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Cadmium-induced teratogenicity in lizard embryos: Correlation with metallothionein gene expression

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ABSTRACT

Cadmium teratogenic effects and metallothionein expression were studied in tissues of lizard embryos at different stages of development. Incubation of eggs in cadmium contaminated soil had no effect on embryo survival, but strongly affected cranial morphogenesis. Cytological analyses demonstrated abnormalities in the development of proencephalic vesicles, mesencephalon and eyes. No defects were observed in somite or limb development. Northern blot analysis demonstrated that MT expression was much stronger in embryos developed in cadmium contaminated soil. In situ hybridization showed an early induction of MT gene expression in developing liver and gut, whereas in brain and eyes the spatial and temporal localization of MT transcripts did not change. A possible correlation between inability to induce MT expression and abnormalities observed in the head region of lizard developing embryos is suggested.

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1. Introduction

During evolution, eukaryotes have developed defence mechanisms against damage caused by adverse environmental stimuli. One cellular mechanism for this involves the induction of detoxifying proteins in response to toxic element exposure. Metallothioneins (MTs), low-molecular weight, cysteine-rich, metal-binding proteins which occur in all eukaryotes investigated (Coyle et al., 2002) comprise one group of such proteins. In addition to their role in immune response and as free radical scavengers (Kumari et al., 1998; Atif et al., 2008; Waeytens et al., 2009) MTs regulate metal homeostasis and detoxify by binding essential and non-essential metal ions (Cobbett and Goldsbrough, 2002; Egli et al., 2006; Bell and Vallee, 2009). MT gene expression is promptly up-regulated by exposure to heavy metals (Carginale et al., 1998; Trinchella et al., 2006; Simoniello et al., 2010a), and the specific MT gene isoforms affected are dependent on metal species, dose, tissue and organism (Carginale et al., 1998; Scudiero et al., 2000, 2005). Induction of MTs provides a mechanism by which the metal can be sequestered in a relatively inert, non-toxic state (Klaassen et al., 2009).

Several studies indicate that MTs also protect embryos from intracellular damage caused by cadmium. In mammals, MTs favour the maintenance of placental integrity, protect against hepatic poisoning and protect trophoblast cells from morphological alteration and apoptosis (Masters et al., 1994; Lee et al., 1996; McAleer and Tuan, 2001). Confirmation of the protective role of MTs comes from the demonstration that transgenic mouse blastocysts overexpressing MT genes are more resistant to cadmium-induced morphological changes than control blastocysts (McAleer and Tuan, 2001).

The intake of cadmium in vertebrates occurs mainly by ingestion of contaminated food and water. Generally, embryonic exposure and uptake varies greatly and is species dependent. In mammals, placental transport during embryogenesis has been demonstrated (Sonawane et al., 1975; Lagerkvist et al., 1992) although the placenta itself, which sequesters most ions, is a primary target for this metal (Bush et al., 2000; Rudge et al., 2009). Reptile and bird embryos have been considered well protected from the external environment and the presence of environmental contaminants in eggs or developing embryos has been attributed to maternal transfer during vitellogenesis and oviductal egg retention (Guirlet et al., 2008). More recently though, it has been demonstrated that metal ions and organic contaminants present in soil may cross the parchment-like shell of reptilian eggs (Marco et al., 2004a,b, 2005; Gómara et al., 2007) and that cadmium ions can interfere in the regulation of gene expression in lizard embryos (Trinchella et al., 2010). However, there is yet no information available on effects of cadmium on organogenesis or on a possible cytoprotective role of MTs during reptilian embryo development. Recent data (Simoniello et al., 2010b) show that MT transcripts are detectable from the early stages of development in lizard embryos. In particular, they are abundant in the neural tube, retina, mesenchyme and in mesodermal derivates. There is lesser abundance in kidney tubules and in endodermal derivatives where they can be detected only immediately before hatching.

In the present study we have examined cadmium cytotoxicity during embryo development in the Italian wall lizard *Podarcis sicula*

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and establish a correlation between cadmium effects and changes in MT gene expression.

2. Materials and methods

2.1. Animal collection and housing

Gravid *P. sicula* females (20 specimens) were captured between May and June from rural sites outside of Naples. Females were kept in a terrarium maintained under conditions of natural temperature and photoperiod in accordance with our institutional guidelines for care and use of laboratory animals. The lizards were fed live mealworms three times a week and water was provided *ad libitum*. All efforts were made to avoid animal stress and to minimize the numbers used. The experiments were carried out in compliance with ethical provisions established by the European Union and authorized by the National Committee of the Italian Ministry of Health for *in vivo* experimentation (Dept. for Veterinary Public Health, Nutrition and Food Safety).

2.2. Egg collection and incubation

Daily check of the terrarium provided freshly laid eggs that were randomly allotted in two groups. Group 1 was transferred to uncontaminated soil (control) whereas group 2 was transferred to soil contaminated with $CdCl_2$ to a final concentration of 50 mg Cd/kg soil. This concentration is above the guideline values for residential soil (http://www.epa.org.nz/contaminants/cadmium-soil.html), but it is regularly found in polluted areas and in soils used in intensive agriculture (Alloway, 1995). Terraria were maintained at natural temperature (range 20–25 °C) and water lost as vapour was reintroduced by daily soil nebulisations with distilled water. Eggs were removed from terraria at regular time intervals from deposition and washed to remove soil traces. Embryos recovered from shells were immediately photographed, staged on the basis of limb paddle development (Dufaure and Hubert, 1961) and processed for cytological and biomolecular investigations. A total of 40 embryos were used.

2.3. Light microscopy

Lizard embryos were fixed in Bouin's solution and processed for paraffin wax embedding according to routine protocols. Sections were stained with haematoxylin–eosin or Mallory's trichrome to show general morphology.

2.4. Total RNA isolation

Total RNA from 20 day old embryos developed under natural (control) and Cd-contaminated (Cd-treated) conditions was extracted according to the Tri-Reagent (Sigma Aldrich) protocol. The two populations of total RNAs were dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at −75 °C. The concentration and purity of RNA samples were determined by UV absorbance spectrophotometry; RNA integrity was checked using formaldehyde-agarose gel electrophoresis (Sambrook and Russell, 2001).

