liver of pregnant rats and their fetuses were used to isolate a total RNA for quantification of *Msx1*, *Cx43*, *Bcl2* and *Bax* genes. The results show the toxic effect of Cd on the pregnant rats and their fetuses, at morphological, physiological and molecular level but, zinc has a very effective protection against cadmium-induced developmental toxicity.

Key words: Cadmium, zinc, rat, organogenesis, gene expression.

INTRODUCTION

Industrial development has brought man into contact with several persistent chemicals, including heavy metals, such as lead, mercury and cadmium (Cd). Cd has been reported to produce several toxic effects in animals and man, while peculiar accumulation kinetics in the kidney cortex mammals has been reported by many authors. Absorption and accumulation of cadmium in tissues is determined by a wide range of factors, like nutritional and

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vitamin status, age and sex (Salvatori et al., 2004). Derived from natural and anthropogenic sources, widespread environmental exposure to arsenic (As) and cadmium (Cd) remains of public health concern due to their potential to cause adverse effects in the human population. In animal models, Cd is a well characterized teratogens inducing embryo-toxicity, including growth effects, mortality and a range of congenital malformations (Salvatori et al., 2004).

Cadmium (Cd) is particularly important as it is the 7th highest priority hazardous substance according to the agency for toxic substances and disease registry. Key sources of Cd in the environment include industrial production of pigments, plastic stabilizers, alloys, nickel-cadmium batteries as well improper discharge of many manufactured products (IARC, 1993). In the Earth's crust, Cd has an average concentration of about 0.1 to 0.2 mg kg^{-1} (Ursínyová and Hladíková, 2000; Lalor, 2008), contaminating the air, food and water, and so increasing the routes of exposure to animals such as ingestion and inhalation (IARC, 1993).

Exposure to this metal can occur in the workplace and in the natural environment because it is utilized in a number of industrial practices and is a contaminant of the environment and dietary products (World Health Organization, 1992). Cd toxicity in humans (Jarup et al., 1998) and experimental animals (Sharma et al., 1991; Brzóska and Moniuszko-Jakoniuk, 2001) has been widely studied and reported. Dietary Cd exposure of mice and rats during pregnancy results in anemia and reduced body weight of pups at birth (Webster, 1976) and distinct changes in trace metal metabolism in pups (Kuriwaki et al., 2005). Cadmium produces oxidative modifications of DNA, such as the formation of 8-hydroxydeoxy-guanosine, and the generation of strand breaks in different cell types, for example, liver and kidney cells (Forrester et al., 2000; Littlefield and Hass, 1995). Oxidative DNA damage produced by cadmium has been associated with an increased production of reactive oxygen species (ROS) (Ochi et al., 1987), and interactions between this metal and DNA repair enzymes (Assmus et al., 2000; Waalkes, 2000). In human lymphoma cells, cadmium has been shown to cause apoptosis by two independent pathways: the Ca^{2+}-cal-pain and the caspase-mitochondria pathways (Li et al., 2000), indicating that apoptosis could play an important role in acute and chronic toxicity from this metal.

It is well known that the metabolism and toxicity of Cd may be modified by many factors, including substances essential for life (Berglund et al., 1994; Brzóska and Moniuszko-Jakoniuk, 1998) as well as very toxic chemical compounds (Brus et al., 1995; Moniuszko-Jakoniuk et al., 2001). One of these substances is zinc. Microelements such as zinc (Zn) play an important role in metabolic pathways affected by Cd. Disturbances in metabolism of these metals in humans and experimental animals were observed after chronic Cd intoxication (Oishi et al., 2000).

Zinc supplementation prior to cadmium administration prevents several of the effects observed when cadmium is added alone (Dreosti, 2001). Thus, it has been shown that zinc inhibits the apoptotic protease caspase-3 (Truong-Tran et al., 2001), stabilizes the structure of p53 and DNA repair proteins (Chai et al., 1999), acts as an antioxidant by decreasing ROS production in cell cultures (Dally and Hartwig, 1997; Szuster-Ciesielska et al., 2000), and prevents the gross teratogenic effects of cadmium by restoring normal development (Warner et al., 1984). One approach to investigating complex developmental processes and coordinating them with genetic regulation has been to disrupt morphogenesis with specific teratogens and study their consequences at the molecular and morphological levels.

