Ecotoxicology and Environmental Safety 72 (2009) 37–44

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Cadmium- and lead-induced apoptosis in mallard erythrocytes (*Anas platyrhynchos*)

D. Romero [a,*], A. Hernández-García [a], C.A. Tagliati [b], E. Martínez-López [a], A.J. García-Fernández [a]

[a] Department of Toxicology, Faculty of Veterinary Medicine, Espinardo Campus, 30100 Murcia, Spain
[b] Departamento de Análisis Clínicos y Toxicológicos, Facultad de Farmacia, Universidad Federal de Minas Gerais, Belo Horizonte, Brazil

ARTICLE INFO

Article history:
Received 21 May 2007
Received in revised form
25 April 2008
Accepted 2 May 2008
Available online 24 June 2008

Keywords:
Lead
Cadmium
Erythrocytes
Flow cytometry
Microscopy
Viability
Apoptosis

ABSTRACT

Cadmium, lead and cadmium–lead (1:10) induced apoptosis were studied using mallard blood cells. The allowable range in concentrations were: 0.01–0.5, 0.1–5.0, and 0.01:0.10–0.50:5.00 mM, for cadmium, lead and cadmium–lead, respectively. The lowest EC_{50} achieved was for cadmium (0.22 ± 0.04 mM). Two doses from each treatment group were chosen to study apoptosis and the presence of metals in cells. The percentage of apoptotic cells increased as the concentration of metals increased. The percentage of cells with intracellular metals was high for both exposure levels and the quantity of intracellular metal was greater for exposure to high concentrations. Morphological alterations for all types of exposure were related to the diverse range of effects that these metals have on membranes. We suggest that the decrease in the number of erythrocytes observed in specimens suffering from lead and cadmium poisoning is related to the induction of apoptosis.

© 2008 Elsevier Inc. All rights reserved.

1. Introduction

Environmental pollution caused by heavy metals is responsible for numerous pathologies in wild species. Lead has been the cause of serious cases of mortality in different types of birds (Eisler, 1988). Cadmium has had serious consequences for the survival and reproduction of birds. Lead causes numerous alterations of a functional and morphological nature in red blood cells (Lumeij, 1985; Grue et al., 1986; Beyer et al., 1988; Pain, 1989; Mateo et al., 2003). Haemolytic anaemia has been described in wild birds poisoned by this element (Mateo et al., 2003), as has an increased fragility of erythrocytes (Moore, 1988; Rosenberg et al., 1998). However, there are few papers describing alterations in the blood cells of birds exposed to cadmium (Cain et al., 1983).

Lead and cadmium have recently been associated with the induction of apoptosis (Oberto et al., 1996; He et al., 2000; Wätjen and Beyersmann, 2004; Banfalvi et al., 2005); and even with oxidative stress phenomena (Hsu and Guo, 2002; Mateo et al., 2003; Bertin and Averbeck, 2006). In studies performed on mammal erythrocytes exposed to lead, Kempe et al. (2005) concluded that apoptosis presumably contributes to a decrease in the life-span of erythrocytes and the development of anaemia in cases of lead poisoning. However, we found no data in the bibliography associating this phenomenon with exposure to cadmium, nor did we find *in vitro* studies using bird erythrocytes.

In birds, erythrocytes can perish rapidly via lysis or pycnosis (Burgoyne, 1999), the latter being the most characteristic expression of programmed cell death (Arends et al., 1990; Walker et al., 1994; Peitsch et al., 1994; Burgoyne, 1999). Erythrocytic pycnosis is extremely simple and mainly due to an energy deficit (Burgoyne, 1999). Most recent experiments reveal that injured (anucleated) erythrocytes display phosphatidylserine on their surface (Berg et al., 2001; Bratosin et al., 2001; Daugas et al., 2001; Lang et al., 2002; Brand et al., 2003), a key feature of apoptosis in nucleated cells (Daugas et al., 2001). The externalization of phosphatidylserine allows us to consider standardized techniques for the study of apoptosis, such as measuring annexin binding via flow cytometry (Van Engeland et al., 1996).

Wild animals are commonly exposed to a mixture of environmental contaminants. However, there are few studies on the toxicity of metal mixtures and they are often based on the frequency of occurrence and the level of contamination in a particular ecosystem (Jadhav et al., 2007).

In the present study, mallard (*Anas platyrhynchos*) erythrocytes were exposed to lead, cadmium and a cadmium–lead combination (1:10) in proportions equal to those found in wild birds in the Region of Murcia (Martínez-López et al., 2004, 2005). The effects of these metals were studied via flow cytometry (Propidium

* Corresponding author. Fax: +34 968364317.
E-mail address: diegorom@um.es (D. Romero).

0147-6513/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2008.05.004

Iodide, Annexin V and Leadmium^{TM} Green staining), optical and transmission electron microscopy (TEM).

2. Material and methods

Studies were conducted in accordance with the national laws for the protection of animals used for experimental and other scientific purposes (Real Decreto 1201/2005).

2.1. Preparation of cultures

Blood samples were taken from a healthy mallard (*A. platyrhynchos*). A combination of trisodium citrate and citric acid (66:44 mmol/L) was utilized as an anticoagulant, at a final citrate–blood concentration of 1:10, according to the INVITTOX protocol no. 37 (1992). Erythrocytes were obtained via density gradient (Percoll 50%, Hamk 10%, MilliQ 40% purified water). The cells were kept in PBS with glucose at 10 mmol/L and +4 °C until utilization. Assays were performed during the first week after obtaining erythrocytes.

2.2. Test chemicals

Lead was used in the form of nitrate and cadmium in the form of chloride (Merk Chemical Co., Darmstadt, Germany). Both were initially prepared in sterile purified water, at 100 mM (lead nitrate) and 10 mM (cadmium chloride). These were used to prepare working dilutions in PBS with glucose at 10 mmol/L. Propidium iodide ($C_{27}H_{34}N_5I_2$) (Sigma, USA) was prepared in PBS at a concentration of 400 μg/ml. To assure that the effects evaluated were in fact the result of exposure to metals, the osmolarity of the culture mediums containing the samples was verified using a Vapro© 5520 osmometer (WESCOR).

2.3. Cytotoxicity assay

All assays were performed in triplicate. The exposure to cadmium, lead and cadmium–lead (1:10) took place in Eppendorf vials in which 100 μl of cell suspension (3.35×10^8 cells) and 1000 μl of glucosated PBS were added, together with different concentrations of metals. At the same time, a negative control was prepared with glucosated PBS. Purified water was used as a positive control. The samples were kept in an incubator (Sanyo MCO-15A) for 24 h at +39 °C in a FALC F205 orbital agitator.