2.5. Northern blot hybridization of RNAs

Total RNA from control and Cd-treated embryos (15 μg each) was size-fractionated by electrophoresis through a 1.2% agarose gel containing 2.2 M formaldehyde. RNAs were blotted on Hybond-N nylon membrane (Amersham Biosciences) and checked by staining the membranes with a methylene blue solution as described (Sambrook and Russell, 2001). The RNAs were probed for metallothionein mRNA by using a cDNA [α-^{32}P]-labelled fragment encoding *P. sicula* metallothionein (Riggio et al., 2003b). The membrane was then rehybridized with a radiolabelled *P. sicula* β-actin probe to correct for equal amounts of RNA. The probes were radiollabeled by the random priming method using a High Prime DNA Labelling Kit (Roche).

Prehybridization and hybridization of the membrane was carried out in 5× SSC, 50% formamide, 5× Denhardt's reagent (where 1× Denhardt's reagent is 0.02% Ficoll 400, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone) and 200 μg/mL fragmented calf thymus DNA at 42 °C for 2 and 20 h, respectively. The membrane was subsequently washed at increasing stringency with a final wash in 0.5× SSC, 0.1% SDS at 60 °C. Hybridization to target RNA was detected by autoradiography. Quantification was carried out by a PhosphorImager apparatus (Storm Imaging System) and expressed in arbitrary units.

2.6. Metallothionein mRNA detection by in situ hybridization

Sections (5–7 μm) were placed on superfrost glass slides (Menzel-Glaser, Germany), fixed in paraformaldehyde 4% in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) pH 7.4 for 20 min and incubated in PK buffer (Tris–HCl, 0.2 M, pH 7.4, EDTA 0.01 M, pH 8, proteinase K, 10 μg/mL, H_2O_{depc}) at 37 °C for 15 min. After washing in PBS, they were incubated at 42 °C for 90 min in a pre-hybridization mix containing formamide, SSC 4× and 1× Denhart's solution. Hybridization was carried out at 42 °C overnight using a dig-labelled cDNA probe encoding *P. sicula* MT (Riggio et al., 2003b). Sections were washed in SSC 2×, in Buffer I (Tris–HCl 0.1 M, pH 7.5, NaCl 0.1 M, H_2O_{depc}) and in Buffer I containing blocking reagent (0.5%). Digoxigenin was revealed by incubating sections overnight with an AP-conjugated anti-dig antibody diluted 1:400. Slides were washed in Buffer I, incubated with levamisole-Tween20 1× for 15 min and exposed with BM-Purple.

Dig-labelled MT cDNA probe was generated by PCR using the DIG High Prime DNA labelling and detection starter kit I (Roche). For negative control, the hybridization solution did not contain MT cDNA probe.

3. Results

3.1. Morphological analysis of embryos

Incubation in Cd-contaminated soil had no visible effect on development below the head region or on embryo survival, but strongly affected cranial morphogenesis. Fig. 1 illustrates malformations of the developing head. The most frequent anomalies were unilateral microphthalmia (Fig. 1C–F), anencephaly (Fig. 1E), exencephaly (Fig. 1F) and varying degrees of midfacial hypoplasia (Fig. 1C–F). Further morphological analyses of these heads revealed the occasional lack of a cranial vault (Fig. 2E–F) and the presence of various deformations at the base of the skull, palate and jaws (Figs. 3B, E, and 4D). Types and frequencies of malformations are summarized in Table 1.

Brain and retinal development was also affected. In the brain, malformations occurred primarily in the mesencephalon and in the proencephalon, and occasionally in the cerebellum (Fig. 2E) but not in the medulla oblongata (Fig. 2D and E). In the mesencephalon (Fig. 2) Cd caused swelling of the ventricle (Fig. 2C) and/or folding of the optic roof with occasional formation of pronounced ridges that invaded the ventricle (Fig. 2D). Asymmetry in the development of the two lobes (Fig. 2E) and of the basal bodies (Fig. 2D) was also observed. In the diencephalon (Fig. 3) Cd induced swelling of the ventricle (Fig. 3B and C) and decreased its wall thickness (Fig. 3B–D). In some cases the hypothalamic roof was interrupted and the vesicle was open dorsally (Fig. 3D); in other cases, the vesicle was severely altered or absent (Fig. 3E). The optic nerves were occasionally interrupted medially at the level of the optic chiasm (Fig. 3E and F). In the telencephalon (Fig. 4) Cd exposed embryos had severe asymmetry of the two

