Cadmium Impairment of Reproduction in the Female Wall Lizard *Podarcis sicula*

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Received 14 February 2011; revised 23 May 2011; accepted 24 May 2011

ABSTRACT: The exposure to environmental toxicants such cadmium (Cd) is an important research area in wildlife protection. In this study, the effect of Cd oral administration on the ovarian structure and function and on reproductive performance of the Italian wall lizard *Podarcis sicula* was studied. *In vivo*, adult female lizards were randomly assigned to three groups. Cd was given with food in single dose and in multiple doses 3 days/week for 4 weeks at dose of 1.0 μg/g body weight. Following euthanasia, the ovaries were removed and analyzed for morpho-functional changes. Results demonstrated that Cd increases prefollicular germ cells number; the evaluation of the number of follicles detects significantly higher number of atretic growing follicles, whereas primary follicles remain unchanged with respect to controls. After Cd treatments, follicles are deformed by the presence of large protrusions and a general dysregulation in the follicle organization is observed. The zona pellucida is also affected. Cd causes alteration in sugar metabolism and in metallothionein gene expression. Finally, Cd administration significantly reduces clutch size and dramatically increases embryo mortality. In conclusion, data here described show that Cd induces morpho-functional alterations in lizard follicles and indicates that these are responsible for a significant impairment of oogenesis. The effects of the dose are time independent, persisting essentially unchanged regardless of single or multiple administration, so it can be concluded that even occasional, sublethal Cd contamination may significantly impair reproductive performance in these animals. © 2011 Wiley Periodicals, Inc. Environ Toxicol 28: 553–562, 2013.

Keywords: cell proliferation; follicular alterations; metallothionein expression; ovarian follicle cells; reptiles; *zona pellucida*

INTRODUCTION

The transition metal, and common environmental pollutant cadmium (Cd) is highly toxic to reproductive tissues, causing organ damage and functional deficiency (Merian, 1990; Saygi et al., 1991; Leoni et al., 2002). In mammals, Cd interferes with gonads directly by affecting structure and steroidogenesis (Laskey and Phelps, 1991; Paksy et al., 1997; Le Guevel et al., 2000; Henson and Chedrese, 2004) and indirectly, via the hypothalamus and hypophysis to alter endocrine control pathways (Paksy et al., 1989; Varga and Paksy, 1991; Mukherjee et al., 1994).

In males, Cd induces testicular necrosis (Lohiya, 1976) and reduces fertility by destroying spermatogenic elements (Foote, 1999), delaying proliferation (Blottner et al., 1999) and by changing pH in the epididymis and vas deferens (Herak-Kramberger et al., 2000).

In females, Cd exposure affects both oocytes and follicle cells to increase follicular atresia (Bires et al., 1995), the incidence of chromosomal anomalies, and to reduce fecundity and egg quality (Watanabe et al., 1979). This latter effect in particular compromises implantation (De et al., 1993), placentation (Wier et al., 1990), and embryo development (Hen Chow and Cheng, 2003). Granulosa cells

This paper is dedicated to Prof. Silvana Filosa, *magister vitae et scientiarum*, by her students in occasion of her retirement.

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Published online 31 August 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/tox.20749

appear to be particularly damaged. Both *in vivo* and *in vitro* the ion alters cell morphology and membrane integrity (Nampoothiri et al., 2007) with reduced gonadotropin binding (Nampoothiri and Gupta, 2006). Cd also impairs progesterone synthesis by inhibiting the expression of StAR and P450scc proteins (Zhang and Jia, 2007). Also, by altering the synthesis and accumulation of hyaluronic acid in the cell matrix, adherence at cell–cell junctions is affected (Mlynarcikova et al., 2004) through Cd induced changes in cytoskeletal and adhesion proteins such as L-CAM and β-catenin (Thompson et al., 2008). These toxic effects indicate that environmental Cd exposure may significantly threaten reproductive performance and therefore population survival of animals in natural habitats. Hence, it is essential to determine effects of this pollutant on wild species.

The studies reported here analyze the effects of Cd on ovarian follicle recruitment and growth in the Italian wall lizard, *Podarcis sicula*. This species has colonized both urban and suburban areas and since it lives in close contact with the soil, it is exposed to metal intoxication by food ingestion and by inhalation.

To test short- and long-term Cd effects on the reproductive axis of these animals, the metal was administered in a single or multiple doses by food, at dosages known to determine liver concentrations comparable to those occurring in animals inhabiting contaminated sites (Trinchella et al., 2006; Mann et al., 2007; Simoniello et al., 2010a). The effects of treatments on reproductive performance were evaluated by analyzing changes in prefollicular oocytes number, in follicle organization, in glycoproteins content and distribution and in follicle cells proliferation (by PCNA immunolocalization). The effects were correlated with changes in the expression of metallothionein (MT), a metal binding protein specialized in chelating both essential (zinc and copper) and highly toxic (Cd and mercury) divalent ions (Palmiter, 1998; Klaassen et al., 2009).

MATERIALS AND METHODS

Animals

Adult females of *P. sicula* were captured on the outskirts of Naples and maintained in animal facilities under a natural photothermal regime, with food and water *ad libitum*. Eggs were obtained at deposition, transferred in clean soil, and left to develop at 26°C in a humidified chamber. All experiments were conducted in accordance with the Guideline for Animal Experimentation of the Italian Department of Health under the supervision of a veterinarian, and organized to minimize stress and the number of animals used.