Following 24 h of exposure, 10 μl of the sample were gathered and transferred to a cytometry tube with 400 μl of PBS, to which 100 μl of propidium iodide (400 μg/ml) were added immediately. Cell viability was measured via flow cytometry using a Beckman Coulter XL cytometer at a wavelength of 488 nm. The EXPO32 ADC program was used for the gathering and analysis of data.

2.4. Study into the induction of apoptosis

In order to evaluate the induction of apoptosis, two doses from each treatment group were chosen, one entitled "*low dose*" and the other "*high dose*". According to the results obtained in the dose–response curve, the "*low doses*" chosen were 0.05, 0.50 and 0.05:0.50 mM for cadmium, lead and cadmium–lead, respectively; and the "*high doses*" were 0.25, 2.50 and 0.25:2.5 mM for cadmium, lead and cadmium–lead, respectively.

The study into the induction of apoptosis was performed using the "Alexa Fluor 488" Annexin V kit (Invitrogen, USA). This fluorochrome (annexin V) binds to the phosphatidylserine of the plasmatic membrane of apoptotic cells. After incubation in the presence or absence of cadmium chloride and/or lead nitrate, 1×10^6 cells were washed in 100 μl of annexin V binding buffer. A solution composed of annexin V conjugate (5 μl) and a solution composed of propidium iodide (1 μl, 400 μg/ml) were added. After 15 min of incubation (room temperature), samples were finally diluted at 1:5 in annexin binding buffer and measured via flow cytometry analysis in a Beckman Coulter XL cytometer with an excitation wavelength of 488 nm.

2.5. The presence of metals in cells

The treatment groups and doses were the same as for the previous section. Leadmium^{TM} Green dye (Invitrogen, USA) is a specific indicator of lead or cadmium in cells. This fluorochrome binds to the cadmium and/or lead inside live cells. After incubation in the presence or absence of cadmium chloride and/or lead nitrate, 1×10^6 cells were washed in 1000 μl of saline solution (NaCl 85%, 37 °C), mixed with a solution composed of Leadmium^{TM} Green conjugate (4 μl), incubated and washed according to the manufacturers instructions. Samples were measured via flow cytometry analysis in a Beckman Coulter XL cytometer, with an excitation wavelength of 488 nm.

2.6. Optical and transmission electron microscopy

Microscopy studies were carried out using the same groups and treatment doses described in the previous sections. Cell morphology was studied using a *Nikon Diaphot-TM* phase contrast optical microscope, with a NIKON Digital Sight DS-L1 image capture and digitalization system. Towards this aim, 10 μl of sample per well were diluted in 500 μl glucosated PBS and transferred to a 24-well dish.

For the ultrastructural morphology study, erythrocytes were collected in one tube alone and centrifuged (50g, 10 min, 2 °C). The sediment was fixed immediately in 3% glutaraldehyde (v/v), post-fixed in 1% osmium tetroxide (v/v) and stained with 4.8% uranyl acetate. Following dehydration, samples were washed in propylene oxide and impregnated with epoxy resins. The semifine sections were contrasted with uranyl acetate and lead citrate for study via microscopy. The microphotographs were taken with a Zeiss EM 10 °C transmission electron microscope.

2.7. Statistical study

The SPSS 15.0 package was utilized for statistical purposes. All values are expressed as means ± standard error of the mean. Statistical analysis was performed via the *T* comparison test. Correlations between doses–response curves and correlations between mortality and apoptosis were analyzed using Pearson's test. Except for when otherwise stated, *p*-values lower than 0.05 were considered statistically significant.

3. Results

The viability of erythrocytes at the beginning of the assays was 99.1–100%. The osmolarity of final samples was between 297 and 306 mmol/kg (lead nitrate), 295 and 301 mmol/kg (cadmium chloride) and between 291 and 299 mmol/kg for the cadmium–lead mixture (1:10). The osmolarity of the negative control culture was 293–317 and 50–58 mmol/kg for the positive control.

The 1:10 cadmium–lead combination was utilized as such since this proportion is equivalent to those found in wild birds in the Region of Murcia (Martínez-López et al., 2004, 2005).

3.1. Cytotoxicity assays

The allowable range in concentrations was established as 0.01–0.5, 0.1–5.0, and 0.01:0.10–0.50:5.00 mM, for cadmium, lead and cadmium–lead (1:10), respectively. The percentages for viability at each concentration tested are displayed in Table 1. The lowest EC_{50} was achieved for exposure to cadmium (0.22 ± 0.04 mM) (Table 1). There was no correlation between cell

Table 1
Percentage of live cells following exposure to lead, cadmium, cadmium:lead (1:10) and effective concentration 50 (EC_{50}) (mean ± SD)

Pb/Cd (mM)[a]	Viability (%)		
	$Pb(NO_3)_2$	$CdCl_2$	$CdCl_2:Pb(NO_3)_2$
0.1/0.01	99.10 ± 0.01	99.25 ± 0.07	99.20 ± 0.52
0.3/0.03	99.25 ± 0.35	98.45 ± 0.50	98.70 ± 0.71
0.5/0.05	98.05 ± 0.91	97.13 ± 0.95	96.70 ± 1.04
0.7/0.07	96.85 ± 3.89	94.80 ± 0.32	97.70 ± 0.98
0.9/0.09	94.10 ± 0.99	88.73 ± 7.85	95.70 ± 1.21
1.0/0.10	95.10 ± 0.90	88.73 ± 4.54	93.93 ± 4.07
1.2/0.12	93.10 ± 1.24	80.37 ± 6.57	93.50 ± 3.25
1.5/0.15	91.18 ± 5.41	67.25 ± 7.11	92.83 ± 2.97
2.5/0.25	82.83 ± 6.31	55.17 ± 9.98	85.80 ± 5.55
3.0/0.30	48.45 ± 7.50	22.33 ± 8.71	55.10 ± 7.56
3.5/0.35	35.75 ± 0.78	14.70 ± 6.41	35.05 ± 6.51
4.0/0.40	30.50 ± 9.33	9.75 ± 5.41	21.37 ± 6.25
4.5/0.45	4.95 ± 0.92	4.87 ± 2.28	11.38 ± 3.78
5.0/0.50	4.60 ± 2.26	5.60 ± 3.11	4.55 ± 1.20
EC_{50}	3.38 ± 0.36	0.22 ± 0.04	0.32:3.20 ± 0.01:0.10

[a] $Pb(NO_3)_2/CdCl_2$.

viability following exposure to each metal separately and following exposure to the mixture.

3.2. The study of apoptosis

Table 2 shows the percentages of both live and apoptotic cells achieved using the annexin V kit. The exposure to *low doses* of metals resulted in a reduced percentage of apoptotic cells, with the highest value being achieved following exposure to lead (18.00±9.87%). Following exposure to *high doses*, the percentage of apoptotic cells increased significantly for all three treatments ($p<0.01$). For the control group, 90.91% (±2.61) of dead cells were undergoing apoptosis. In the group exposed to low concentrations, the percentages of dead and apoptotic cells were: 95.94±0.64% (lead), 83.36±6.59% (cadmium) and 84.57±1.86% (cadmium–lead). For the group exposed to high concentrations, the percentages of dead and apoptotic cells were as follows: 99.30±0.37% (lead), 78.52±10.20% (cadmium) and 98.24±0.76% (cadmium-lead).