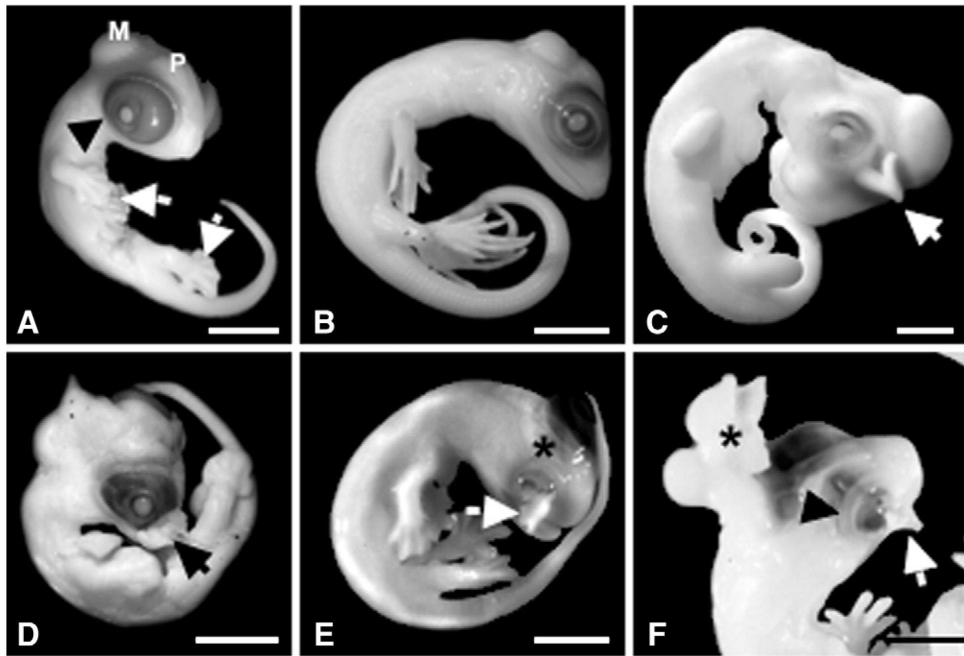

Fig. 1. Morphological alterations observed in *P. sicula* embryos incubated in cadmium-contaminated soil. (A) Control embryo at 25 days post deposition (pd). The eye (arrowhead) is well developed, pigmented, with lens differentiated; mesencephalon (M) and proencephalon (P) are prominent; limb paddles (arrows) show developing fingers. (B) Control embryo at 40 days pd. The eye has completely developed eyelid, the encephalon has become less prominent, digits are fully separated. (C) Cd-treated embryo at 15 and (D) 20 days pd. Malformed eye with fleshy growths (arrow). (E) Cd-treated embryo, 25 days pd. The encephalon (*) and the eye are missing, a large fleshy growth (arrow) is present. (F) Cd-treated embryo, 35 days pd. The encephalon is extruded (*), the eye is poorly developed (arrowhead) and shows a fleshy growth (arrow). Bars: (A) 2 mm; (B) 2.5 mm; (C) 0.5 mm; (D, E) 2 mm; (F) 2 mm.

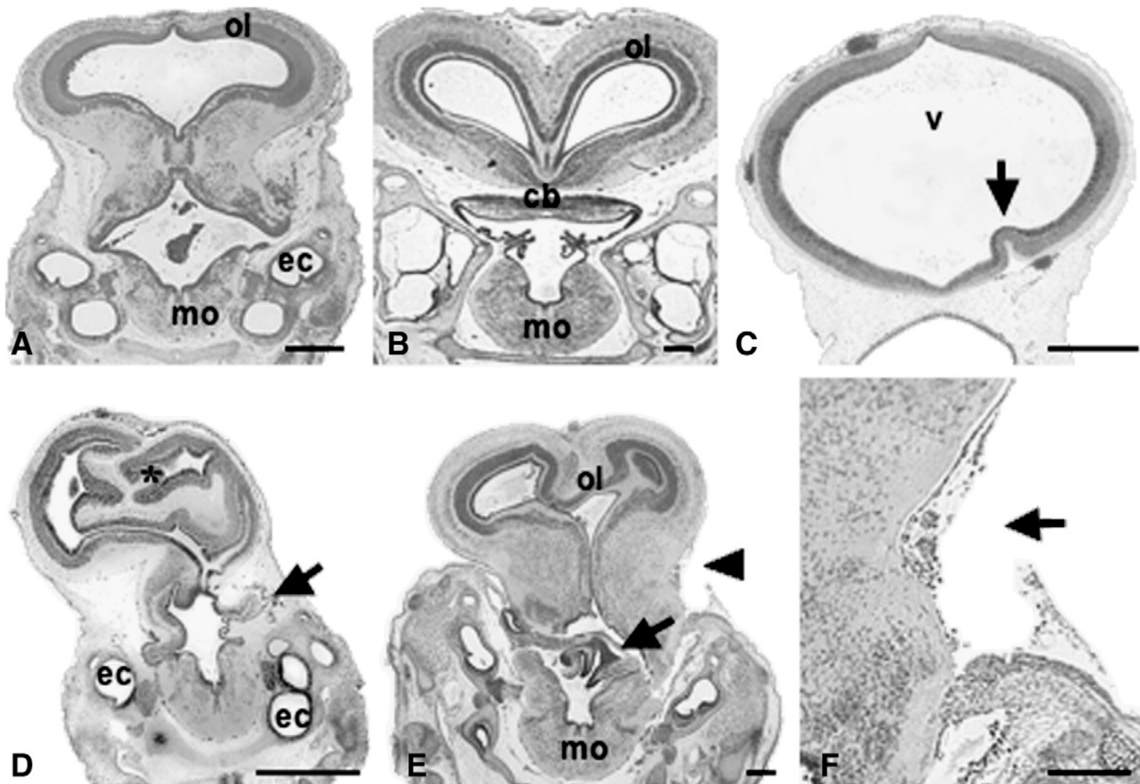

Fig. 2. Cross sections at mesencephalic level of control (A, B) and Cd-treated *P. sicula* embryos (C–F). (A) 20 days pd. Optic lobes (ol), medulla oblongata (mo), ear canals (ec). (B) 40 days pd. Fully developed optic lobes (ol), cerebellum (cb) and medulla oblongata (mo). Ear canals (ec). (C-D) Cd-treated embryos, 20 days pd. (C) Swollen mesencephalic ventricle (v) and small ridge in the wall (arrow). (D) Folding of the optic roof (*) invading the ventricle. Note the asymmetry of the basal bodies (arrow) and the ear canals (ec) (E) Cd-treated embryos, 30 days pd. Marked asymmetry of the optic lobes (ol). Note the abnormal cerebellum (arrow) and the absence of the cranial vault (arrowhead) resulting in the extrusion of mesencephalon. The medulla oblongata (mo) is normally developed. (F) Detail of the interruption (arrow) of the cranial vault. Bars: (A) 0.35 mm; (B) 0.19 mm; (C) 0.2 mm; (D) 0.8 mm; (E) 0.2 mm; (F) 0.06 mm.