Treatments

Acclimatized animals were randomly allotted to three experimental groups. Group I and II ($n = 30$ in each group) were fed with insect larvae injected with an appropriate volume of Cd chloride (Sigma, St Louis, MO) solution so to obtain a final dosage of 1 μg Cd/gr lizard body mass. Group I was fed once (single treatment) while group II was fed with contaminated larvae every other day for 28 days (multiple treatment). Group III was left untreated ($n = 10$, controls). Sampling was carried out in triplicate (treatments) or on single animals (controls) at regular time intervals up to 30 days. No mortality was recorded during the experiments.

Light Microscopy

Ovaries were fixed in Bouin's solution and processed for wax embedding according to routine protocols. Sections were stained with hematoxylin–eosin or Mallory's trichrome to show general morphology. Periodic acid/Schiff (PAS) was used to highlight mucin and glycogen. Sections were oxidized in 0.5% periodic acid solution for 10 min, rinsed in double distilled water and stained with Schiff's reagent in the dark for 45 min. Reaction was blocked by repeated washing in 2.5% sodium bisulphite in 0.05 N HCl (Bonucci, 1981). Sections were counterstained with hematoxylin.

Glucid residues were analyzed by staining sections with the lectins WGA (*Triticum vulgaris* agglutinin) for D-*N*-acetyl-glucosamine (glcNAc)2 and UEA-1 (*Ulex europaeus* agglutinin), for L-fucose. Slides were incubated in 0.3% hydrogen peroxide (H_2O_2) in PBS (0.2 M, pH 7.2–7.4) for 45 min and microwaved at 750 W for 3 min in citrate buffer (0.01 M, pH 6.0) then washed in PBS and incubated with biotinylated lectins at a concentration of 10 mg/mL in PBS for 2 h at room temperature. After rinsing in 0.5% BSA in PBS, they were treated with Avidin–Biotin–Peroxidase Complex (Dako, Glostrup, Denmark) 1 h at room temperature. After rinsing in PBS, lectin binding sites were visualized with DAB and made permanent by mounting. Negative controls were prepared by incubating slides with the lectins and the specific competing sugar or by omitting the AB-Complex from the reaction. Control samples were completely unstained (data not shown).

Embryos were obtained by gently opening the eggshell membrane under a dissecting microscope. They were examined, photographed and fixed as described for ovaries.

Germ cells were counted in serially sectioned germinal beds, two small areas located close to the ovarian hilum which contain 200–1000 germ cells in all prefollicular stages (Filosa, 1973). The reduced number of cells and the fact that they are gathered in the two germinal beds that appear in no more than 10–15 sections made possible to count directly their number without making complicated estimation. Counting was carried out exclusively on females receiving a single treatment. All numerical data are given as means ± standard deviation and significance was tested by 1-way ANOVA at 95% confidence.

Immunolocalization of PCNA Antigen

Sections were washed in serum to reduce nonspecific binding and incubated overnight at 4°C with a polyclonal primary antibody (Novocastra, Newcastle Upon Tyne, UK) diluted 1:50 in PBS buffer. Antibody binding was detected by a secondary antirabbit antibody (1:100 in phosphate buffer) conjugated to peroxidase, incubated with a tertiary anti-PAP antibody (1:100 in phosphate buffer) and revealed with DAB. Sections were then counterstained with hematoxylin and mounted. Negative controls were prepared by omitting the primary antibody; antibody specificity was tested by western blotting in preliminary experiments (data not shown).

Metallothionein mRNA Detection by *In Situ* Hybridization

Sections were washed in PBS (pH 7.4), fixed in paraformaldehyde (4% in PBS) 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 prehybridization mix containing formamide, SSC 4× and Denhart's solution 1×. Hybridization was carried out at 42°C overnight using a dig-labeled cDNA probe prepared from *P. sicula* MT (Riggio et al., 2003). Sections were then washed in SSC 2×, SSC 1×, SSC 0.5×, in Buffer One (Tris-HCl 0.1 M, pH 7.5, NaCl 0.1 M, H_2O_{depc}) and in Buffer Two containing the blocking reagent (0.5% in Buffer One). Digoxigenin was revealed by incubating sections overnight with an AP-conjugated antidig antibody diluted 1:400 in Buffer Two. Slides were then washed in Buffer One, incubated with levamisole-Tween20 1× in a staining mix (1 mL BM-Purple, 10 μL levamisole-Tween20 100×) for 15 min. Reactions were stopped in TE 1×.

COMET Assay

The single cell gel electrophoresis assay (also known as COMET assay), was performed as described by Singh et al.1988. Briefly, the ovaries were dissected and placed in sterile PBS. Follicles were manually isolated and dissociated by vortexing; resulting cells were recovered by centrifugation at 4000 rpm for 5 min (De Caro et al., 1998) at 4°C and resuspended in fresh PBS. 30 μL of a cell suspension (≈200,000 cell/mL) were mixed with 70 μL of 1% low melting agarose and poured on fully frosted slides previously coated with a layer of normal melting agarose. Subsequently, the slides were exposed to a lysis solution (2.5 M NaOH, 0.1 M EDTA, 10 mM Tris and 1% Triton X-100, pH 10) for 1 h at 4°C, rinsed with distilled water, placed in the electrophoresis buffer (0.5M NaCl 1 mM EDTA, 100 mM Tris, pH 9) for 20 min and electrophoresed at 25 V and 300 mA for 20 min. After running the slides were neutralized in 0.4 M Tris buffer (pH 7.5), stained with ethidium bromide (5 μg/mL) and observed using a fluorescence microscope.

