CARYOLOGIA Vol. 59, no. 4: 403-408, 2006

Cadmium accumulation induces apoptosis in *P. Lividus* embryos

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Abstract — Cadmium is a heavy metal considered one of the most toxic pollutants both in terrestrial and aquatic ecosystems, which causes a series of toxic effects in differents organisms. We have previously studied the effects of a continuous exposure to subacute/sublethal cadmium concentration in *Paracentrotus lividus* embryos, a very suitable model system for testing how specific stresses can affect development and protein synthesis (Roccheri, 2004).
In this paper we demonstrate by Atomic Absorption Spectometry that when sea urchin embryos were treated since fertilization with 100 μM $CdCl_2$, the metal is accumulated during time in the cells. We found by TUNEL assays on whole mount embryos that prolonged exposure to cadmium induces severe fragmentation of DNA. We demonstrate that cleaved caspase-3 is involved in apoptosis execution induced by cadmium.

Key words: apoptosis, cadmium, development, sea urchin.

INTRODUCTION

Cadmium is a potent cell poison, causing different types of damage, including cell death and it is a highly toxic environmental pollutant released from the smelting and refining of metals. Its very long biological half-life (30 years) is responsible for the almost irreversible accumulation in human organs (Nordberg 1984), thus causing several adverse effects. Experimental evidences show that cadmium is bioaccumulated by marine mollusks including oysters (Roesijadi 1996).

Although cadmium toxicity is well proved, its cytotoxic effect is controversial, given that some authors have indicated that cadmium can kill cells after a prolonged exposure (Kaji 1993), while others emphasize that it is a carcinogen in animals and humans (Jin & Ringertz 1990; Koizumi & Li 1992; Kazantzis 1992). Numerous pathologies, like teratogenesis and carcinogenesis, due to cytotoxic concentrations of the ion, have been described both in invertebrates both in higher organisms (Sunderman 1992; Waalkes and Rehm 1998).

Several investigations have demonstrated that the Cd cytotoxicity is associated with ROS which has also been demonstrated to perform certain functions in the early stages of apoptosis. An increase of ROS induces an increase of proapoptotic molecules levels in the cytosol (Shih 2004; Buttke and Sandstorm 1994; Pourahmad and O'Brien 2000).

Some authors have suggested that cadmium induces apoptosis in several cell-types (Habeebu 1998; Ishido 1999; Tsangaris & Tzortzatou-Stathopoulou 1998; Kim 2000; Risso De Faverney 2001; Kondoh 2002). Although Cd has been demonstrated to induce toxicity through apoptotic cell death, the precise pathway remains poorly understood.

Sea urchin embryos are a suitable model system for studying the responses to different stresses, through activation of putative mechanism of defence and/or cell death. It is known that sea urchin embryos are able to undergo to apoptosis, both in physiological conditions and in response to chemical and physical stress (Roccheri 2002, 1997; Vega and Epel 2004; Pellerito 2005; Lesser 2003).

In a previous paper (Agnello 2006) we have demonstrated that 1mM $CdCl_2$ induces massive apoptosis 24 hours after treatment. The present work is aimed to understanding if cadmium is accumulated during time, whatever concentration is, provoking the same apoptotic events.

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MATERIALS AND METHODS

Embryo culture and treatment - Gametes were collected from gonads of the sea urchin *P. lividus* harvested from the West coast of Sicily. Eggs were fertilized at a concentration of 5000/ml and grown under gentle rotation at 18°C in millipore filtered sea water (MFSW) containing antibiotics (50mg/L streptomycin sulfate and 30mg/L penicillin). Just after fertilization, embryos were continuously cultured in the presence of 1 mM, 100 µM or 10 µM $CdCl_2$. Development was monitored by optical microscopy.