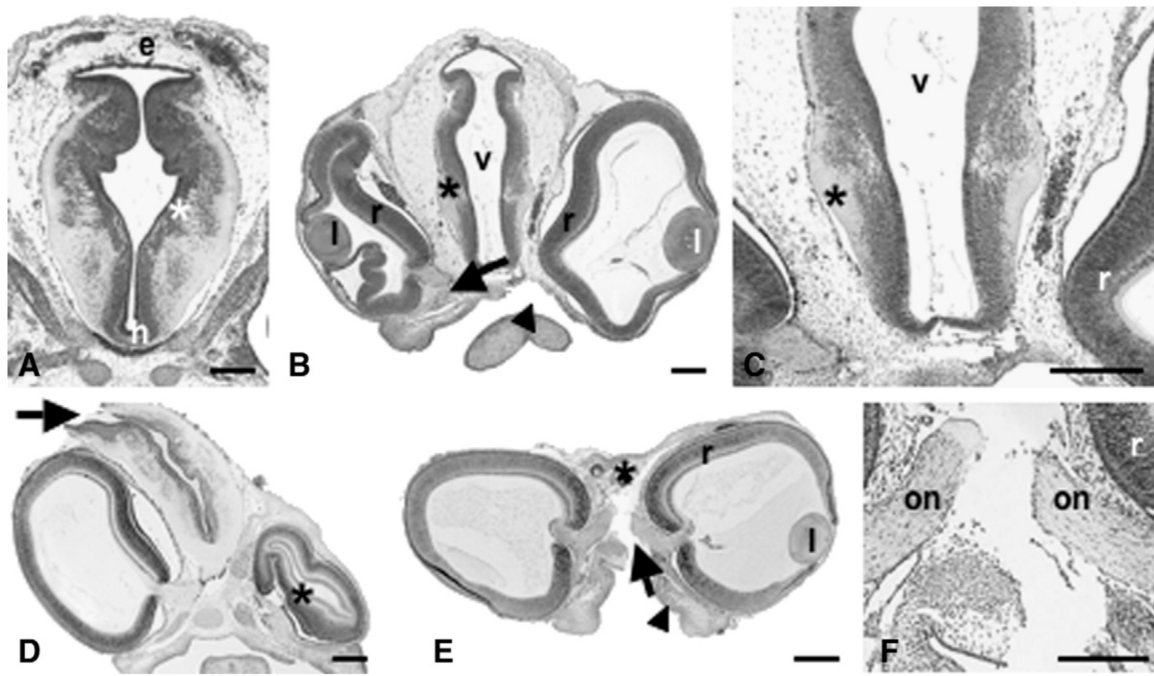

Fig. 3. Cross sections at diencephalic level of control (A) and Cd-treated *P. sicula* embryos (B–F). (A) 20 days pd. Thalamic vesicle with thick walls (*), expanded epithalamus (e) and a still undifferentiated hypothalamus (h). (B) Cd-treated embryo, 20 days pd. Swollen ventricle (v) and reduced wall thickness (*). Notice the unilateral folding of the retina (r) invading the optic cup, and the root of the optic nerve (arrow). The skull base is altered (arrowhead). (C) Detail showing dramatic reduction of the white matter in the ventral part of the thalamus (*). (D) Cd-treated, 20 days pd. Asymmetric diencephalon with ventricle open dorsally (arrow). Unilateral microphthalmia (*). (E) Cd-treated embryo, 20 days pd. Absence of the diencephalon (*). The optic nerves (arrow) are interrupted medially at the level of the optic chiasm; the palatal vault is missing (arrowhead). (F) Detail of figure E showing the interrupted optic nerves (on). r: retina, l: lens. Bars: (A) 0.19 mm; (B) 0.2 mm; (C) 0.17 mm; (D, E) 0.2 mm; (F) 0.17 mm.

hemispheres (Fig. 4C) and poor development of the pallium with dorsomedial interruption (Fig. 4C–E). The olfactory bulbs also showed asymmetric disruptions (Fig. 4F).

In the eye, altered retinal folding over the optic cup was observed (Fig. 3B, D, and Fig. 4C and D). These alterations were unilateral and appeared to be independent of developmental stage. Retinogenesis