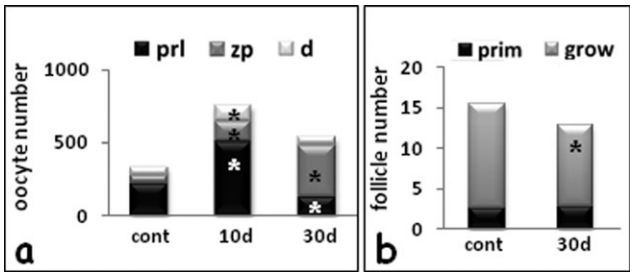

Fig. 1. Effects of a single injection of Cd on the number of prefollicular germ cells (a) and follicles (b). (a) After 10 days from treatment the number of oocytes is 2.3-fold higher than in controls due to the increase in prelepto-leptotene (prl), zygo-pachytene (zp), and early diplotene (d) oocytes number. After 30 days, the number of oocytes is about 1.6-fold higher than in controls. The number of prelepto-leptotene oocytes is significantly reduced, that of zygo-pachytene oocytes is increased and that of diplotene oocytes is unchanged with respect to controls. (b) Growing follicles number decreases after 30 days from Cd treatment; primary follicles number remains unchanged. $p < 0.05$ (*).

RESULTS

Cadmium Increases Prefollicular Germ Cells Number

Data are summarized in Figure 1. Germ cells in animals exposed to single treatment indicate that Cd administration stimulates oocyte recruitment from the pool of oogonia [Fig. 1(a)]. Total oocytes number increased from 326 ± 58 in controls to 751 ± 97 (10 days from treatment) and 533 ± 49 (30 day from treatment). The distribution of oocytes in the different stages of meiotic prophase also changes significantly. The number of prelepto-leptotene oocytes increases on day 10 (495 ± 94) and decreases on day 30 (125 ± 34) compared to control values (214 ± 28). Zygotene and pachytene oocytes also increase in number on day 10 (139 ± 28) and day 30 (350 ± 54) compared to control (58 ± 17) while early prefollicular diplotene oocytes show a significant increase in number on day 10 (117 ± 21) and return to control value (54 ± 9) on day 30 (58 ± 17).

Primary follicles number remains unchanged (2.5 ± 0.4) with respect to controls (2.7 ± 0.3), while the number of growing follicles significantly decreases from 12 ± 0.6 to 10 ± 1.5 on day 30 due to the occurrence of follicular atresia [Fig. 1(b)].

Cadmium Impairs Fecundity

Cd administration significantly reduces clutch size from 14.5 ± 1.2 eggs for controls to 5.7 ± 0.6 and 0.9 ± 0.3

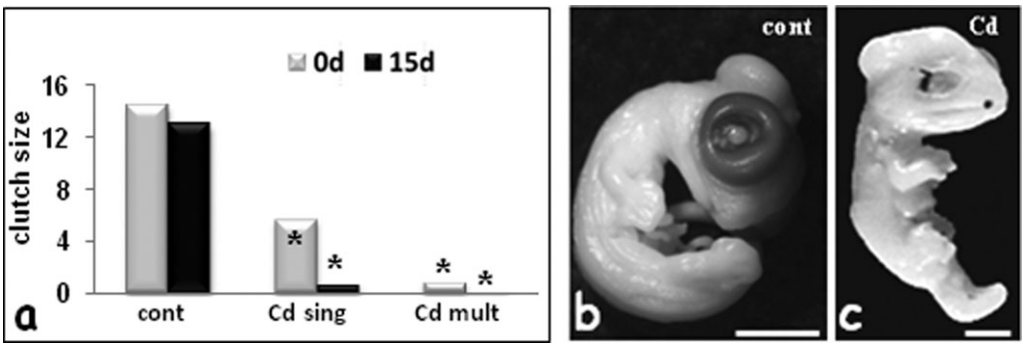

Fig. 2. Effects of a single or multiple injections of Cd on fecundity. (a) Effects on clutch size: the ion significantly ($p < 0.05$, *) decreases the number of vital eggs at deposition (0 days) and 15 days after deposition. (b) Regularly developed 15 days embryo obtained from a control female. (c) Embryo recovered from a treated female showing anopthalmia and defects in limb and tail development. Bars: 2 mm.

eggs for single and multiple treatments, respectively [Fig. 2(a)]. Embryo mortality also dramatically increases after treatments. Seven days post deposition in controls 13.0 ± 1.3 eggs are still developing while after Cd the average number has fallen to 0.7 ± 0.6 and 0.2 ± 0.02 (single and multiple treatments, respectively).

The few embryos recovered from treated mothers show very severe deformities [Fig. 2(c)] with respect to controls [Fig. 2(b)]; head, limbs, and tail are scarcely developed, the brain is often extruded from the braincase and the eye cup is deformed and/or abnormally pigmented. All control embryos developed regularly until birth.