3.3. Study into the presence of intracellular metal

Table 3 shows the percentage of both live cells and of those with intracellular metal, achieved with the Leadmium™ Green kit. The percentage of cells with metal in their interior was high for both types of exposure, there being no significant statistical differences between exposures to high and low concentrations, nor between metals, for each treatment group. The percentage of live cells (propidium iodide staining) was, for all treatments, lower than those obtained in dose–response and annexin V studies. Likewise, Table 3 displays the intensity of the fluorescence, which is equal to the quantity of fluorochrome and as such, equal to the quantity of metal inside the cell. The intensity was greater in cases of exposure to high concentrations, there being statistically significant differences with respect to the quantity of intracellular metal following exposure to low concentrations.

3.4. Optical microscopy

The control culture cells were of an elliptical morphology and had an elongated centralized nucleus (Fig. 1a), with a small percentage of small round cells of an orange hue (5–10%). The cells from cultures treated with low concentrations of metals (Fig. 1b–d) maintained their elliptical morphology, although there was a small percentage of round cells (1–5%). A greater quantity of cells was observed with an orange hue with respect to controls (25–30%), with scarcely any cellular agglomeration. There was an obvious presence of deformed cells in the cases of exposure to cadmium and cadmium–lead (Figs. 1c and d). Following exposure to high concentrations (Figs. 1e–g), images similar to those described for low concentrations were observed, albeit with a greater quantity of refringent, rounded and deformed cells grouped in masses, thus being impossible to count.

3.5. Transmission electron microscopy

The control cells were of a diverse morphology, predominantly elongated and without organelles (Fig. 2), although some cells displayed "lumpy" structures in their cytoplasm. Nuclei were round in shape with a surrounding halo and evenly distributed chromatin. In cells exposed to 0.05 mM cadmium chloride (Fig. 3a) the nuclei were similar to those of the control group, although some displayed large vacuoles and electrodense structures of varying morphology and position. The perinuclear halo had a greater width than those of the control group and "lumpy" structures were observed surrounding the cells. The cells exposed to 0.5 mM lead nitrate (Fig. 3b) displayed diffuse, occasionally eccentrically displaced nuclei, with electrodense structures similar to those described for exposure to cadmium. Large creases were observed in the cytoplasmic membrane, which on occasion encapsulated electrodense structures in the form of lumps. In the case of exposure to 0.05:0.5 mM cadmium chloride–lead nitrate (Fig. 3c) highly similar images were found to those described for

Table 2
Cell viability (propidium iodide) and apoptosis (annexin V) in mallard erythrocytes exposed to high and low concentrations of cadmium, lead and cadmium:lead (mean±SD)

Treatment (mM)	% live cells	% apoptotic cells
Control	97.5±1.21	3.49±0.89
Low concentrations		
$Pb(NO_3)_2$ (0.5)	92.07±3.36	18.00±9.87
$CdCl_2$ (0.05)	92.23±4.50	10.87±7.05*
$CdCl_2$:$Pb(NO_3)_2$ (0.05:0.5)	95.30±0.66	4.80±1.08
High concentrations		
$Pb(NO_3)_2$ (2.5)	79.50±0.71**	51.50±3.25***
$CdCl_2$ (0.25)	56.60±6.98***	46.83±9.97***
$CdCl_2$:$Pb(NO_3)_2$ (0.25:2.5)	84.33±1.66***	61.57±9.15***

* $p<0.1$.
** $p<0.05$.
*** $p<0.01$.

Table 3
Cell viability (propidium iodide), presence of metals within cells and the intensity of the fluorescence in mallard erythrocytes (Leadmium™ Green) exposed to high and low concentrations of cadmium, lead and cadmium:lead (1:10) (mean±SD)

Treatment (mM)	% live cells	% cells with lead and/or cadmium	Intensity of the fluorescence[a]
Control	96.25±3.04	1.10±1.27	–
Low concentrations			
$Pb(NO_3)_2$ (0.5)	80.40±2.26	81.65±1.20	3.30±0.20
$CdCl_2$ (0.05)	85.97±3.15	87.23±2.61	3.97±0.12
$CdCl_2$:$Pb(NO_3)_2$ (0.05:0.5)	77.70±7.45	77.93±2.72	3.73±0.51
High concentrations			
$Pb(NO_3)_2$ (2.5)	66.17±7.54	83.37±5.67	7.50±0.95**
$CdCl_2$ (0.25)	49.70±2.17	73.57±3.84	8.23±1.68**
$CdCl_2$:$Pb(NO_3)_2$ (0.25:2.5)	73.83±6.60	82.70±4.55	5.80±0.52**

[a] A dimensional parameter.
** $p<0.05$.