Determination of the metal - In order to measure cadmium content 100 mg of embryos were digested with 1 ml of 70% HNO_3 and 0.5 ml of 30% H_2O_2 at 150°C in oil bath. The dried residues were reconstituted in 0.2% HNO_3 and analyzed for cadmium contents by Atomic Absorption Spectrometry using a Perkin-Elmer apparatus model 5100 equipped with Zeeman graphite furnace.

TUNEL assay - Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (Promega) was performed on whole mount embryos. Embryos were fixed with a solution of 0,1% formaldehyde in MFSW, washed several times and permeabilized with cold methanol, washed with MFSW and PBST (0.15 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.15 M NaCl, 0.05% Tween 20) and then incubated for 60 min. at 37° C with 50 µl of a solution containing the following reagents: equilibration buffer, TdT enzyme (10 U), and fluorescent nucleotide mix (5 µ*M fluorescein 12-dUTP). Negative controls embryos were incubated in the same mixture but without the enzyme; positive controls embryos were pretreated with DNAase (10 µg/ml) before the TdT assay. Nuclei were stained by incubation with propidium iodide (2µg/ml). The reaction was interrupted by incubating the embryos with NaCl/Sodium-Citrate (300mM/30mM) for 15 min, followed by observations under confocal microscope (Olympus FV 300 with a He-Ne 543 nm laser).*

Embryo fixation - Embryos, at pluteus stage (38 hours of development), were fixed for 12–16 h with Bouin fixative (15:5:1 of picric acid solution, 37% formaldehyde, glacial acetic acid, respectively), kept at 4°C in 70% ethanol after serial dehydratation.

Immunocytochemistry - Samples of about one thousand embryos were analyzed. After washings in 100% ethanol and in 100% methanol containing 2 mM EDTA, the embryos were rehydrated with decreasing ethanol concentrations, washed 3 times with PBST and incubated for 12 hours with the following antibodies, diluted in PBST in the presence of 3% BSA: polyclonal anti cleaved-caspase-3 (Cell Signaling; 1:100 dilution) and monoclonal anti pro-caspase-3 (Santa Cruz; 1:50 dilution). In the negative controls the primary antibodies were omitted. The embryos were washed 3 times with PBST and then incubated for 1 h with the secondary antibodys respectively anti-rabbit and anti-mouce IgG alkaline phosphatase (AP) conjugated (1:1000 dilution; Promega). The antibody excess was eliminated by washing embryos 3 times with PBST and then with TBS (150 mM NaCl, 10mM Tris-HCl pH 8). Staining was performed in AP buffer (100mM NaCl, 100mM Tris–HCl, pH 9.5, 50mM $MgCl_2$) containing NBT/BICP (Boehringer Mannheim). The reaction was blocked with 50% ethanol. Whole embryos resuspended in 80% glycerol in PBST were finally mounted on coverslips, observed under an Axioskope MC80 microscope (Zeiss) and photographed with 100 Asa Kodak Gold film.

RESULTS

We have previously demonstrated that in *Paracentrotus lividus* embryos the intracellular quantity of cadmium highly increased during exposure time and causes severe DNA fragmentation and occurrence of apoptotis. In this work we have investigated the effects of embryo exposition to lower cadmium concentration but for prolonged times.

In order to study cadmium effect on embryo development, we performed the following experiments. Embryos just after fertilization were cultured in presence of three different cadmium concentrations: 1mM, 100µM and 10µM. Treatment with 1mM cadmium chloride for 24h provokes remarkable developmental delay and morphological aberrations (fig.1 A and B); in addition treatments with lower concentrations (100µM and 10µM) for 38 hours or 7 days cause severe morphological abnormalities (see fig. 1, D and F respectively).