Cadmium Changes MT Expression

In situ hybridizations carried out to identify cells involved in MT expression reveal that in controls MT mRNA is present in the cytoplasm of all small stem cells and, to a lesser extent, in the cytoplasm of large pyriforms [Fig. 3(a)]. After Cd treatment MT mRNA transcripts are concentrated in the cytoplasm of scattered pyriforms where they form an intensely stained cap; occasionally labeled small cells are also visible [Fig. 3(b,c)].

Cadmium Impairs Follicle Organization

In untreated animals, growing oocytes are round and enveloped by a thick epithelium [Fig. 4(a–c)]. After Cd treatments, follicles are deformed by the presence of large protrusions [Fig. 4(e)]. These usually contain an apparently normal oocyte cytoplasm and are enveloped by a regularly arranged epithelium, basal membrane, and theca [Fig. 4(e)]. Cytological analyses reveal that single and multiple treatments induce essentially comparable effects on the ovaries. These include an increased vascularization of the theca [Fig. 4(d)], the occurrence of atresia among the early previtellogenic follicles and alterations in follicle organization [Fig. 7(d)].

In other follicles, however, epithelial thickening is present with protrusion into the oocyte cytoplasm [Fig. 4(e,f)] or, less often, into the theca [Fig. 4(g)]. These form masses filled with loosely adherent [Fig. 4(f)] or strictly packed

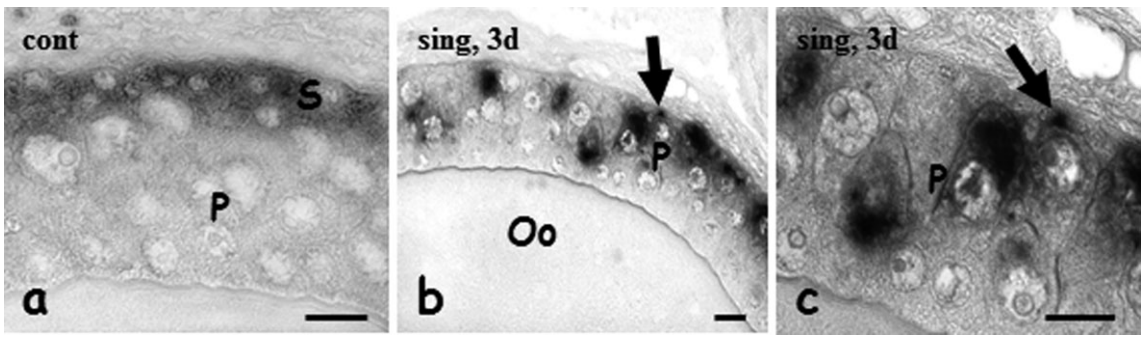

Fig. 3. MT mRNA expression in untreated (a) and Cd treated (b,c) ovarian follicles. (a) Messenger is present in small cells (S) and, to a lesser extent, also in the cytoplasm of pyriform cells (P). (b) Intense labeling on scattered pyriforms; occasional S cells are also labeled (arrow). The oocyte (Oo) cytoplasm is unlabeled. (c) Detail of fig. b. Labeled S cell (arrow). Cont: control; Sing: single Cd dose; d: days from beginning of treatment. Bars: (a–c) 15μm.