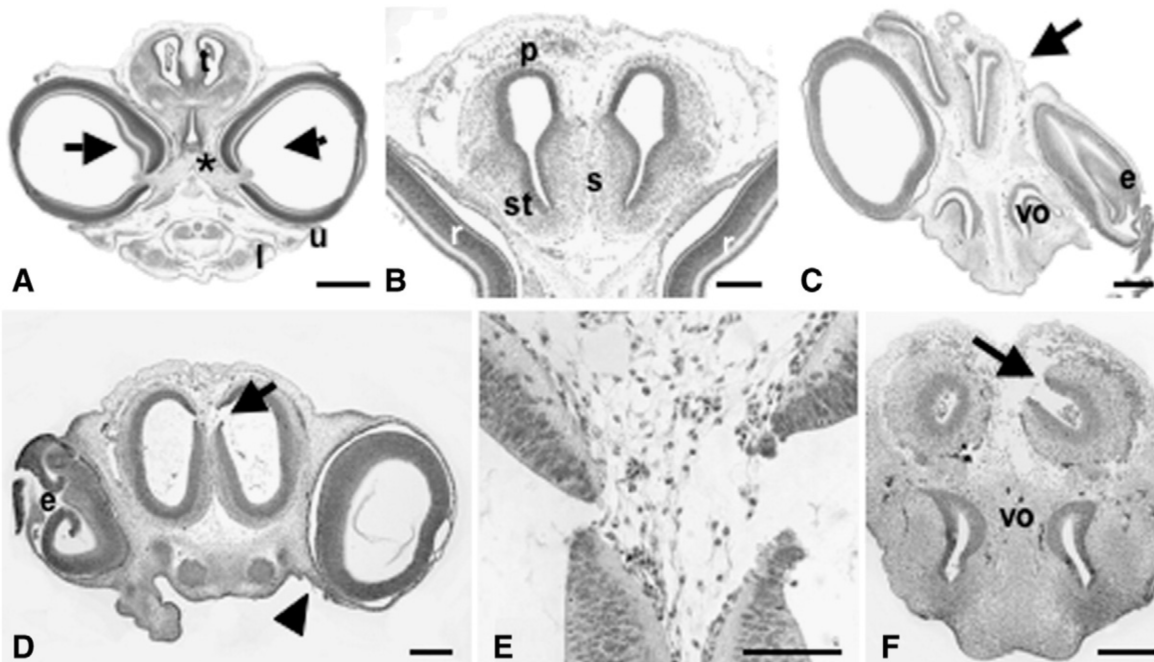

Fig. 4. Cross sections at telencephalic level of control (A–B) and Cd-treated *P. sicula* embryos (C-F). (A) Embryo at 20 days pd. Eyes with retina starting differentiation (arrows). Note the well developed upper (u) and lower (l) jaw, the optic chiasm (*) and the anterior telencephalon (t) with ventricles. (B) Telencephalic hemispheres at 20 days pd. Note the well developed septum (s) and striatum (st) and the stratified retina (r); the pallium (p) is still undifferentiated. (C) Cd-treated embryo, 20 days pd. Asymmetry and poor development of the telencephalic hemispheres (arrow) and of the eye that shows a folded retina (e). Vomeronasal organ (vo). (D) Cd-treated embryo showing a malformed eye (e) and a telencephalic vesicle with a thin wall interrupted at the level of the medio-dorsal pallium (arrow). Note also the malformation of the viscerocranium (arrowhead). (E) Detail of the pallium at the level of the interruption. (F) Asymmetric disruption of the olfactory bulbs (arrow). vo: vomeronasal organ. Bars: (A) 0.37 mm; (B) 0.12 mm; (C) 0.3 mm; (D) 0.15 mm; (E) 0.056 mm; (F) 0.2 mm.

Table 1
Main gross morphological abnormalities in lizard embryos after 30-days in ovo incubation in soil contaminated with 50 mg/kg cadmium.

Abnormality	Percentage of affected embryos
Anencephaly	5%
Defects of the cranial part of the neural tube	
Telencephalon and diencephalon	*60%*
Exencephaly	30%
Mesencephalon	*70%*
Exencephaly	45%
Eye abnormalities	50%
(microphthalmia, retinal malformations)	
Facial abnormalities	20%
Limb anormalities	1%
Trunk abnormalities	0%

however was not affected as at all stages development and differentiation of the retina appeared comparable to those observed in controls at the same stage (Fig. 3D and E). Lenses were always structurally normal.

No effects of Cd on development of the trunk organs were seen. Lung, kidney, liver and gut of Cd-treated embryos showed size, shape and histological organization comparable to those of control embryos (Fig. 5). However, an increase in vascularization and edema was often observed. No morphological changes were observed in the development of the spinal cord (Fig. 5G).

3.2. Metallothionein expression pattern in cadmium contaminated embryos

Densitometric analyses of Northern blots showed an appreciable amount of MT mRNA in untreated embryos. Increased amounts were seen in embryos developed in Cd contaminated soil, suggesting the induction of MT gene expression by heavy metals (Fig. 6).

In situ hybridization experiments did not reveal significant changes in MT mRNA localization in developing brain and retina of control and Cd-treated embryos. Encephalic areas and optic cups carrying severe alterations showed considerable amount of MT transcripts whose localization followed the same spatio-temporal pattern found in intact structures (Fig. 7). Labelling was concentrated in the ventricular zones of encephalic vesicles (Fig. 7A–I) and in the

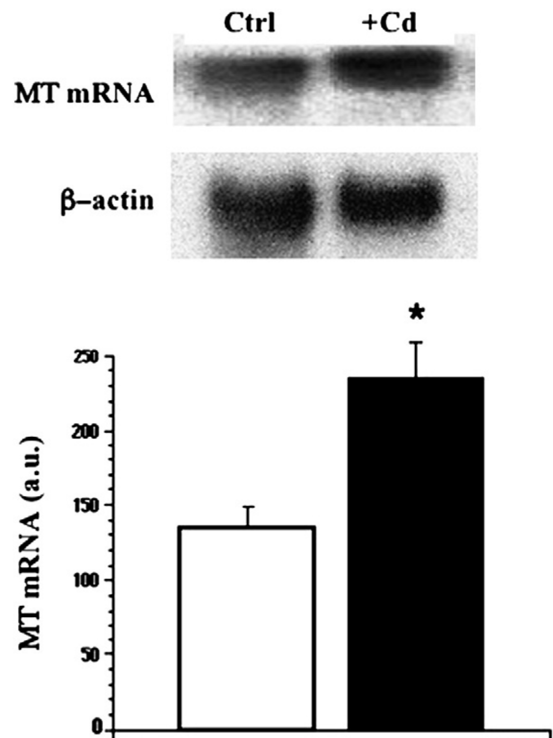

Fig. 6. Metallothionein mRNA content in control and Cd-treated *P. sicula* embryos at 25 days post deposition. (A) Northern blots showing MT mRNA (upper panel) and beta-actin mRNA (lower panel) in control and Cd-treated embryos. (B) Histogram showing the the quantitative analysis of MT mRNA present in control and Cd-treated embryos by Image Quant Software (Molecular Dynamics); the relative intensities of MT mRNA have been normalized by β-actin mRNA levels. (*) Difference significant at $p<0.05$ probability level; (a.u.) arbitrary units.