In the present paper, we studied the abnormal morphology in dam and foetus induced by cadmium, in addition, alteration in the expression levels of selected genes by using the Real-PCR technique. The genes under study (*MSX1*, *CX43*, *Bcl-2* and *Bax*) were selected according to their implication in organogenesis and in the apoptotic pathway.

MATERIALS AND METHODS

Adult male and female Sprague Dawley rats were used in this study, with weight of 150 to 200 g, obtained from the animal house of National Organization for drug control and research (NODCAR). Animals were kept under standard conditions and allowed free access to food and water.

The reproductive cycles of every rat which were kept in cages at room temperature, were followed for 15 days. Every female rat determined to be in oestrus or pro-oestrus phase of their cycles were mated with male rats in the same cage for one day and those which had sperm in their vaginal smears were considered to be zero day of pregnancies.

40 pregnant rats (Sprague-Dawley) were divided into four groups (10 each); the first group served as the control (G1), the second group (G2) received 61.3 mg/kg cadmium chloride (Cadmium chloride as monohydrate obtained from LOBA Chemise) dissolved in distilled water daily from 7th to 16th day of gestation (organogenesis period) by oral tube. Group 3 (G3) was administrated a solution of 25 mg/kg zinc chloride (zinc chloride purified obtained from LOBA Chemise) dissolved in distilled water orally from 1st day to 20th day of pregnancy. Group 4 were administrated a solution of cadmium chloride (61.3 mg/kg) and zinc chloride (25 mg /kg) daily from 7th to16th day of gestation.

All pregnant females were observed daily throughout gestation for mortality and general appearance. Maternal body weights were measured on gestational day 0, 6, 9, 12, 15 and 20. At the 20th day of gestation, blood samples were collected from the eye, using orbital sinus technique (Sanford, 1954). The blood was collected and allowed to clot, then serum was separated by centrifugation at 3000 rpm for 20 min and the clear non haemolysed serum was collected, divided into several aliquots and stored at -20°C until assayed. Serum aspartate transaminase (AST) and alanine transaminase (ALT) were determined calorimetric according to the method of Reitman and Frankel (1957). Serum, urea and creatinine were determined according to Bartel (1972). All of the pregnant rats were sacrificed by ether anaesthesia at the 20th day of gestation and foetuses were removed from the uterus. The implantation sites, corpora lutea, living, dead and reabsorbed foetuses were counted

Table 1. Oligonucleotide primers used for real-time RT-PCR analysis.

Gene	Primer	Product size (bp)
GAPDH	F: GGCTCTCTGCTCCTCCCTGTTCTA R: TGCCGTTGAACTTGCCGTGG	242
Msx1	F: GCCTGCACCCTACGCAAGCA R: AGCAGGCGGCAACATTGGCT	261
Cx43	F: TCCTTTGACTTCAGCCTCCAAGGAG R: GCAGACGTTTTCGCAGCCAGG	279
Bcl2	F: CTG GTG GAC AAC ATC GCT CTG R: GGT CTG CTG ACC TCA CTT GTG	228
Bax	F: TTCATC CAGGAT CGA GCA GA R: GCA AAG TAG AAG GCA ACG	263

and recorded. All living foetuses were weighed and evaluated for externally visible abnormalities. 50% of the foetuses were fixed in 96% ethanol and their soft tissues were removed in 1.0% KOH solution. After staining with Alcian blue- Alizarin Red-S combined technique, skeletal system was examined (Mcleod, 1980). Data obtained from the treated and the control groups were compared statistically by analysis of variance (ANOVA) test.