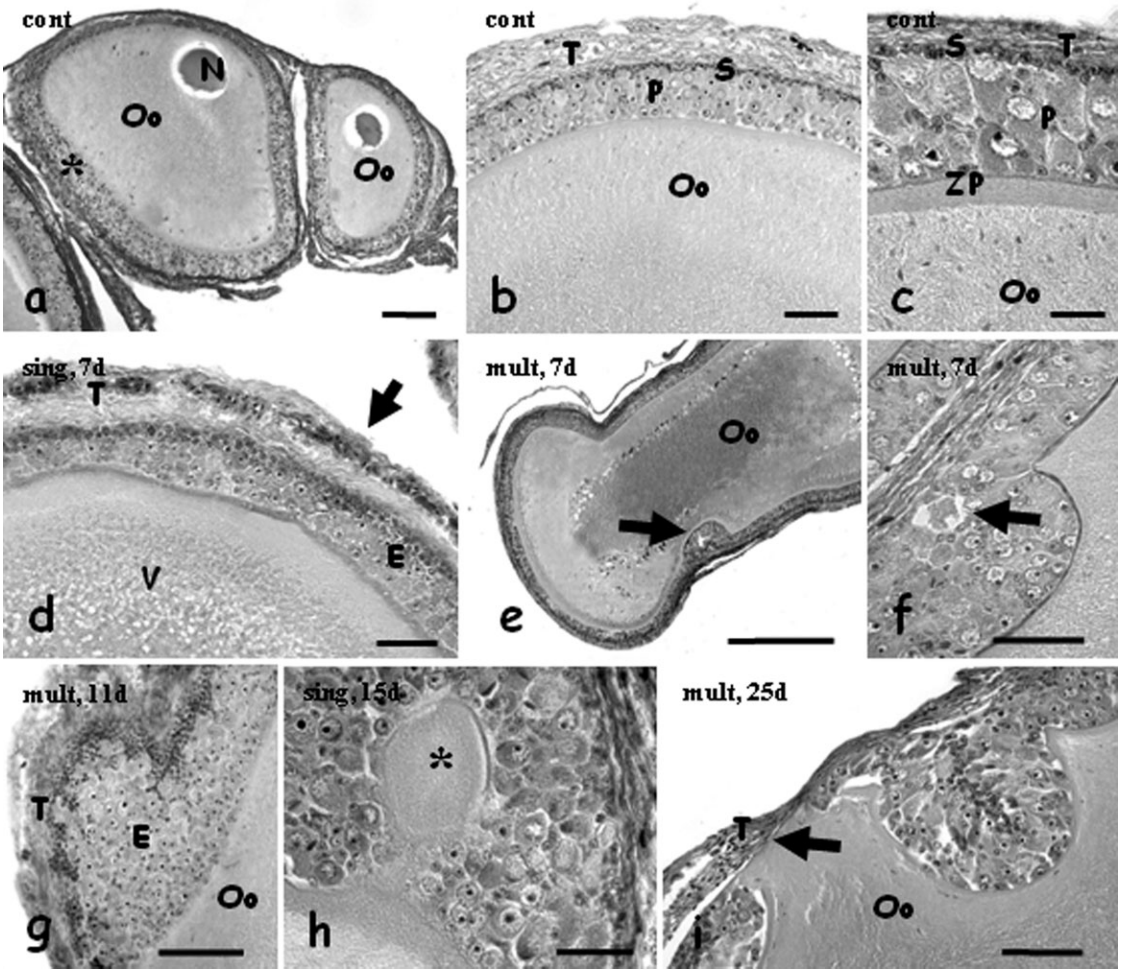

Fig. 4. Effects of Cd on ovarian follicles morphology. (a–c) controls; growing oocytes (Oo) surrounded by a thick follicular wall (*). Oocyte nucleus (N). (b,c) Details show the theca (T), the outer layer of epithelial stem cells (S), and the differentiated pyriforms (P). ZP: zona pellucida. (d–i) Cd treated animals. (d) Follicle with highly vascularized theca (arrow). Notice the presence of vesicles (V) in the oocyte cytoplasm. (e) Deformed follicle with a patch of altered epithelium (arrow). (f) Detail of the epithelial thickening showing the loosely adherent cells (arrow). (g) Epithelial thickening (E) protruding in the theca. Note the strictly packed cells. (h) Thickening containing a small bleb of oocyte cytoplasm (*). (i) Absence of follicular epithelium: the oocyte membrane (arrow) is in direct contact with the theca (T). Hemalum-eosin staining; Cont: control; sing: single Cd dose; mult: multiple Cd doses; d: days from beginning of treatment. Bars: (a,b, d, f,g, i) 50 μm; (c,e) 20 μm; (h) 30 μm.

[Fig. 4(g)] cells and may contain small blebs of oocyte cytoplasm [Fig. 4(h)]. Thickenings are often characterized by an overdeveloped germ layer [Fig. 4(g)]. In few cases, the epithelium is interrupted and the oocyte membrane is in direct contact with the theca [Fig. 4(i)].

Significant alterations are also observed at the level of the oocytes where Cd induces hydropic swelling [Fig. 5(a)] with cytoplasmic vesicles containing a clear [Fig. 5(b)] or a filamentous [Fig. 5(c)] material. Analyses with lectins indicate that they do not contain PAS [Fig. 5(d)], WGA [Fig. 5(e,f)], or UEA [Fig. 5(g,h)] positive sugar residues. In the cytoplasm of cortical oocytes, however, Cd induces the formation of many small granules positive to PAS [Fig. 5(d)], WGA [Fig. 5(e)], and LEA [Fig. 5(h)] staining.

The zona pellucida (ZP) is also affected by Cd treatment. In controls [Fig. 6(a)] it is interrupted only by intercellular bridges connecting the cytoplasm of pyriforms cells to that of the oocytes (Andreuccetti et al., 1978). Following Cd administration, the ZP frequently shows the presence of small vesicles [Fig. 6(c)] or becomes disorganized with patches of altered epithelium [Fig. 6(d)]. These alterations are particularly evident after PAS staining. In controls, the