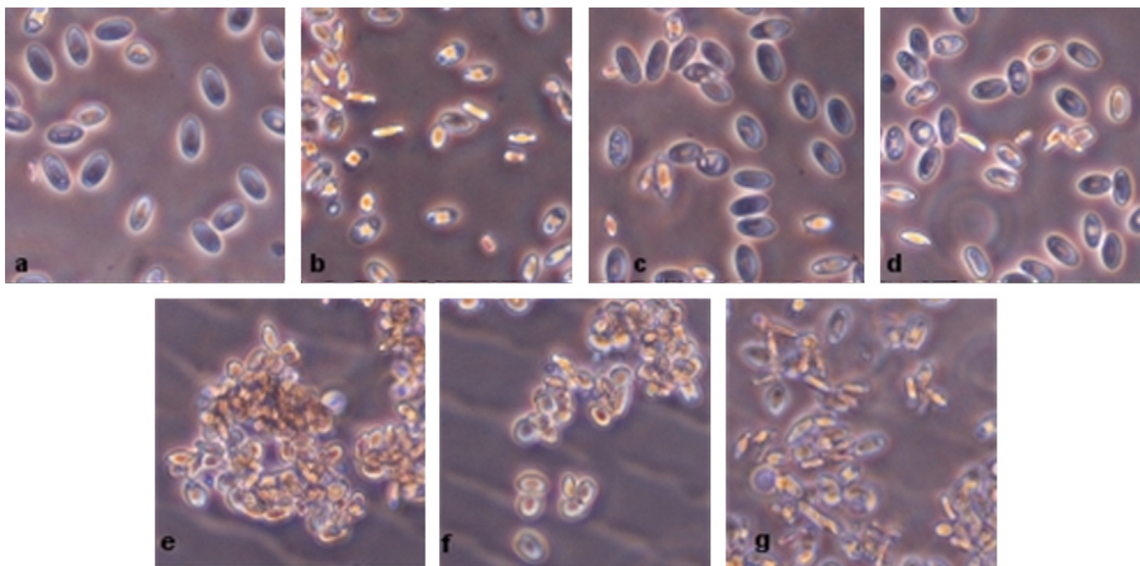

Fig. 1. Mallard erythrocytes (200 ×). Control culture (a): cells of an elliptical morphology with a central nucleus. Low number of small cells with an orange hue. Cultures exposed to $CdCl_2$ (0.05 mM) (b), $Pb(NO_3)_2$ (0.5 mM) (c) and $CdCl_2$:$Pb(NO_3)_2$ (0.05:0.5 mM) (d): cells of an elliptical morphology, cells of an orange hue and small size and deformed cells. Cultures exposed to $CdCl_2$ (0.25 mM) (e), $Pb(NO_3)_2$ (2.5 mM) (f) and $CdCl_2$:$Pb(NO_3)_2$ (0.25:2.5 mM) (g): cells of an orange hue, small in size with lumpy formations.

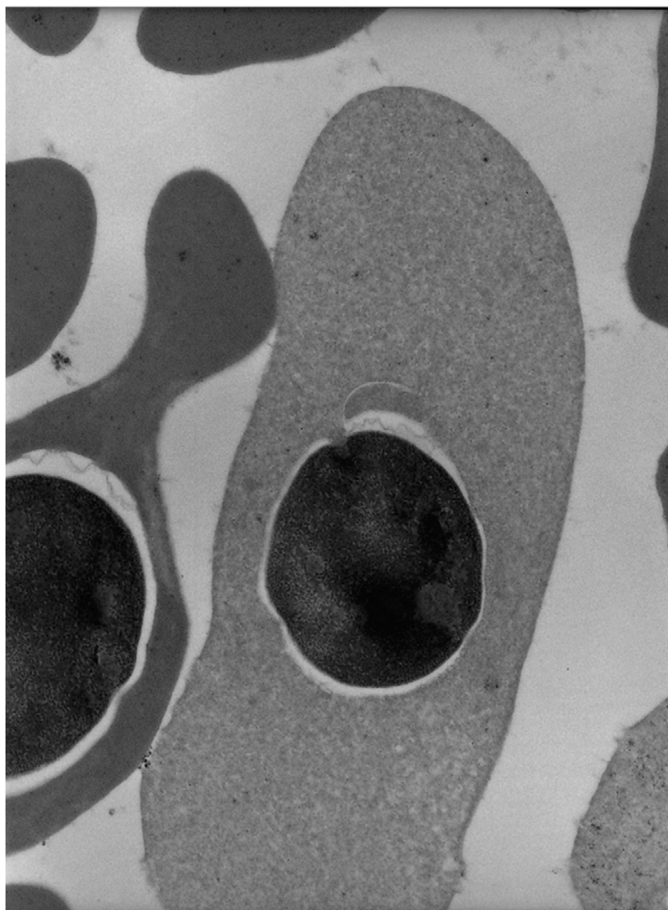

Fig. 2. Mallard erythrocyte control culture (12,500 ×). Cell with an elliptical morphology and centralized nucleus with surrounding halo, no organelles.

exposure to lead, although there were also cells with lumpy extracellular structures and apparently normal nuclei, similar to those described for the case of exposure to cadmium. Following exposure to 0.25 mM $CdCl_2$ (Fig. 4a) cells were observed with a morphology similar to that of the control group, albeit with externalized cytoplasmic and nuclear content, vacuolated cytoplasm and evenly distributed chromatin. In the case of erythrocytes exposed to 2.5 mM $Pb(NO_3)_2$ (Fig. 4b), aggregates appeared with electrodense lumps and an apparent loss of nuclear content. Large accumulations of electrodense lumps were observed surrounding the erythrocytes exposed to 0.25:2.5 mM of the cadmium–lead mixture (Fig. 4c), together with the apparent displacement of cytoplasmic and nuclear content.

4. Discussion

Evaluating the osmolarity of cultures exposed to metals allowed us to discard effects derived exclusively from osmotic pressure. Furthermore, the use of orbital agitators throughout the entire course of the experiment allowed for an even exposure to be applied over the entire culture, thus avoiding the deposition of both cells and metals.

4.1. Control cultures

It is known that bird erythrocytes are capable of maintaining their functional state under *in vitro* conditions (Viscor et al., 1987), which makes them an excellent material for researching the effects of environmental contaminants such as lead and cadmium in wild birds. Mallard erythrocytes display a high percentage of cell viability at the moment blood samples are collected (99.1–100%), which was maintained through to the end of the assays (99.1–99.25%). The low percentage of apoptotic cells (3.49%) could be considered physiological, which coincides with that described by Bratosin et al. (2001) in human blood, who stated it was normal to find 1% senescent erythrocytes in peripheral circulation. The morphological study showed a cell population with a reduced number of pycnotic cells, a process made evident by the existence of an orange hue, as described by Burgoyne (1999). The ultrastructural study showed cells with similar characteristics to those