RNA isolation and real-time reverse transcription polymerase chain reaction

Total RNA was extracted from liver of mothers and their fetuses using analytic jena bio solution (innuPREP RNA Mini Kit. Germany). For reverse transcription (RT), first strand complementary DNA (cDNA) was synthesized from RNA by using a cDNA synthesis kit (RevertAidTm First Strand cDNA Synthesis Kit, Fermentas,) according to the manufacturer's instructions. After RT at 42°C for 60 min, polymerase chain reaction (PCR) was performed using a Jena Bioscience PCR-101 Taq Master Mix (Jena bioscience, Germany) according to the manufacturer's protocol. The specific primer pairs used in this study are listed in Table 1. Serially, diluted cDNA samples were used as standards. After an initial denaturation step of 5 min at 95°C, 35 cycles of amplification for Msx1 and Cx43, Bcl2 and Bax primer pair, were carried out. Each cycle included a denaturation step, 30 s at 95°C; an annealing step, 30 s at 56°C; and an elongation step, 30 s at 72°C. Final elongation temperature was 72°C for 5 min. Relative levels of gene expression were measured by QuantiTect SYBR Green PCR kit (Qiagen, Clinilab, Egypt) according to the manufacturer's instructions using Mx3000 instrument (Stratagene). The expression levels of Msx1, Cx43, Bcl2 and Bax genes were normalized to the level of *GAPDH* gene expression in each sample.

RESULTS

Morphological parameters

Among the experimental groups, food intake decreased in group treated with cadmium (G2) than that of the other groups. The average maternal body weight showed a steady increase during the gestation period, while the rate of increase during the gestation period was found to be relatively less in groups G2 and G4 compared to control and G3 group (Table 2). No abortion was recorded among mothers of the control and G3 groups. However, the rate of abortion in G2 was high than that of G4. The effect of cadmium on the pregnant rats was indicated by reduction in the uterine weight of pregnant rats and increase in the resorption rates of fetuses (Table 2, Figures 1a, b and c). The uterine weight of G2 was very low compared to that of G4. Cd resulted in abortion and resorption. By co-administration of zinc, the toxicity of Cd in pregnant rats decreased. Body weight of pregnant rats was reduced by Cd while relative weight of liver and kidney was increased (Table 5).

In the present study, Cd reduced growth parameters of the offspring (Table 3) and increased percentage of fetal malformation (paralysis of forelimbs and external hematomas) by co-administration of zinc; no significant decrease in growth parameters were observed meanwhile percentage of malformation decreased in fetuses

Skeletal examination

Fetal skeletal abnormalities were most obvious during embryogenesis period (7th to 14th day of gestation) (Figures 2a, b, c and d; Figures 3a and b).

The skeletal defects observed in fetuses included incomplete ossification of skull bones, sternum, ribs, vertebrae, forelimbs bones, pelvic girdle and hind limbs bones (Figure 2c).

The major skeletal defects were observed mainly in pelvic girdle and hind limbs bones in the form of shortness and partial ossification of ilium ischium, pubis, femur, tibia and fibula (Figure 3b). Shortness of the 13th