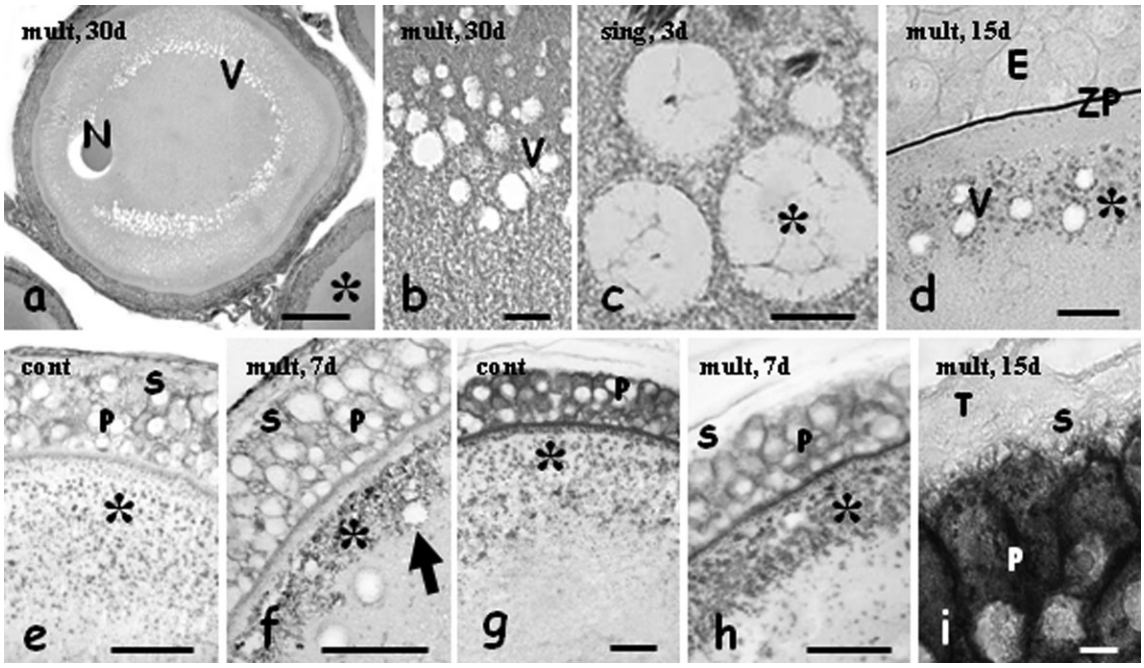

Fig. 5. Effects of Cd on oocyte cytoplasm. (a) Vacuolated cytoplasm (V) in a treated oocyte. Oocyte nucleus (N), non vacuolated oocyte cytoplasm (*). (b) Detail of vesicles (V) containing a clear material. (c) Detail of vesicles containing a filamentous material (*). (d) PAS stains the zona pellucida (ZP) and the small granules present in the oocyte cortex (*) but not the oocyte cytoplasm, the large vesicles (V) and the epithelium (E). (e,f) Labeled WGA stains follicle cells cytoplasm (S and P cells) and the dense granules dispersed in the cortical oocyte cytoplasm (*). Notice how these latter significantly increase in number after treatment. Large vesicles (arrow) remain unstained. (g–i) Labeled UEA stains pyriform cells cytoplasm (P) but not small (S) cells. Labeling is also present on the dense granules (*), particularly numerous in treated oocytes. (i) Detail showing the labeled P cells and the unlabeled S cells. Theca (T). (a–c): hemalum-eosin, (d): PAS staining, (e,f): staining with POX-labeled WGA, (g,h): staining with POX-labeled UEA. Cont: control; sing: single Cd dose; mult: multiple Cd doses; d: days from beginning of treatment. Bars: (a) 150 μm; (b) 20 μm; (c,i) 10 μm; (e–h) 50 μm.

ZP is uniform and intensely stained [Fig. 6(e)] but after Cd treatment, vesicles [Fig. 6(f)] and disorganized areas [Fig. 6(g)] are clearly recognizable.

Treated epithelia show increased apoptotic pyriforms. In controls, apoptosis occurs exclusively in the late previtellogenesis stage when occasional regressing pyriform cells can be observed [Motta et al., 2001; Fig. 7(a)]. In treated animals, apoptotic pyriform cells become very frequent [Fig. 7(b–d)] and large disorganized areas appear in the epithelium [Fig. 7(e)]. In treated animals, apoptosis also occurs in cells of primary and early previtellogenic follicles [Fig. 7(f,g)] in which cell regression is normally absent. The presence of atretic follicles is also observed in treated [Fig. 7(g)] but not in control ovaries (Andreuccetti et al., 1990).

Increased apoptosis is accompanied by an increased proliferation rate in small stem cells as demonstrated by PCNA immunolocalization. This is evident in primary follicles [Fig. 7(j)] and also in growing follicles [Fig. 7(k,l)] in which proliferation is usually rare [Fig. 7(h,i)]. In both controls and treated follicles, PCNA positive reaction is localized in the oocytes nuclei [Fig. 7(h)].

DISCUSSION

Environmental Cd is readily taken up and distributed by the blood stream to the ovaries of all animals studied, with the subsequent appearance of acute disorder (Szczerbik et al., 2006; Massanyi et al., 2007; Nad et al., 2007). In Podarcis, a lacertid reptile, Cd accumulation in ovaries also occurs (Trinchella et al., 2006; Simoniello et al., 2008, 2010b) and this is accompanied by severe damage in both follicular epithelial cells and oocytes. The effects of the dose tested were time independent, persisting essentially unchanged regardless of single or multiple administrations. Cd stimulates stem cell proliferation in the epithelium as demonstrated by the increased number of cells positive for the