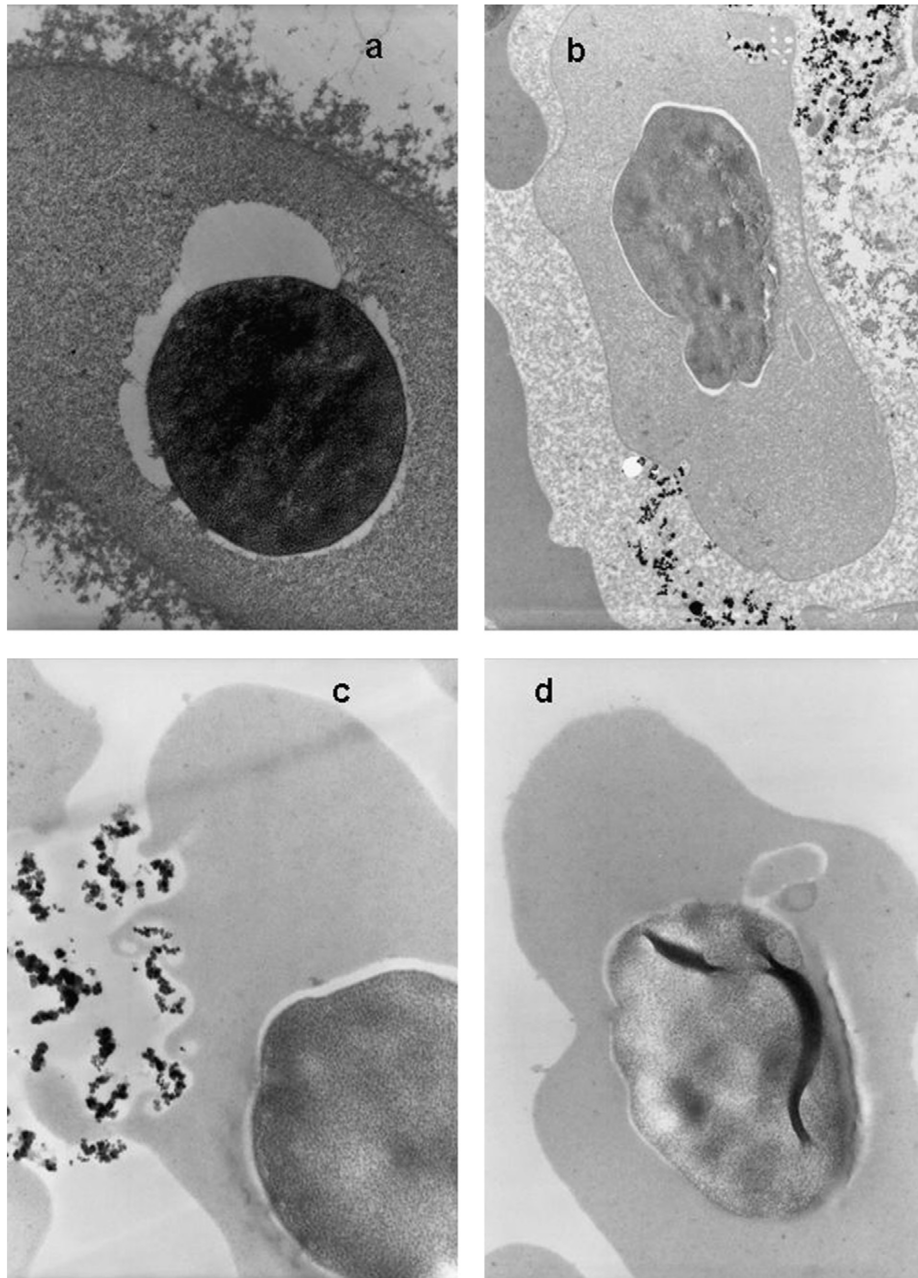

Fig. 3. Mallard erythrocytes exposed to low concentrations of metals. Culture exposed to $CdCl_2$ (0.05 mM) (a: 16,000 ×): ample halo surrounding the nucleus and lumps on the outside of the plasmatic membrane. Culture exposed to lead (0.5 mM) (b: 8000 ×): electrodense lumps in the cytoplasm. Culture exposed to cadmium–lead (0.05:0.5 mM) (c: 25,000 ×): electrodense lumps on the cytoplasm edge; cells with electrodense structures in the nucleus (d: 24,600 ×).

described by other authors for non-poisoned bird erythrocytes (Burgoyne, 1999).

4.2. Cytotoxicity assays

The erythrocytes were more sensitive to cadmium ($EC_{50} = 0.22 \pm 0.04$ mM) than they were to lead ($EC_{50} = 3.38 \pm 0.36$ mM). We found no correlation between the dose–response curves for each metal and that of the mixture. As such, we cannot confirm that the effects of exposure to said mixture are attributable to either metal in particular. As occurred in the present study, other *in vitro* research using different cell cultures showed a lower EC_{50} for cadmium than for lead, although we found no research using either a mixture of both metals or bird erythrocytes in the bibliography.

4.3. Exposure to low concentrations of metals

Although the percentage of apoptotic cells was low (less than 20% for both metals, Table 2), this could be said to be expected, given the capacity of both metals to induce such a phenomenon (Oberto et al., 1996; He et al., 2000; Wätjen and Beyersmann, 2004; Banfalvi et al., 2005). Following exposure to said concentrations most of the dead cells had undergone apoptosis, a fact made evident by the presence of both fluorescent markers (propidium iodide and annexin V), although the percentage was noticeably lower in the case of exposure to cadmium and cadmium–lead. A low percentage of smaller cells with an orange hue were observed under optical microscopy (Figs. 1b–d), this having been described by other authors as the typical image of apoptosis in birds (Burgoyne, 1999). The aforementioned cells