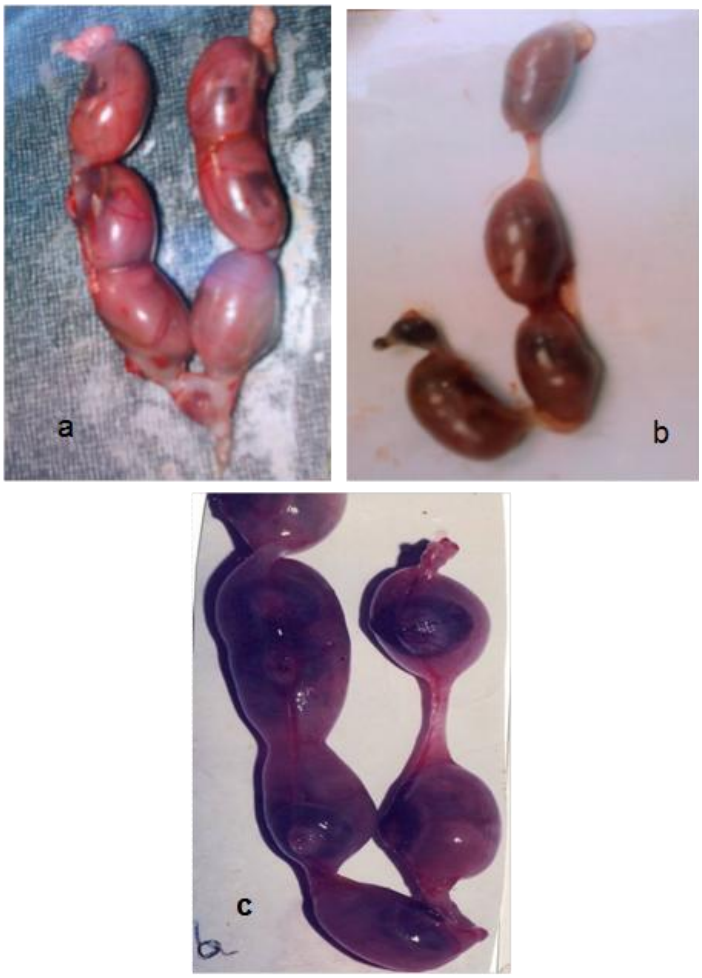


Figure 1. Uterus of control pregnant rat (a), uterus of pregnant rat treated with Cd (b) and uterus of pregnant rat treated with Cd and Zinc (c).

rib was observed in some fetuses (Figure 2c). Incomplete ossifications of vertebral column were observed mainly in sacral and caudal vertebrae (Figure 3b). Forelimbs bones were less affected than hind limbs bones. Abnormalities detected in forelimbs bones were in the form of partial ossifications of hummers, ulna, radius and missed ossifications of metacarpals and phalanges. However, the group treated with Cd and zinc showed that zinc improved the defects of skeletal system.

Liver and kidney function

A significant increase in blood urea and serum creatinine was found in pregnant rats administrated cadmium (G2). By co-administration of zinc, a significant decrease was observed (Table 4). The same trend was observed in the liver enzymes in cadmium treated group (G2) which was slightly decreased by co-administration of zinc (G4) (Table 4).

Quantitative real-time PCR confirmation of selected transcripts

Four genes (*Msx1*, *Cx43*, *Bcl2* and *Bax*) were quantified in independent samples from the four groups (G1, G2, G3 and G4) by quantitative real-time PCR (Figures 4 and 5).

Regarding the expression level in the cadmium treated pregnant rats (Figure 4), the lowest relative expression of Cx43 (0.03) was found in group G2, followed by Bcl2 and Msx1 (0.07, 0.23) respectively. On the opposite direction, Bax recorded the highest level (0.23) in this group compared to the control (G1). The effect of zinc (G3) was very clear in our experiment, where it resulted in a high expression for Msx1, Cx43 and Bcl2 and reduced the expression for Bax to minimum level compared to control group. The protective effect of zinc against cadmium (G4) was greater in Cx43 (3.6 fold), followed by Msx1 (2 fold) and Bcl2 (1.8 fold) and finally Bax (0.65 fold) compared to cadmium group (G2).