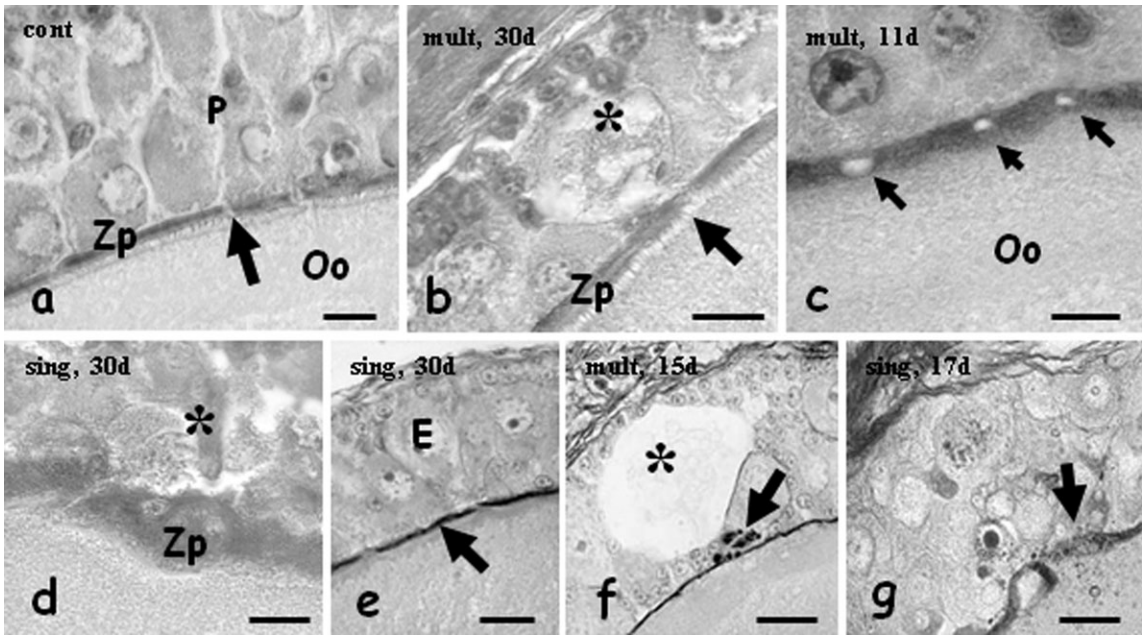

Fig. 6. Effects of Cd on the zona pellucida (ZP). (a) In controls the ZP is interrupted by the intercellular bridges (arrow) connecting the cytoplasm of the pyriform cells (P) with the oocyte (Oo) cytoplasm. (b) Normal ZP with a bridge (arrow) and a degenerating pyriform (*). (c) ZP with vesicles (arrows). (d) Irregular ZP close to disorganized epithelial cells (*). (e) PAS positive ZP (arrow). The epithelial cells (E) and the oocyte cytoplasm are almost unstained. (f) ZP forming large PAS positive vesicles (arrow); notice the regressing pyriform (*). (g) Disorganized ZP (arrow). (a–d): hemalum-eosin staining; (e–g): PAS staining. Cont: control; sing: single Cd dose; mult: multiple Cd doses; d: days from beginning of treatment. Bars: (a–c,e) 10 μm; (d) 5 μm; (f,g) 10 μm.

PCNA following treatment. This effect contrasts with that reported in other cell systems in which Cd delays proliferation and stops cell cycle progression (Blottner et al., 1999; Yang et al., 2004; Xie and Shaikh, 2006). The mechanism by which Cd induces proliferation in *P. sicula* remains unknown. One possibility, however, is that it has mimicked the endogenous gonadotropins (Simoniello et al., 2010b) or estrogens (Jang et al., 2003) by acting, like other metallic endocrine disrupters, as a metalloestrogen (Henson and Chedrese, 2004; Darbre, 2006).

The intense cell proliferation induced by Cd would explain the formation of thickening in the follicular epithelium. The increased number of cells could have generated mechanical forces, which have induced epithelial bending outward or inward to invade either the theca or the oocyte cytoplasm. As Cd interferes with cell adhesion molecules, these movements, as well as the formation of large protrusion from oocytes, may have been favored by a reduced adherence among follicle cells and/or between follicle cells and the basal membrane (Paksy et al., 1997). A role may have been also played by disruption of cytoskeletal organization by Cd. Oocyte shape depends on a criss-crossed protein network composed of actins, tubulins, cytokeratins, and spectrins (Maurizii et al., 1997; Ricchiari et al., 2004), whose structure and function can be altered by Cd (Lau and Chiu, 2007; Thompson et al., 2008). Whatever the mechanism of their formation might be, the epithelial thickening and the oocyte protrusions appear to significantly alter the 3-dimensional architecture of the follicle and this is suggestive of a significant impairment of proper oocyte growth and maturation.

Further impairment of oocyte functionality could come from the hydrotropic swelling (Simoniello et al., 2008, 2010b) resulting from the formation of large cytoplasmic vesicles and by alterations of the ZP. This barrier between oocyte and follicle cells has a fundamental role in sperm recognition and binding (Wassarman, 1989). Impaired sperm–egg interaction and general fecundity may be manifest as treated animals show significantly reduced clutch size and a low rate of embryo survival compared to untreated animals.

An unexpected result from this study is the decreased MT mRNA expression in small cells as MTs are proteins involved in Cd detoxification (Trinchella et al., 2006; Klaassen et al., 2009). Therefore, an increased expression in the small cells was anticipated. Being located in the outer epithelium in close contact with the vascularized theca they are likely exposed to the highest Cd concentrations.

