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In vitro evaluation of cell death induced by cadmium, lead and their binary mixtures on erythrocytes of Common buzzard (*Buteo buteo*)

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ABSTRACT

Cadmium and lead are persistent and ubiquitous metals that can cause several deleterious effects in living beings. Apoptosis and necrosis are two types of cell death that can be found after *in vivo* and *in vitro* exposure to these metals. In this study, isolated red blood cells from living captive Common buzzard (*Buteo buteo*) were exposed *in vitro* to different concentrations of lead, cadmium, and the mixture lead–cadmium in a proportion of 1:10 (similar to that found in previous field studies). Data obtained from dose–response curves were used to evaluate the interactive effects of metal mixtures on cell viability. In general, except for the exposure to NOEC, additivity was the most frequently observed response. As described in human, after *in vitro* exposure, lead was highly accumulated in buzzard erythrocytes, while cadmium accumulation was scarce. Finally, the type of cell death (apoptosis or necrosis) induced by the exposure to different concentrations of these heavy metals and their mixtures was evaluated in the red blood cells. Apoptosis was found to be the main type of cell death observed after cadmium and/or lead exposure. However, this exposure caused an increase in lysis or necrosis, especially if red blood cells were exposed to high doses.

1. Introduction

Cadmium (Cd^{2+}) and lead (Pb^{2+}) are considered persistent and ubiquitous environmental contaminants, and present in all living beings. Lead-induced mortality has been reported in a variety of birds (see review Franson and Pain (2011)), and cadmium exposure has been associated to altered survival and reproduction of birds (see review Wayland and Scheuhammer (2011)). In these reviews, both metals have been described as responsible for several deleterious effects, such as tissue damage, immune deficiencies, altered behaviour, and haematological alterations.

Studies about toxicity assessment after *in vivo* exposure of wild birds to these metals have been carried out, being Mallards (*Anas plathyrynchos*) (Di Giulio and Scanlon, 1984; Mautino and Bell, 1987; Sanderson, 2002) and raptors, such as American kestrels (*Falco sparverius*), Red-tailed hawks (*Buteo jamaicensis*) or Turkey vultures (*Cathartes aura*) (Carpenter et al., 2003; Custer et al., 1984; Hoffman et al., 1985; Redig et al., 1991) the main species studied. However, the use of protected wild animals for scientific purposes is nowadays not allowed for ethical and legal reasons in Spain, and the use of alternative methods should be developed (Real Decreto 53/2013). In this sense, cell cultures such as erythrocytes obtained from different wild bird species could be used to design experimental tests of toxicity. Therefore, the *in vitro* exposure of these erythrocytes to lead and cadmium could provide useful information about the effects of these heavy metals in these species, and by extrapolation, in others.

It is well known that erythrocytes continuously suffer several physiological stress situations during their limited lifespan (approximately 120 days in vertebrates). Apoptosis is considered an important physiological mechanism to remove injured erythrocytes prior to haemolysis, which puts life and death of the erythrocyte at a central position in the study of organism homeostasis (Bosman et al., 2005; Föller et al., 2008; Lang et al., 2005). Lang et al. (2012) have recently reviewed the apoptosis in erythrocytes and they concluded that a myriad of xenobiotics are involved in the pathophysiology of several clinical conditions with suicidal erythrocyte death, and an excessive apoptosis in erythrocytes most likely also plays a role in other, yet unrecognized, clinical conditions.

Lang et al. (2005) suggested the term "eryptosis" to distinguish the death of erythrocytes from apoptosis of nucleated cells. Because mammal erythrocytes are devoid of nuclei and mitochondria, they lack crucial elements in the mechanisms of

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apoptosis. In spite of this, some of the typical features of apoptotic nucleated cells, such as cell shrinkage, membrane blebbing and phosphatidylserine externalization, have been described in erythrocytes. Even though avian erythrocytes are nucleated, the lack of DNA-dependent functions like transcription and translation (Burgoyne, 1999) could indicate a strong selective effect on oxygen transport, the main function of erythrocytes (Quintanar-Escorza and Calderón-Salinas, 2006). In addition, Burgoyne (1999) described two types of avian erythrocyte death: Lysis, as an abrupt death that is not accompanied by alteration of the nucleus, and programmed death, which is followed by pyknosis but not by DNA degradation, being the latter process described in other apoptotic cells (Bagchi et al., 2000). Erythrocyte death could be induced by osmotic shock, oxidative stress or energy depletion (Lang et al., 2005).

Cadmium and lead have been related to alterations like eryptosis (Kempe et al., 2005; Sopjani et al., 2008) and pyknosis (Gill and Pant, 1986; Hiraga et al., 2008; Romero et al., 2009) in mammals and nucleated erythrocytes. These alterations contribute to the decrease of erythrocytes life-span and the development of the anaemia observed after Cd^{2+} or Pb^{2+} intoxication (Fox et al., 1971; Hoffman et al., 1985).