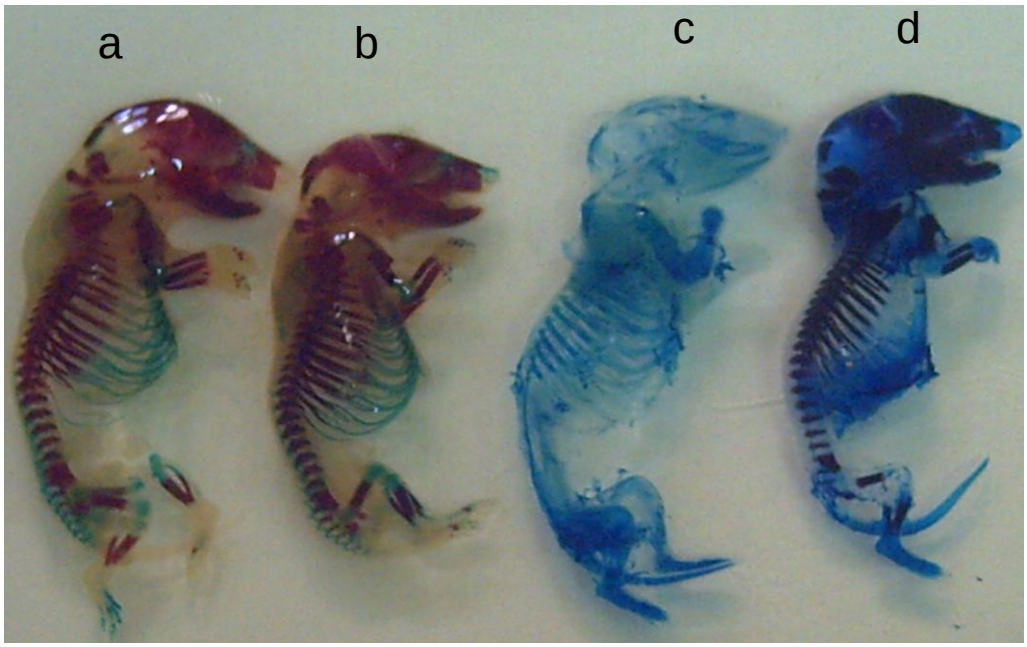


Figure 2. Skeletal system of fetus at the 20th day of gestation. **a.** Control. **b.** Maternally treated with zinc during organogenesis period. **c,** Maternally treated with Cd. **d.** Maternally treated with Cd and zinc.

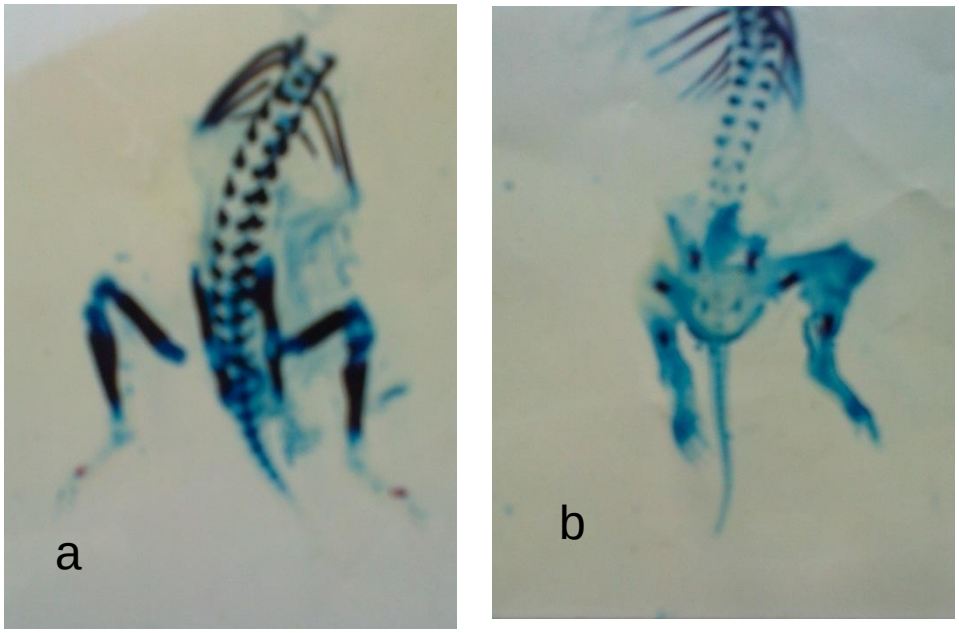


Figure 3. Hind limb and vertebral column of control fetus (a) and of fetus maternally treated with Cd (b).

In the foetuses samples (Figure 5), cadmium has resulted in the same trend as found in mothers, where the lowest relative expression of Cx43 (0.08) was found in group G2, followed by Bcl2 and Msx1 (0.16 and 0.42), respectively. On the opposite direction, Bax recorded the highest level (0.70) in this group compared to the control (G1). Regarding the effect of zinc (G3), it has shown also the same trend as a high expression for Msx1, Cx43 and Bcl2 was observed and reduction in the expression for Bax to minimum level was observed compared to control group. The protective effect of zinc against cadmium (G4) showed a slightly different trend compared to mothers,

Table 2. The effects of Cadmium and /or zinc on the pregnant rats.