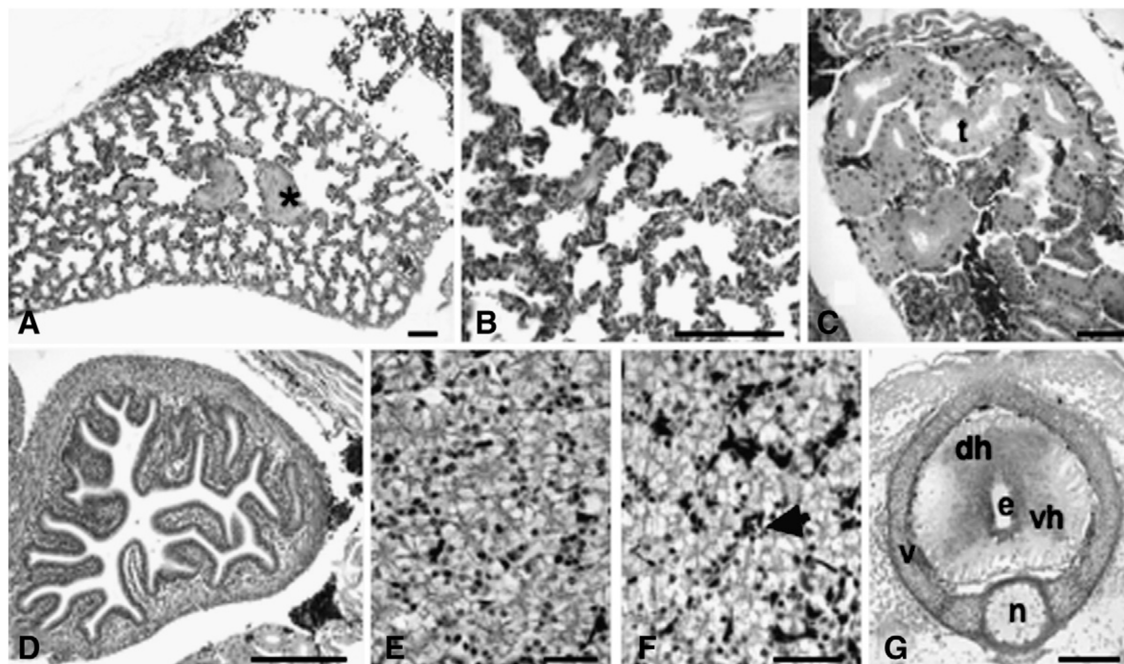

Fig. 5. Trunk organs in Cd-treated *P. sicula* embryos at 25 days post deposition. (A–B) Lung with well developed pulmonary wall and septa (*). (C) Kidney with differentiated tubules (t). (D) Cross section of gut with thick and differentiated mucosa. Liver from control (E) and Cd-treated (F) embryos; the parenchyma shows a preserved architecture but increased vascularization and edema. Swollen sinusoids (arrow). (G) Spinal cord from a Cd-treated embryo; dorsal (dh) and ventral horns (vh) of the gray matter are regularly arranged around the ependymal canal (e). Developing vertebra (v); notochord (n). Bars: (A, B) 0.1 mm; (C) 0.05 mm; (D) 0.12 mm; (E, F) 0.075 mm; (G) 0.1 mm.

undifferentiated retina (Fig. 7M); the retinal stratification resulted in a redistribution of MT mRNA that concentrated in the ganglion cells layer and in the inner and outer nuclear layers, both in control (Fig. 7L) and Cd-treated embryos (Fig. 7N). Interestingly, in Cd-treated embryos no induction of MT gene expression was found in areas that were unlabelled in control embryos, such as the anterior vesicles of 40-days old embryos (Fig. 7J and K).

Developing trunk organs showed variable experimental effects. For kidney (Fig. 8A) and lung (Fig. 8B), the timing and localization of MT transcripts in control embryos did not differ from those incubated in Cd contaminated soil. In contrast, in gut and liver a marked increase in MT mRNA expression was seen in Cd-treated embryos. In intestinal mucosa (Fig. 8C and D) and liver sinusoids (Fig. 8E–G) particularly, the hybridization signal appeared in 10 day old embryos but in control