These results suggest that higher cadmium concentration for shorter times causes similar morphological damages than the treatment with lower concentration for longer time, and then the occurrence of abnormalities depends both on

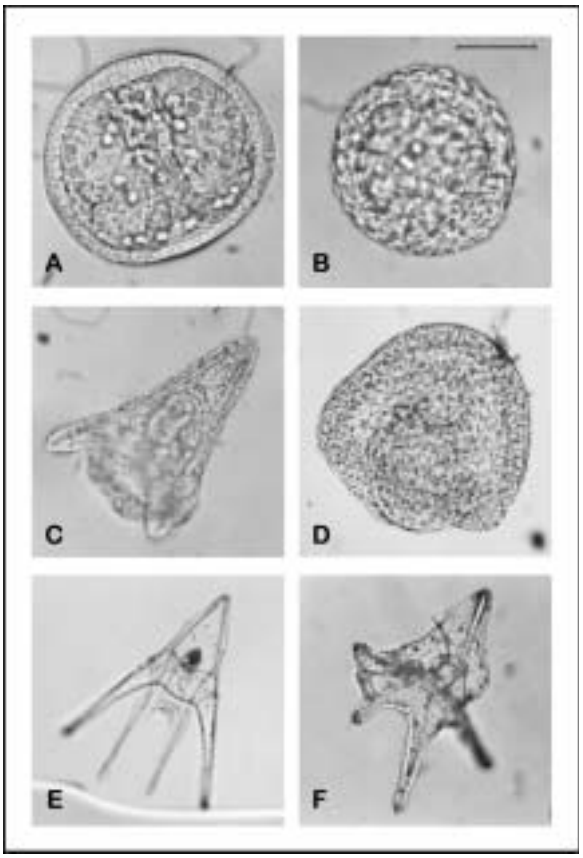

Fig. 1 — Sea urchin embryos treated with cadmium: morphological observation by optical microscopy of sea urchin embryos exposed continuously to 1mM $CdCl_2$ for 24h (B), 100µM $CdCl_2$ for 38h (D) or 10µM $CdCl_2$ for 7 days (F). Control embryos after 24h (A), 38h (C) and 7 days (E) of development. Bar=60µm (A, B, D); 40 µM (C, E, F).

cadmium concentration and on exposure time. In order to measure the ion content, embryos were incubated soon after fertilization with 100 µM $CdCl_2$ for a time range between 3h and 48h, and the amount of metal in 100 mg of embryos was measured by AAS. Results shown in fig.2 demonstrated that cadmium was accumulated during time.

It is noteworthy that the amount of incorporated cadmium exponentially increases during time probably because the presence of the pluteus intestine facilitates the metal entry.

To understand if the accumulation of cadmium and the morphological aberrations are related to damages of DNA, we performed TUNEL assays on whole mount embryos. Results suggest that 38 hours of exposure triggers severe fragmentation of DNA (fig.3 A) demonstrating that this event is related to the exposure time. Neglegible DNA fragmentation in the arms and in the intestine cells of control plutei (fig. 3 D) is due to the physiological apoptosis, a process implicated in the development (Roccheri *et al*. 2002).

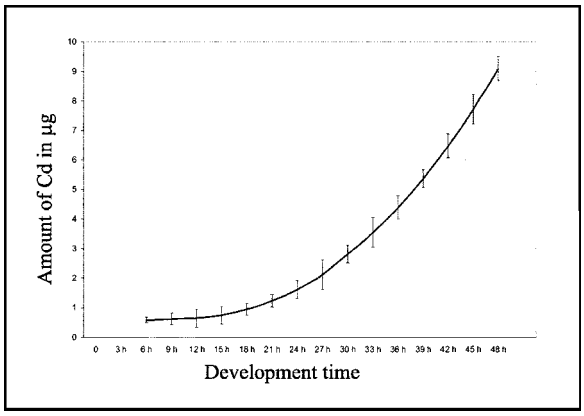

Fig. 2 — **Cadmium accumulation during time**. The embryos were cultured in 100µM $CdCl_2$. Cadmium content was tested by Atomic Absorption Spectrometry (AAS), measuring the metal amount in 100 mg of embryos. Graph was obtained by the mean of three experiments; standard deviations are indicated.