Group	No. of pregnant	No. of aborted (%)	No. of sacrificed (%)	Average wt. of pregnant rats			Uteri			
				At 1st day	At 20th day	Average increase (%)	Total No. of uteri	Without resorption (%)	Partially resorbed (%)	Average wt. of uteri ±SE
Control G1	10	-	10 (100)	150.2	208	45.8±0.59 (30.5)	10	10 (100)	-	35.53±2.84
Cadmium G2	10	2 (20)	8 (80)	149.8	179.7	29.9±0.43 (20)	8	4 (50)	4 (50)	20±2.81
Zinc G3	10	-	10 (100)	139.8	189.7	49.9±0.42 (35.7)	10	9 (90)	1 (10)	39.5±1.53
Zinc+ cadmium G4	10	1 (10)	9 (90)	169.5	201.3	31.8±0.23 (18.76)	9	6 (66.67)	3 (33.33)	30.04±1.09

Table 3. The effects of zinc and /or cadmium on the fetuses.

Group	Number of sacrified	Number of implantation/mother	% of living fetuses	% of resorbed fetuses	Average fetal body weight (g)	Average fetal length (mm)	% of malformed fetuses	% of hematomas
Control	10	74 (7.4)	74 (100)	-	3.97±0.012	4.1±0.002	-	-
Zinc	10	77 (7.7)	75 (97.4)	2 (2.6)	3.89±0.003	3.95± 0.002	9 (12)	6
Cadmium	8	53 (6.625)	40 (75.47)	13 (24.53)	3.01±0.007	3.23±0.004	21 (52)	24
Cadmium + Zinc	9	62 (6.88)	56 (90.32)	6 (9.68)	3.51±0.02	3.53±0.01	11 (19.64)	13

where the expression was greater in Cx43 (2.5 fold), followed by Bcl2 (2.25 fold) and Bax (0.51 fold) and finally Msx1 (1.6 fold) compared to cadmium group (G2).

We can conclude that, the effect of cadmium and the protective effect of zinc at molecular level are transferred from treated mothers to their foetuses and follow the same trend.

DISCUSSION

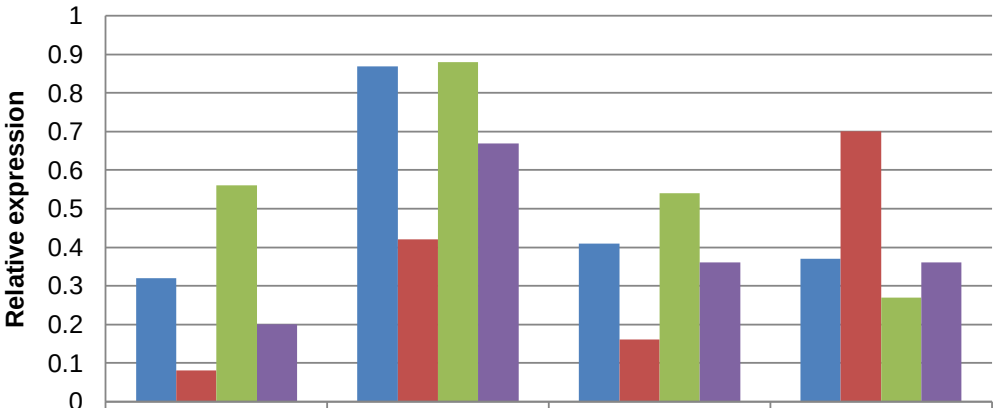
Cd induced nephrotoxicity in pregnant rats which was reduced by co-administration of zinc. In rabbits, Cd treatment resulted in increase of blood urea. Simultaneous administration of zinc prevented Cd induced uremia (Stowe et al., 1972). In uterus, exposure of cadmium leads to toxic renal effects in adult offspring (Jacquillet et al., 2007). Chronic Cd exposure can cause renal proximal tubular dysfunction resulting from the release of Cd metallothionein from the liver and its accumulation and degradation in the renal tubular epithetlial cells. Pretreatment with zinc can protect against acute Cd nephrotoxicity (Tang et al., 1998). Messaoudi et al. (2009) demonstrated the beneficial effects of selenium and zinc combination in the treatment of Cd nephrotoxicity. In Wistar rats, cadmium reaches the placenta or embryo at an organogenetically sensitive time (day 9 of gestation), and zinc may protect the embryo by decreasing the exposure to cadmium this time (García and González, 2010). Cadmium administrated to pregnant mice increased primary DNA damage and activated the apoptotic pathway. These effects could be ameliorated by zinc pretreatment so the mechanism of cadmium teratogenicity could be related to zinc metabolism (Fernández et al., 2003).