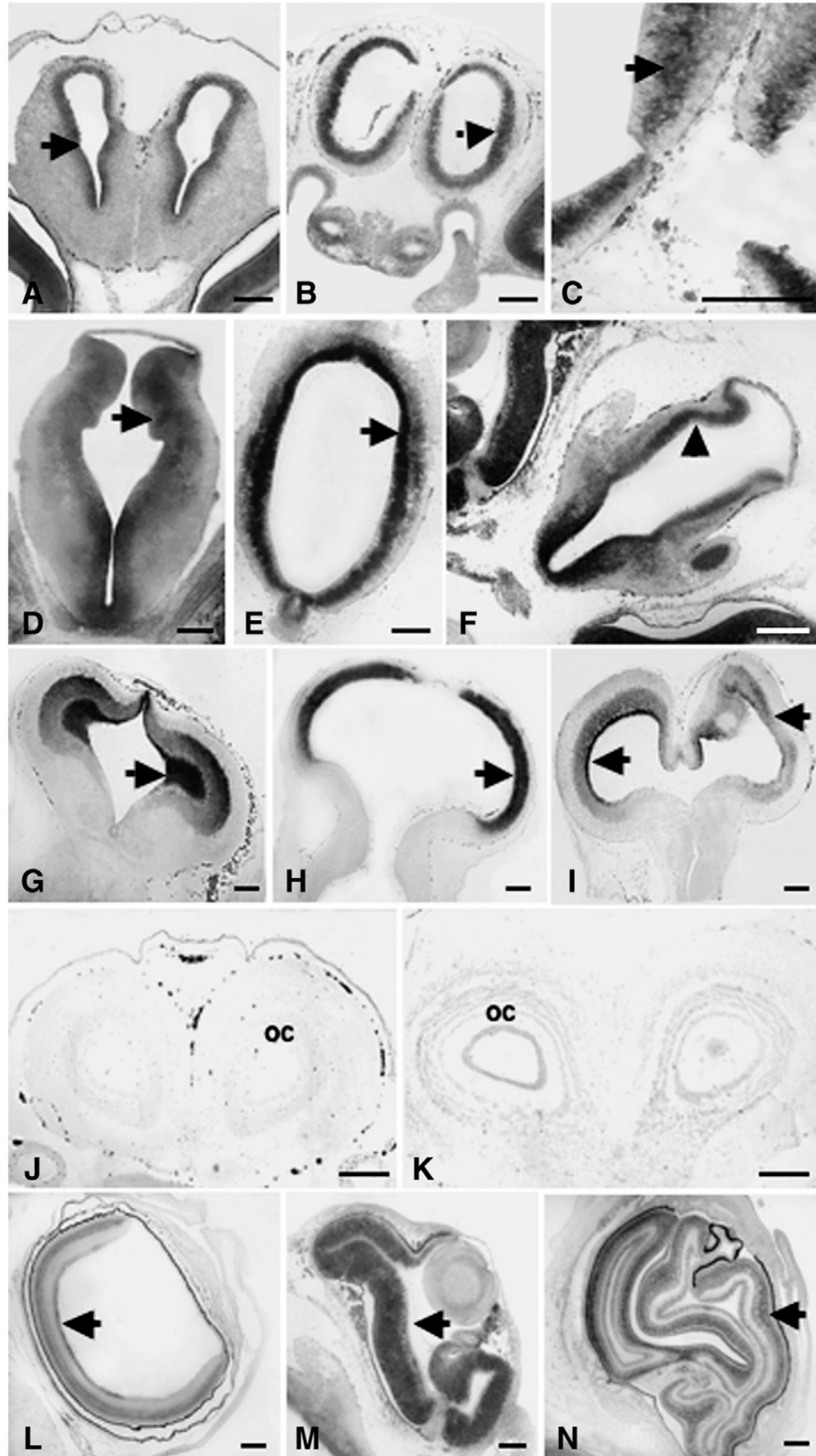

Fig. 7. Metallothionein mRNA localization in brain and eye of control and Cd-treated *P. sicula* embryos. In early embryos the MT mRNA is present in the ventricular zones (arrows) in controls (A, D, G) and Cd-treated telencephalon, diencephalon and mesencephalon (B-C, E–F, H–I). In differentiated vesicles (40 days pd) the optic cortex (oc) of the mesencephalon is unlabelled in both control (J) and Cd-treated (K) embryos. MT mRNA is present in the retina (arrows) of control (40 day pd, L) and Cd-treated (20 and 40 days pd, M and N) embryos. Bars: (A, B) 0.1 mm; (C) 0.05 mm; (D, E) 0.1 mm; (F) 0.2 mm; (G, H, I) 0.1 mm; (J, K) 0.2 mm; (L, M, N) 0.1 mm.

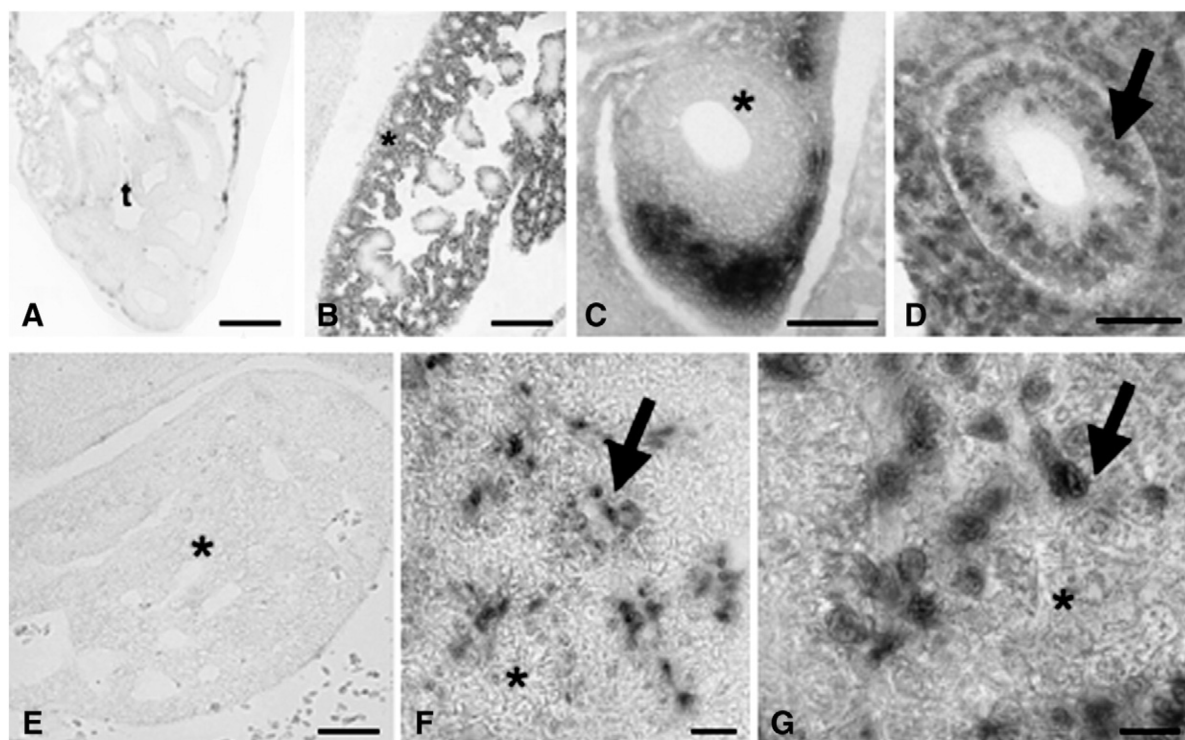

Fig. 8. Metallothionein mRNA localization in gut and liver of control and Cd-treated *P. sicula* embryos. (A) Cd-treated embryo at 20 days pd. Kidney with unlabelled tubules (t). (B) Cd-treated embryo at 40 days pd. Lung with labelled wall. (C) Control embryo at 10 days pd. The gut mucosa is completely unlabelled (*). (D) Cd-treated embryo at 10 days pd. The gut mucosal cells show intensely labelled nuclei (arrow). (E) Control embryo at 10 days pd. Unlabelled liver parenchyma (*). (F) Cd-treated embryo at 10 days pd. A significant labelling is observed over several sinusoidal cells (arrows). Unlabelled liver parenchyma (*). (G) Detail of figure F. Bars: (A, B, C, D, E) 0.05 mm; (F) 0.02 mm; (G) 0.01 mm.

embryos MT mRNA was detectable only after 40 days and immediately before hatching (Simoniello et al., 2010b), respectively.

4. Discussion

The buffering capacity of soil has long been considered able to limit the accumulation of harmful contaminants to which terrestrial animals would be naturally exposed. Consequently, a historical research focus has largely been on pollution effects in aquatic environments. More recently however, data on soil accumulation of heavy metal pollutants have been published. For example, cadmium concentrations greater than 600 mg/kg topsoil in some areas have been reported (Stafilov et al., 2010). With this awareness, there is a growing research effort directed towards understanding the effects of cadmium on organisms such as plants and invertebrates which are closely dependent on the soil (Kammenga et al., 2000; Chabicovsky et al., 2004; Spurgeon et al., 2005; DalCorso et al., 2008; Veltman et al., 2008; Verbruggen et al., 2009; Peralta-Videa et al., 2009; Farinati et al., 2010).