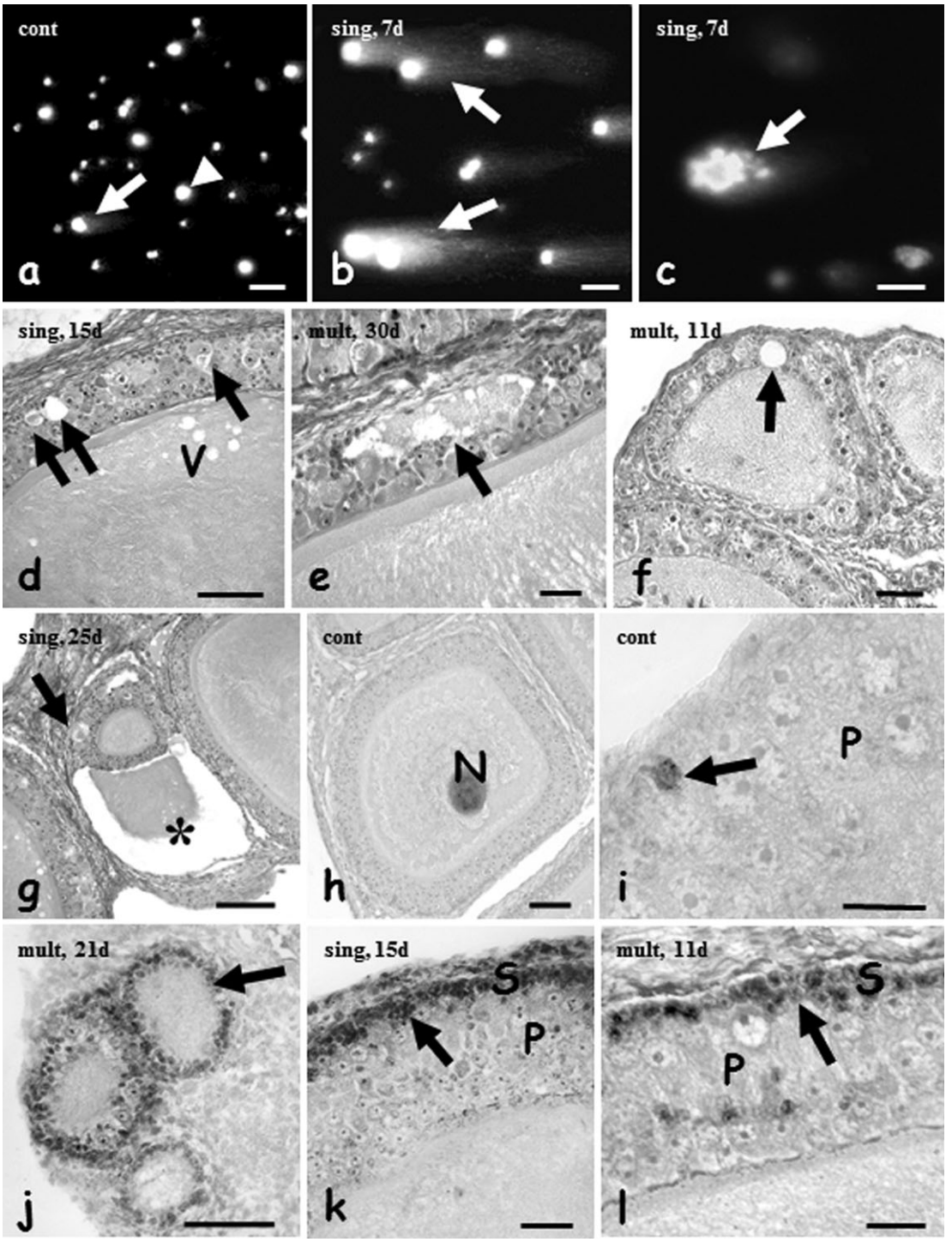

Fig. 7. Effects of Cd on follicle cell proliferation and death. (a–c) COMET assays. (a) Control epithelium shows occasional apoptotic cells (arrow). Non apoptotic cells (arrowhead). (b) Cd induces apoptosis in most follicle cells (arrow). (c) Detail of a cell nucleus forming apoptotic bodies (arrow). (d) Treatment significantly increases the number of apoptotic pyriforms (arrows). Vesicles (V). (e) Extensive cell regression causes the appearance of disorganized areas (arrow) in the epithelium. (f) Apoptotic cell (arrow) in epithelium of a primary follicle. (g) Atretic follicle (*). Notice the presence of an apoptotic cell (arrow) in the epithelium of a very small adjacent follicle. (h–l) PCNA immunolocalisation. (h–i) Controls. Labeling on oocyte nucleus (N) and cytoplasm of occasional small follicle cells (arrow). (j–l) Cd treated females. Labeling is on all small (S) follicle cells (arrows). Pyriforms (P) are always unlabeled. (a–d) Hemalum-eosin staining; Cont: control; sing: single Cd dose; mult: multiple Cd doses; d: days from beginning of treatment. Bars: (a,d,g,h) 50 μm; (b,c,e,f, i–l) 20 μm.

Whether Cd has down-regulated MT expression or simply induced a massive translation reducing stored cytoplasmic mRNA is still unclear. Previous investigations have demonstrated a significant increase in ovarian MT proteins (Riggio et al., 2003) after Cd exposure but whether this is due to the translation of messenger present in small cells or to *de novo* synthesis by the pyriforms remains to be investigated.

In conclusion, our data show that Cd induces morpho-functional alterations in lizard follicles and indicates that these are responsible for a significant impairment of oogenesis. If we also consider that the same Cd treatments have dramatic effects on embryo development (Simoniello et al., 2011), it can be seen that even occasional, sublethal Cd contamination may significantly impair reproductive performance in these animals. Further studies will be required to assess the ecological impact of these effects in regions where Cd pollution is expected to be widespread.

The authors gratefully thanks Dr. Edward Howell for critical reading of the manuscript.

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