Salvatori et al. (2004) showed that Cd treatment during organogenesis was not able to induce maternal toxicity; induced external malformations; increased significantly fetus anomalies and malformations, with reduced metacarpus ossification and cleft palate. Haldsrud and Krokje (2009) found that exposure to high concentration of combination of Copper and cadmium produced a significant increase in the occurrence of DNA strand break and addition of low zinc to the mixture of Cadmium and copper restored DNA damage level back to that of control.

The effects of Cd on prenatal hepatocytes was studied by Bruscalupi et al. (2009) which found that fetal hepatocyte are less sensitive to Cd toxicity and the adverse effects of the metal are always better counteracted by fetal cells. Abrahim et al. (2010) added more proof that Cd exposure has a genotoxic effect and early detection of Cd

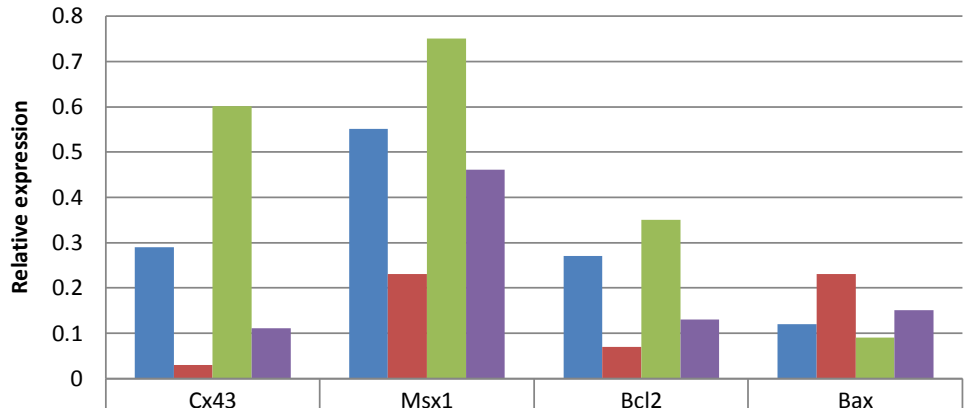
Table 4. Effects of zinc and /or cadmium on liver and kidney functions of pregnant rats (mean ±SE).

Group	Liver	Kidney	Spleen
Control	3.64 ± 0.065	0.621 ± 0.012	0.426 ± 0.076
Cadmium	4.95 ± 0.061	0.701 ± 0.021	0.367 ± 0.043
Zinc	3.73 ± 0.041	0.602 ± 0.02	0.432 ± 0.034
Cadmium+ zinc	3.95 ± 0.053	0.671 ± 0.031	0.0421 ± 0.012



	Cx43	Msx1	Bcl2	Bax
G1	0.32	0.87	0.41	0.37
G2	0.08	0.42	0.16	0.7
G3	0.56	0.88	0.54	0.27
G4	0.2	0.67	0.36	0.36

Figure 4. Expression level of selected genes in pregnant rats at different groups; control (G1), zinc treated (G2), cadmium treated (G3) and both cadmium and zinc treated (G4).



	Cx43	Msx1	Bcl2	Bax
G1	0.29	0.55	0.27	0.12
G2	0.03	0.23	0.07	0.23
G3	0.6	0.75	0.35	0.09
G4	0.11	0.46	0.13	0.15

Figure 5. Expression level of selected genes in rat fetuses of treated mothers at different groups; control (G1), zinc treated (G2), cadmium treated (G3) and both cadmium and zinc treated (G4).

induced mutagenecity is required.

Hepatic dysfunction has been observed after cadmium poisoning and liver function tests may be effective for diagnosis of cadmium effects. Increased activities of serum GOT and serum GPT have been found, and this in in accordance with the results of Nomiyama (1980) and Weigel et al. (1984) who studied the effects of 7.15 ppm dietary cadmium in male Wister rats for 40 and 60 days and found that that the activities of serum GOT and GPT were increased indicating disturbed hepatic functions. On the same side, Jihan et al. (2009) concluded that selenium and zinc protect the liver against oxidative stress induced by Cd.