As the eggs of oviparous terrestrial vertebrates have been considered to be protected by shells, the potential risks to development and survival from cadmium contaminated soils have largely concerned juveniles and adults only. Studies on teratogenic effects of cadmium on embryos therefore have been mainly undertaken on maternal transfer mechanisms (Sato et al., 1997; Nagle et al., 2001; Guirlet et al., 2008) or on shell-deprived embryos (Thompson and Bannigan, 2001, 2007; Thompson et al., 2005; Cullinane et al., 2009). However, recent studies have demonstrated that the flexible reptilian eggshell does not fully protect embryos from environmental contaminants (Marco et al., 2004a, 2005; Gómara et al., 2007), and that cadmium, as well as other trace elements (Marco et al., 2004b), can cross the shell of *P. sicula* eggs (Trinchella et al., 2010; this paper).

In this study no in ovo mortality was observed in embryos incubated in Cd contaminated soil at levels compatible with environmental pollution. However, embryos at different developmental stages showed severe malformations which would be incompatible with the survival after the hatching. These observations, together with impaired fecundity of Cd-treated *P. sicula* females (unpublished data), suggest a loss of reproductive performance and a likely decline in the numbers of individuals for wild populations living in polluted areas. Because almost all reptilian species lay their eggs in subterranean nests, the rapid decline in many reptilian populations observed in recent years might be partly ascribed to developmental failures in offspring in these increasingly polluted environments.

In *P. sicula* embryos, cadmium caused damage to head skeletal structures and the prosencephalon similar to that observed in mammalian embryos. In mammals, Cd toxicity has been often associated with the alteration of bone metabolism and osteoporosis (Kazantzis, 2004). In *P. sicula*, however, the bone defects are apparently localized only in the craniofacial region, with limb and backbone normally developed. This observation suggests a specific Cd mediated interference in neural-mesodermal tissue signalling (Morriss-Kay, 2001) rather than a generalized mechanism of Cd dependent osteoporosis, which should have affected other parts of the skeleton.

Embryonic *P. sicula* brain development was damaged in the forebrain vesicles while the medulla and spinal cord retained their typical organizational structure. This could be attributed to the fact that the different encephalic structures organise at different times and the complex architecture of the prosencephalic vesicles is completed only in the later stages. In reptiles, females retain their eggs for some days in the reproductive tract thereby extending the gestation period (Porter, 1972). As a consequence, embryos at oviposition have already reached the gastrula stage, and this could explain the absence of early-stage developmental abnormalities observed in these embryos. Developmental stage-dependent susceptibility to teratogen is regarded as one of the essential characterizations of developmental toxicity (Pérez-Coll and Herkovits, 1990; Castañaga et al., 2009). In mammals, it has been demonstrated that proliferating matrix cells in

the developing brain are particularly sensitive to teratogenic insults when the undifferentiated ventricular cells start to differentiate into cerebral cortical neurons (Kameyama, 1983, 1991).

Recently, we have demonstrated that cadmium modifies the expression of many genes of *P. sicula* embryos developed under the same conditions described in this work (Trinchella et al., 2010). In particular, cadmium affects the expression of genes involved in molecular pathways associated with membrane trafficking, signal transduction, neuronal transmission and regulation of gene transcription. No changes are observed in the expression of early master genes controlling body patterning. However, Cd-induced changes in the expression of these genes could explain many of the morphological alterations displayed by lizard embryos as described in this paper. Remarkably, similar damage (in particular, exencephaly and eye malformations) can be observed in mammalian and amphibian embryos exposed to the valproic acid, an antiepileptic drug capable of altering the expression of the same cadmium-responsive genes (Menegola et al., 1996; Krätke and Kirschbaum, 1996; Pennati et al., 2001; Massa et al., 2005; Di Renzo et al., 2010).

An interesting aspect concerning the modulation of gene expression in Cd-treated embryos is that the localization of MT transcripts in the malformed encephalic areas did not change although we noted a general increase of MT gene expression in these embryos. In both 40 day old control and Cd-treated embryos no MT transcripts were present in the telencephalon, diencephalon and mesencephalon. The failure of Cd to induce *de novo* MT transcripts in these vesicles could be due to a physiological mechanism of organ-specific gene silencing that cadmium fails to remove. In zebrafish embryos, it has been demonstrated that at the gastrula stage, Cd-induced MT expression could be detected only after a concomitant treatment of embryos with 5′-deoxy-azacytidine, suggesting DNA methylation as an epigenetic control of stage-specific gene regulation (Riggio et al., 2003a). At this time however we cannot exclude the possibility that the amount of cadmium ions reaching the brain vesicles is sufficient to exert a toxic effect but not to initiate or increase the expression of the embryonic MT gene in these structures.

In contrast, a marked Cd induction of embryonic MT gene was found in liver sinusoids and gut mucosa of 10 day old embryos. Interestingly, in liver of both control embryos and adults, MT gene expression was detectable only in Kupffer cells, whereas in sinusoids, MT transcripts were always absent (Simoniello et al., 2010a,b). Additionally, under natural conditions, liver and gut mucosa show detectable amount of MT transcripts only in pre-hatching embryos (Simoniello et al., 2010b). These data suggest a Cd-induced temporal and spatial shift in regulation of embryonic MT gene expression in these tissues.

It is noteworthy that the earlier Cd-induced MT gene expression in liver and gut seems to act by preventing morphological alterations of the developing visceral organs. The abundance of MT transcripts observed in the early stages of embryonic development in the brain and in the eyes does not provide for protection against the toxic effects of cadmium. Possibly these messengers are translated in gut and liver but not in brain or proteins translated from these transcripts are engaged in functions other than heavy metal detoxification.

Together, these data suggest a possible correlation between Cd-inability to induce MT expression and the morphological alterations observed in lizard embryos and underline the importance of carrying out further investigations on MT protein synthesis in the lizard embryo to clarify the physiological role of MT in different tissues during reptilian embryonic development and to ascertain its ability to act as a detoxifying agent.

Furthermore, these data clearly emphasize the importance of considering soil pollution for environmental risk assessment of flexible-shelled reptiles. The awareness of the risks should lead to investigate the potential teratogenic effects of other environmental pollutants (pesticides, fertilizers) on terrestrial oviparous vertebrates.

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