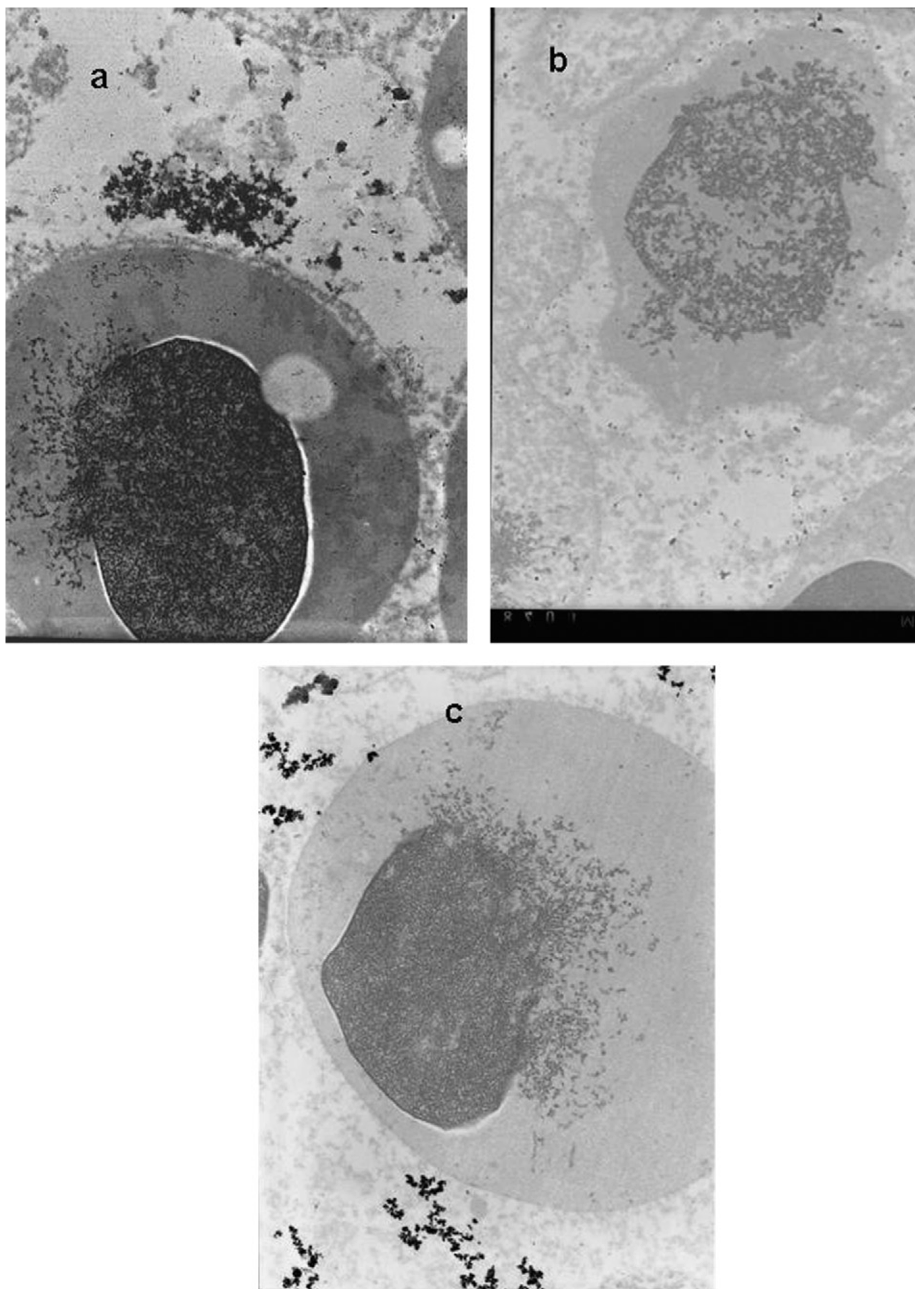

Fig. 4. Mallard erythrocytes exposed to high concentrations of metals: (a) culture exposed to 0.25 mM cadmium chloride (10,000 ×). (b) Erythrocytes exposed to 2.5 mM lead nitrate (10,000 ×). (c) Erythrocytes exposed to 0.25:2.5 mM cadmium chloride:lead nitrate (10,000 ×). In all cases there was a breakage in the nuclear membrane, externalization of its content into the cytoplasm and lumpy structures surrounding the cytoplasmic membrane.

could correspond to the low percentage of annexin V positive cells (Table 2). Likewise, a greater number of deformed cells were observed following exposure to cadmium and cadmium–lead (Figs. 1b and d). Both metals are capable of producing alterations in membranes due to free radicals (Skoczynska and Smolik, 1994; Jan and Frantisek, 2000), but this result could be directly related to the capacity of cadmium to produce alterations in the molecular structure of the bilipidic layer and in the membrane proteins (Lundbaek et al., 1996; Suwalsky et al., 2004). Perhaps these cadmium-associated alterations accelerate cell lysis, which would justify the lower percentage of cells with both fluorochromes in cases of exposure to cadmium and cadmium–lead. At such low concentrations, apoptosis could be an important phenomenon in the prevention of hemolysis, as stated by Lang et al. (2005), although less effective when the medium contains cadmium.

However, the hypothesis that apoptosis is the principal cell-death mechanism at low doses does not correlate with observations on an ultrastructural level, since none of the typical images of apoptotic cells described by Burgoyne (1999) were found. The large orifices found in both the nucleus and cytoplasm of the cells exposed to both metals, could be the consequence of the alterations described in the membranes by various authors (Rosenberg et al., 1998; Goyer, 1996; Hamada et al., 1998; Jan and Frantisek, 2000; Mojzis and Nistiar, 2001); and in turn, could be the cause of the appearance of lumpy structures surrounding the cytoplasmic membrane (Fig. 3) as well as of cell lysis. New experiments are being designed in order to examine whether the

cause of death in these cells was due to lysis or whether, on the contrary, they might undergo phagocytosis upon externalizing phosphatidylserine.

Moreover, in cases of exposure to lead and the lead–cadmium combination, lumpy structures were seen to penetrate the cell from the outside (Figs. 3b and c). Electrodense structures were also observed in the nucleus of cells in all cases, although more frequently in the case of exposure to lead and cadmium–lead. The aforementioned structures do not correspond to those described in nuclei by Burgoyne (1999) as pycnosis and nucleolytic pycnosis. It is known that lead induces the formation of intranuclear inclusion bodies (Goyer, 1996; Vicente-Ortega et al., 1996; Romero et al., 2004). It is possible, as occurs in other cells exposed to lead, that said inclusion bodies are formed, which might transform the image of the apoptotic cells, making them differ from those described by Burgoyne (1999). In the case of cadmium, despite having been detected on a nuclear level both *in vivo* (Matssura et al., 1991; Marshall et al., 1994) and *in vitro* (Hamada et al., 1994), only occasionally have intranuclear inclusion bodies been seen to be formed (Romero et al., 2003), which might justify the lower frequency of electrodense structures in the nuclei. The elongated nuclear structures (Fig. 2c) could be deformities and folds in the nuclear membrane, since lead is capable of deforming erythrocytes (Terayama, 1993; Baranowska-Bosiacka and Hlynczak, 2003).

4.4. Exposure to high concentrations of metals

The higher concentrations produced an increase in the percentage of dead cells, while at the same time increasing considerably the percentage of apoptotic cells (Table 2). The statistical analysis showed a concentration-dependent correlation between mortality and apoptosis ($r = 0.699$, $p < 0.01$), which clearly indicates that the apoptotic phenomenon is closely linked to the death of the erythrocytes exposed to the metal in question. It is known that the presence of a greater quantity of metal in the culture medium causes an increase in the percentage of cells with annexin (Kempe et al., 2005). The latter authors described this phenomenon in human erythrocytes exposed to lead, although the range utilized by said authors (0.1–3 μM) was narrower than that utilized in the present study (500–2500 μM). Interspecific differences or the existence of nuclei in bird erythrocytes could explain the difference in sensitivity. Furthermore, we found no data in the bibliography regarding the induction of apoptosis by cadmium in erythrocytes. Nonetheless, research conducted on other cell cultures show that cadmium is capable of destroying cells due to necrosis at high doses and due to apoptosis at low doses (El-Azzouzi et al., 1994), with the latter being more effective (Hamada et al., 1996). This does not coincide with our observations, since the increase in concentrations favoured the induction of apoptosis, with a positive correlation between mortality and concentration-dependent apoptosis. Given that the majority of dead cells also contained annexin, one could postulate that apoptosis precedes lysis, a fact described previously with regard to necrosis (Habeebu et al., 1998; Aydin et al., 2003). As in the case of exposure to low concentrations, new experiments could shed light on whether these cells perish due to lysis or whether apoptosis, and subsequently phagocytosis, are allowed to run their course.

Observations made via optical microscopy (Figs. 1e–g) show cells with the same type of alterations described for low concentrations, albeit in greater number, as well as the tendency to agglomerate, which could be related to the higher percentage of apoptotic cells (Table 2).

TEM (Figs. 4a–c) reveals the same alterations as the case of exposure to low concentrations, albeit to a greater degree and mainly in the form of a loss of cytoplasmic and nuclear content. As occurs for low doses, these alterations could be related to the diverse range of effects that these metals have on membranes, thus being the cause of said lysis.