To verify if DNA fragmentation was related to apoptotic events, we performed immunocytochemical experiments in whole mount embryos treated with 100µM $CdCl_2$, reacting with anti-cleaved-caspase-3 and anti-pro-caspase-3 antibodies. It is known in fact that the execution of programmed cell death is usually operated by a proteolytic cascade involving caspase activation. Results showed the presence of cleaved caspase-3 (fig. 4 A) and not of procaspase-3 in treated embryos (fig. 4 C), while in the control embryos we observed opposite results, as suggested by the presence of uncleaved pro-caspase-3 (fig. 4 D).

In conclusion the results suggest that cadmium induces apoptosis through the cleavage of caspase-3, by a pathway different than the one activated during the physiological development.

DISCUSSION

The toxic effects of the heavy metals have been studied since many years in marine invertebrate embryos, which are subjected to the risk of contamination in their habitat. The echinoderm embryos and larvae represent a widely used experimental model, given their high sensitivity to chemical and physical environmental changes, and their peculiar position in the marine trophic chain, where pelagic larvae are part of the diet of several planctonic and benthonic organisms. Sea urchin embryos, like many other organisms, are

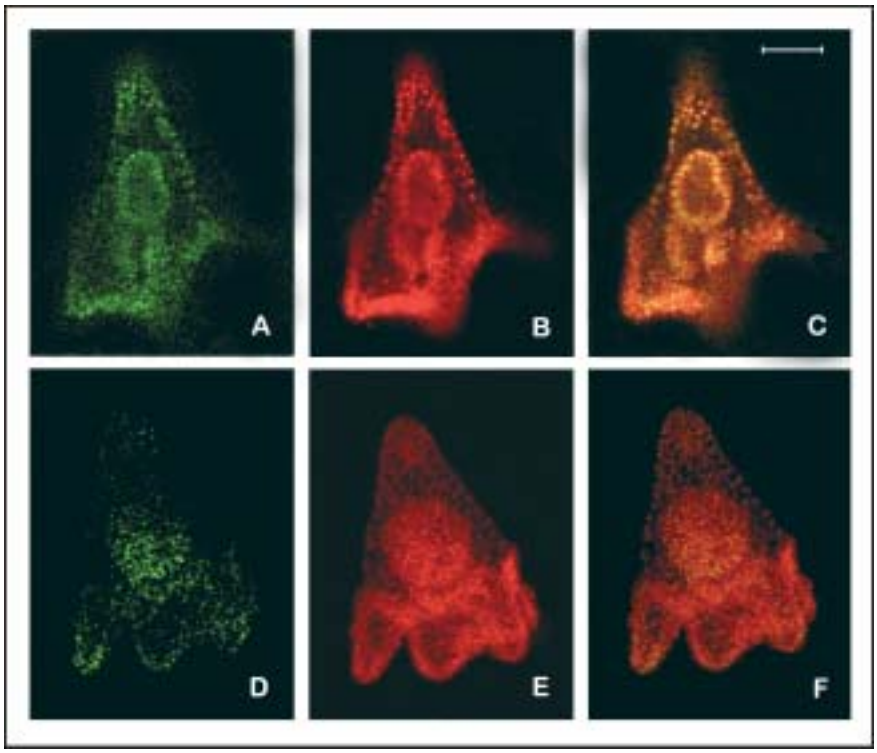

Fig. 3 — **DNA fragmentation:** Whole mounts of pluteus embryos observed after TUNEL assay. The images obtained from confocal laser scanning microscopy show equatorial sections. Green, DNA fragmentation (A,D); red, nucleic acids (propidium iodide) (B,E); merge of green and red (C,F). Embryos treated for 38h with 100 μM $CdCl_2$ (A-C); control embryos (D-F); Bar=40μm.