Cd studies (GD8.0, Cd 4mg/kg) suggest that peak cellular morphological changes (increased pyknotic nuclei) that occur 10 to 12 h correlated with subsequent alterations in neural tube development (Webster and Messerle, 1980). Under these conditions, As and Cd exposures can affect DNA-damage, cell cycle arrest, oxidative stress and cell death pathways (Pulido and Parrish, 2003), yet the means by which these metals interfere with signaling pathways and subsequent morphogenesis remains unresolved. Investigations suggest additional effects such as glucose impairment and disruption in ion (Zn) or folic acid regulation also may be potential mechanisms of metal-induced embryo toxicity (Fernandez et al., 2007; Hill et al., 2009; Robinson et al., 2010).

Our data demonstrates the importance of Cx43 for limb development, as confirmed before using antisense oligonucleotides inhibition of Cx43 expression in the chick embryo which resulted in limb malformations, including truncation of the limb bud, fragmentation into two or more domains, or complete splitting of the limb bud into two or three branches (Becker et al., 1999; Green et al., 1994). These data implicate that Cx43 plays a very important role in osteogenic function and stated that, the genetic deletion of Cx43 resulted in skeletal ossification abnormalities. Therefore, the lack of Cx43 causes a generalized osteoblast dysfunction and this is in accordance with what was reported by Lecanda et al. (2000). Other role was found also for Msx1 as an important gene for embryo development as reported by Tesfaye et al. (2010) and El-Sayed et al. (2011). In addition, Msx genes may modulate the regulation of type I collagen possibly affecting the formation of extracellular matrix (ECM) development (Dodig et al., 1996; Alappat et al., 2003). Moreover, mice with homozygous mutations in both Msx1 and Msx2 die in late gestation with severe craniofacial malformations, including exencephaly, cleft palate, agenesis of teeth, and unossified calvarial bones (Bei and Maas, 1998; Satokata et al., 1994). Our results concerning expression level of both Cx43 and Msx1 are in accordance with what was stated before about the importance of these genes in organogenesis and embryo development.

Bcl-2 and Bax produce mitochondrial-related proteins with antagonistic effects; the former having an anti-apoptotic activity and the latter a pro-apoptotic activity. Bcl-2 was significantly down regulated, more than three fold, as opposed to Bax, the expression levels of which were significantly up regulated in G2 compared to the control (G1). Interactions between Bcl-2 and Bax regulate cytochrome c release from mitochondria and establish baseline sensitivity to apoptotic stimuli. In this study we observed a significant downregulation of Bcl-2 and a concurrent upregulation of Bax at the transcription level. It is thus likely that a decreased Bcl-2/Bax ratio promotes apoptosis signaling activation (Oltvai et al., 1993).

The injection of zinc before cadmium treatment can keep the expression levels of the genes under study at basal levels. Zinc supplementation thus maintained the expression Bcl-2/Bax ratio (Fernández et al., 2003).

In general toxicological studies, four common predominant mechanisms have been proposed regarding metal toxicity: 1) alterations in cell proliferation pathways; 2) inhibition of major DNA repair systems; 3) interference with cellular redox regulation/generation of oxidative stress, and 4) induction of apoptosis or cell death (Pulido and Parrish, 2003).

Conclusion

From the results obtained in this study, it appears that Cd has toxic effects on pregnant rats by reduction of body weight, increased percentage of abortion and resorption and produced elevation of both liver and kidney functions. As regards to its toxic effects on the fetuses, Cd caused fetal growth retardation and congenital abnormalities. Moreover, from our findings, it could be suggested that, the supplementation with zinc during gestation in conditions of exposure of Cd, is an effective therapy which could reduce Cd induced toxicity.

In addition, we have clearly shown that, cadmium can be a potent inducer of primary DNA damage in embryonic cells, and that it can activate several transcription factors implicated in the apoptotic pathway, (Bcl-2, Bax) and organogenesis and embryo development (Cx43, Msx1). We have also shown that, treatment with zinc could ameliorate the effects induced by cadmium, supporting previous data implicating that the mechanisms of cadmium teratogenicity are in some way related to zinc homeostasis. However, future studies may be able to answer many such unanswered questions in the mechanism involved in the beneficial effect of Zinc against Cd deleterious effects.

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