4.5. The presence of metals in cells

The cultured cell viability in the Leadmium™ Green assays (Table 3) was lower than that obtained for the dose–response (Table 1) and annexin assays. It is known that these metals affect the membrane structures via different mechanisms (Rosenberg et al., 1998; Goyer, 1996; Hamada et al., 1998; Jan and Frantisek, 2000; Mojzis and Nistiar, 2001). Perhaps the weakened state of the cells following exposure to the metals, together with the necessary handling for carrying out the Leadmium™ Green technique accelerates the death process, something much more evident at higher concentrations (Table 3).

In the treated cultures, at both high and low concentrations, all the viable cells contained lead and/or cadmium. At high concentrations, the percentage of cells containing metal was even higher than that of viable cells. Taking into account the fact that Leadmium™ Green is a fluorochrome which penetrates live cells exclusively (Bradford et al., 2005), this would justify the aforementioned hypothesis that the cells were alive at the time they were exposed to the fluorochrome and that they perished due to treatments subsequent to being marked.

The fact that the cultures exposed to higher concentrations of lead, cadmium and cadmium–lead displayed a more intense fluorescence (2.27 times greater in the case of lead, 2.08 times greater for cadmium and 1.55 times more for exposure to cadmium–lead) (Table 3) could justify an intracellular dose-dependent effect.

5. Conclusion

In view of these results, we would suggest that the decrease in the number of erythrocytes observed in specimens suffering from lead and cadmium poisoning could be related to the induction of apoptosis, this becoming more evident as the quantity of metal present within cells increases. Nonetheless, more experiments are required in order to discern whether the cell death is caused by lysis or whether it is a consequence of incomplete apoptosis due to the absence of phagocytes in *in vitro* assays.

Acknowledgments

This study received backing from CICYT/FEDER Project CGL2004-5959 (MASCA'2004). Thanks to Pedro María Mojica and Pilar Gómez Ramírez for collecting blood samples. Thanks also to the Consellería de Territori i Habitatge de la Generalitat Valenciana for providing said samples.

References

Arends, M.J., Morris, R.G., Wyllie, A.H., 1990. Apoptosis: the role of the endonuclease. Am. J. Pathol. 136, 593–608.
Aydin, H.H., Celik, H.A., Deveci, R., Terzioglu, E., Karacali, S., Mete, N., Akarca, U., Batur, Y., 2003. Characterization of the cellular response during apoptosis induction in cadmium-treated Hep G2 human hepatoma cells. Biol. Trace Elem. Res. 95, 139–153.
Banfalvi, G., Gacsi, M., Nagy, G., Kiss, Z.B., Basnakian, A.G., 2005. Cadmium induced apoptotic changes in chromatin structure and subphases of nuclear growth during the cell cycle in CHO cells. Apoptosis 10, 631–642.
Baranowska-Bosiacka, I., Hlynczak, A.J., 2003. The effect of lead ions on the energy metabolism of human erythrocytes in vitro. Comp. Biochem. Physiol. C 134, 403–416.