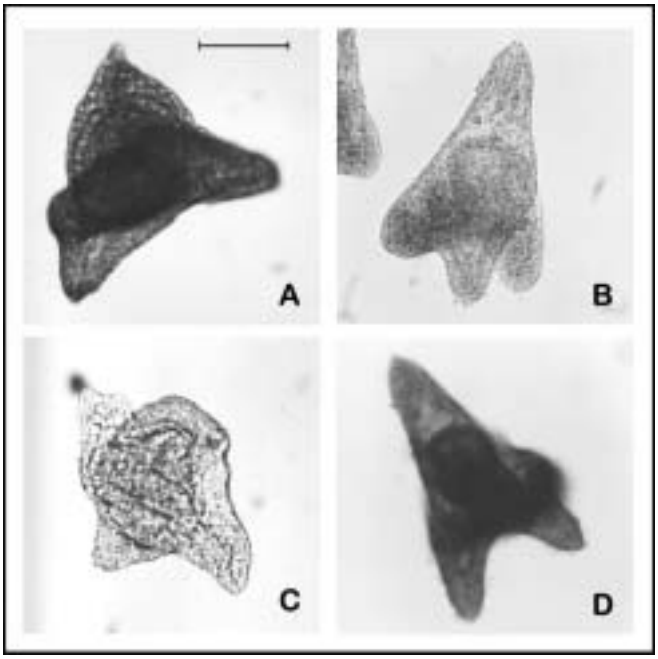

Fig. 4 — **Immunolocalization of cleaved-caspase-3 and pro-caspase-3**. Embryos treated with 100μM $CdCl_2$ whole mounted and incubated respectively with anti cleaved-caspase-3 antibody (A) and with anti pro-caspase-3 antibody (C). Control embryos (38h of development) incubated with anti cleaved-caspase-3 (B) and anti pro-caspase-3 antibodies (D). Bar=40μm

able to respond to many stresses activating the synthesis of highly conserved proteins in the evolution, the heat shock proteins (Hsps) (ROCCHERI 1981) and/or the metallothioneines (RUSSO 2003; SCUDIERO 1995). We have previously demonstrated that the ability to synthesize Hsps represents for sea urchin embryos a protective general strategy against several stress agents (ROCCHERI 1981; 1988; 1993; CASANO 1998). During Hsps induction cells are refractory to the toxic effects of several agents, and the protecting effect is partially due to the inhibition of apoptosis (SAMALI and COTTER 1996). As we have demonstrated, the programmed physiological cell death is involved during sea urchin development and metamorphosis (ROCCHERI 2002), other than in the response to specific external stimuli. Although Cd has been demonstrated to induce apoptotic cell death in several cell-types, the precise pathway remains poorly understood. Is well known that apoptotic cell death can be induced through distinct pathways based on caspase-3 activation and culminating in cell death (KISCHKEL 1995; FULDA 2001).

In this paper we demonstrate that in embryos treated since fertilazation whit 100 μM $CdCl_2$ the amount of incorporated cadmium exponentially increased during time, probably because the newly formed intestine facilitates metal internalization. In addition, we show that treatment with lower cadmium concentration for longer times causes similar morphological damages than with higher concentration for shorter times. We can then hypothesize that the occurrence of abnormalities would depends both on cadmium concentration and on exposure time. The increasing accumulation suggests that even very small amounts of cadmium in the environment could cause the increase of the metal levels inside the cell up to cytotoxic values. Therefore the accumulation of the ion in the embryos would probably induce the severe DNA fragmentation observed. We demonstrate that the DNA damages are, in this case, a final event of apoptosis induction through the activation of caspase-3. In addition the results suggest that cadmium induced apoptosis follows a different pathway than the one activated during physiological development, which does not implicate caspase-3 cleavage.

Acknowledgment — We thank G. Morici for the computing assistance. We are deeply indebted to prof. Silvana Filosa (Univ. of Napoli) for achievement of AAS experiments. This work was supported by grants from MURST (ex 60% and COFIN 2004 prot. 2004057282_003).

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