Berg, C.P., Engels, I.H., Rothbart, A., Lauber, K., Renz, A., Schlosser, S.F., Schulze-Osthoff, K., Wesselborg, S., 2001. Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. Cell Death Differ. 8, 1197–1206.
Bertin, G., Averbeck, D., 2006. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). Biochimie 88 (11), 1549–1559.
Beyer, W.N., Spann, J., Sileo, L., Franson, J.C., 1988. Lead poisoning in six captive avian species. Arch. Environ. Contam. Toxicol. 17, 121–130.
Bradford, J., Godfrey, W., Martin, V., Gee, K., 2005. Intracellular detection of lead and cadmium with a fluorescent probe using flow cytometry. Experimental Biology Meeting, San Diego.
Brand, V.B., Sandu, C.D., Duranton, C., Tanneur, V., Lang, K.S., Huber, S.M., Lang, F., 2003. Dependence of *Plasmodium falciparum* in vitro growth on the cation permeability of the human host erythrocyte. Cell. Physiol. Biochem. 13, 347–356.
Bratosin, D., Estaquier, J., Petit, F., Arnoult, D., Quatannens, B., Tissier, J.P., Slomianny, C., Sartiaux, C., Alonso, C., Huart, J.J., Montreuil, J., Ameisen, J.C., 2001. Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. Cell Death Differ. 8, 1143–1156.
Burgoyne, L.A., 1999. The mechanism of pyknosis: hypercondensation and death. Exp. Cell Res. 248, 214–222.
Cain, B.W., Sileo, L., Franson, J.C., Moore, J., 1983. Effects of dietary cadmium on mallard ducklings. Environ. Res. 32 (2), 286–297.
Daugas, E., Cande, C., Kroemer, G., 2001. Erythrocytes: death of a mummy. Cell Death Differ. 8, 1131–1133.
Eisler, R., 1988. Lead hazards to fish, wildlife, and invertebrates: a synoptic review. US Fish Wildl. Serv. Biol. Rep. 85.
El-Azzouzi, B., Tsangaris, G.T., Pellegrini, O., Manuel, Y., Benveniste, J., Thomas, Y., 1994. Cadmium induces apoptosis in a human T cell line. Toxicology 88 (1–3), 127–139.
Goyer, R.A., 1996. Toxic effects of metals. In: Klaassen, C.D. (Ed.), Casarett & Doull's. Toxicology. The Basic Science of Poisons, fifth ed., pp. 691–736.
Grue, C.E., Hoffman, D.J., Beyer, W.N., Franson, L.P., 1986. Lead concentrations and reproductive success in European starlings *Sturnus vulgaris* nesting within highway-nesting barn swallows. Condor 86, 383–389.
Habeebu, S.S., Liu, J., Klaassen, C.D., 1998. Cadmium-induced apoptosis in mouse liver. Toxicol. Appl. Pharmacol. 149, 203–209.
Hamada, T., Tanimoto, A., Iwai, S., Fujiwara, H., Sasaguri, Y., 1994. Cytopathological changes induced by cadmium-exposure in canine proximal tubular cells: a cytochemical and ultrastructural study. Nephron 68, 104–111.
Hamada, T., Sasaguri, T., Tanimoto, A., Arima, N., Shimajiri, S., Abe, T., Sasaguri, Y., 1996. Apoptosis of human kidney 293 cells is promoted by polymerized cadmium-metallothionein. Biochem. Biophys. Res. Commun. 219, 829–834.
Hamada, T., Tanimoto, A., Arima, N., Ide, Y., Sasaguri, T., Shimajiri, S., Sasaguri, Y., 1998. Altered membrane skeleton of red blood cells participates in cadmium-induced anemia. Biochem. Mol. Biol. Int. 45 (4), 841–847.
He, L., Poblenz, A.T., Medrano, C.J., Fox, D.A., 2000. Lead and calcium produce rod photoreceptor cell apoptosis by opening the mitochondrial permeability transition pore. J. Biol. Chem. 275, 12175–12184.
Hsu, P.C., Guo, Y.L., 2002. Antioxidants nutrients and lead toxicity. Toxicology 180, 33–44.
INVITTOX protocol no. 37, 1992. Red blood cell test system. ⟨http://www.invittox.com⟩.
Jadhav, S.H., Sarkar, S.N., Aggarwal, M., Tripathi, H.C., 2007. Induction of oxidative stress in erythrocytes of male rats subchronically exposed to a mixture of eight metals found as groundwater contaminants in different parts of India. Arch. Environ. Contam. Toxicol. 52 (1), 145–151.
Jan, M., Frantisek, N., 2000. Cadmium-induced changes in cation-osmotic haemolysis in rats. Environ. Toxicol. Pharmacol. 8, 79–81.
Kempe, D.S., Lang, P.A., Eisele, K., Klarl, B.A., Wieder, T., Huber, S.M., Duranton, C., Lang, F., 2005. Stimulation of erythrocyte phosphatidylserine exposure by lead ions. Am. J. Physiol. Cell. Physiol. 288, C396–C402.
Lang, K.S., Roll, B., Myssina, S., Schittenhelm, M., Scheel-Walter, H.G., Kanz, L., Fritz, J., Lang, F., Huber, S.M., Wieder, T., 2002. Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. Cell. Physiol. Biochem. 12, 365–372.
Lang, K.S., Lang, P.A., Bauer, C., Duranton, C., Wieder, T., Huber, S.M., Lang, F., 2005. Mechanisms of suicidal erythrocyte death. Cell. Physiol. Biochem. 15 (5), 195–202.
Lumeij, J.T., 1985. Clinicopathologic aspects of lead poisoning in bird: a review. Vet. Quart. 7 (2), 133–138.
Lundbaek, J.A., Birn, P., Girshman, J., Hansen, A.J., Andersen, O.S., 1996. Membrane stiffness and channel function. Biochem. J. 35, 3825–3830.
Marshall, A.T., Schroen, C., Condron, R.J., 1994. X-ray micro-analysis of renal proximal tubules in cadmium-treated rats. J. Submicrosc. Cytol. Pathol. 26, 59–66.
Martínez-López, E., Martínez, J.E., María-Mojica, P., Peñalver, J., Pulido, M., Calvo, J.F., García-Fernández, A.J., 2004. Lead in feathers and δ-aminolevulinic acid dehydratase activity in three raptor species from an unpolluted Mediterranean forest (Southeastern Spain). Arch. Environ. Contam. Toxicol. 47 (2), 270–275.
Martínez-López, E., María-Mojica, P., Martínez, J.E., Romero, D., Calvo, J.F., García-Fernández, A.J., 2005. Cadmium in feather of adult and blood of nestlings of three raptor species from a nonpolluted Mediterranean forest, Southeastern Spain. Bull. Environ. Contam. Toxicol. 74, 477–484.
Mateo, R., Beyer, W.N., Spann, J.W., Hoffman, D.J., Ramis, A., 2003. Relationships between oxidative stress, pathology and behavioral signs of lead poisoning in mallards. J. Toxicol. Environ. Health A 66 (14), 1371–1389.
Matssura, K., Takasugi, M., Kunifugi, Y., Horie, A., Kuroiwa, A., 1991. Morphological effects of cadmium on proximal tubular cells in rats. Biol. Trace Elem. Res. 31, 171–182.
Mojzis, J., Nistiar, F., 2001. Lead-induced changes of cation-osmotic hemolysis in rats. Gen. Physiol. Biophys. 20, 315–319.
Moore, M.R., 1988. Haematological effects of lead. Sci. Total Environ. 71, 419–431.
Oberto, A., Marks, N., Evans, H.L., Guidotti, A., 1996. Lead (Pb^{2+}) promotes apoptosis in newborn rat cerebellar neurons: pathological implications. J. Pharmacol. Exp. Ther. 279, 435–442.
Pain, D.J., 1989. Haematological parameters as predictors of blood lead indicators of lead poisoning in the black duck (*Anas rubripes*). Environ. Pollut. 60, 67–81.
Peitsch, M.C., Muller, C., Tschopp, J., 1994. DNA fragmentation during apoptosis is caused by frequent single strand cuts. Nucleic Acids Res. 21, 4206–4209.
Real Decreto, 1201/2005. de 10 de octubre, sobre protección de los animales utilizados para experimentación y otros fines científicos. BOE 252, 34367–34391.
Romero, D., Gómez-Zapata, M., Luna, A., García-Fernández, A.J., 2003. Morphological characterisation of BGM (*Buffalo Green Monkey*) cell line exposed to low doses of cadmium chloride. Toxicol. In Vitro 17, 293–299.
Romero, D., Gómez-Zapata, M., Luna, A., García-Fernández, A.J., 2004. Morphological characterization of renal cell lines (BGM and VERO) exposed to low doses of lead nitrate. Histol. Histopathol. 19, 69–76.
Rosenberg, C.E., Peri, S.I., Arrieta, M.A., Fink, N.E., Salibian, A., 1998. Red blood cell osmotic fragility in *Bufo arenarum* exposed to lead. Arch. Physiol. Biochem. 106, 19–24.
Skoczynska, A., Smolik, R., 1994. The effects of combined exposure to lead and cadmium on serum lipids and lipid peroxides level in rats. Int. J. Occup. Med. Environ. Health 7, 263–271.
Suwalsky, M., Villena, F., Norris, B., Cuevas, F., Sotomayor, C.P., 2004. Cadmium-induced changes in the membrane of human erythrocytes and molecular models. J. Inorg. Biochem. 98, 1061–1066.
Terayama, K., 1993. Effects of lead on electrophoretic mobility, membrane sialic acid, deformability and survival of rat erythrocytes. Ind. Health 31, 113–126.
Van Engeland, M., Ramaekers, F.C., Schutte, B., Reutelingsperger, C.P., 1996. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 24 (2), 131–139.
Vicente-Ortega, V., Martínez-García, A.F., Cremades-Campos, A., Rodríguez-Vicente, J., Calderón-Rubiales, F., Martínez-Díaz, F., 1996. Ultrastructural investigation of lead-induced intranuclear inclusion bodies in mice. Ultrastruct. Pathol. 20, 263–273.
Viscor, G., Martinez, I., Riera, M., Palomeque, J., 1987. On the preservation of avian blood cells. Rev. Esp. Fisiol. 43, 469–475.
Walker, R.P., Weaver, V.M., Lach, B., LeBlanc, J., Sikorska, M., 1994. Endonucleases activities associated with high molecular weight and internucleosomal DNA fragmentation in apoptosis. Exp. Cell Res. 213, 100–106.
Wätjen, W., Beyersmann, D., 2004. Cadmium-induced apoptosis in C6 glioma cells: influence of oxidative stress. Biometals 17, 65–78.