Although most experimental studies in birds have evaluated the individual effects of these metals, environmental exposure to these contaminants occurs seldom isolated (Bae et al., 2001). Previous studies on raptors as sentinel animals of environmental contamination detected both heavy metals in blood of adults (García-Fernández et al., 1996, 1997) and nestlings (Martínez-López et al., 2004, 2005) exposed to background levels of pollution, being the cadmium blood concentrations 10-fold lower than those for lead, independently of the age and period of study. Studies using contaminants mixtures are often based on the frequency of occurrence and the level of contamination in a particular ecosystem (Jadhav et al., 2007). While the binary mixture cadmium–lead has been studied in mammals (ATSDR, 2004), literature in birds is scarce (Romero et al., 2009) and null in raptor species. For example, apoptosis in mallard erythrocytes was suggested after *in vitro* exposure to lead and cadmium (Romero et al., 2009).

In general, the concept of "additivity" is assumed for exposure to low levels of the components in a chemicals mixture (Bae et al., 2001), although it is well known that interactions (synergism, antagonism, and additivity) are also possible when the mixture is composed of metals (Ren et al., 2004).

The main aim of this study was to evaluate *in vitro* effects induced by lead, cadmium, and their mixtures in avian erythrocytes isolated from healthy Common buzzards (*Buteo buteo*). The mixtures were prepared in a proportion of cadmium:lead 1:10, according to the monitoring field studies carried out previously on raptor species, Common buzzard included, from uncontaminated areas (Martínez-López et al., 2004, 2005). Raptor erythrocytes were used to evaluate the type of death cell and the interactive effects induced by the exposure to these heavy metals.

2. Materials and methods

Studies were conducted in agreement to the Spanish national laws for the protection of animals used for experimental and other scientific purposes (Real Decreto 53/2013).

2.1. Preparation of the cell cultures

Blood samples were taken from healthy Common buzzards kept in captivity in the Wildlife Rehabilitation Centre "Santa Faz" (Alicante, Spain). The health status of the birds was clinically evaluated by a veterinarian prior to blood sampling. This clinical exploration includes the evaluation of general body conformation, posture, attitude, stimulus response, character of respiration. Also it includes exploration of the feathers, skin, beak, eyes, ears, cere, nares, oral cavity, bones, muscles (especially breast muscle), wings, faeces, abdomen and vent. Besides, a plasma biochemistry analysis was done in every individual to check the normal health status and ensure that birds did not suffer any subclinical pathology.

Throughout the study, blood samples from four different common buzzards were collected, but, for each experiment, only cells from the same individual were used. According to INVITOX Protocol No. 37 (1992), a combination of trisodium citrate and citric acid (66:44 mmol/L) was used as anticoagulant, at a final citrate–blood proportion of 1:10. Erythrocytes were obtained via density gradient (Percoll 57%, Hamk 10%, MiliQ 33% purified water). The cells were kept in phosphate-buffered saline (PBS) with 10 mmol/L glucose and refrigerated at +4 °C until utilization.

2.2. Test chemicals

Lead and cadmium (Merck Chemical Co., Darmstadt, Germany) were initially prepared in sterile purified water, at 100 mM (lead nitrate) and 10 mM (cadmium chloride). These stock solutions were used to prepare working solutions in PBS with 10 mmol/L glucose. To assure that the effects evaluated were due to the exposure to metals, the osmolarity of the culture mediums that contained the samples was verified using a Vapro© 5520 osmometer (WESCOR).

2.3. Cytotoxicity assay

All assays were performed in four replicates within the week after blood sampling. Four replicates of each concentration level and each contaminant/mixture were analysed, and each replicate was obtained from different blood samples from different buzzards.

The exposure to cadmium, lead and cadmium:lead (1:10) was carried out in Eppendorf vials, in which 100 µl of cell suspension (about 3.35×10^6 cells), 1000 µl of glucosated PBS and different concentrations of metals were added. At the same time, a negative control sample with glucosated PBS was prepared. The samples were kept in an incubator (Sanyo MCO-15A) for 24 h at 39 °C in a FALC F205 rotating plate.

After 24 h of exposure, 10 µl of the sample were transferred to a cytometry tube with 400 µl of PBS and 1 µl of propidium iodide was added immediately. Propidium iodide ($C_{27}H_{34}N_5I_2$) (Sigma, USA) was prepared in PBS at a concentration of 400 µg/ml. Cell viability was measured via flow cytometry using a Beckman Coulter XL cytometer at 488 nm . The EXPO32 ADC program was used for the gathering and analysis of data.

2.4. Assessment of interactive responses to binary mixtures

The method used to assess the interactive toxicity of binary combinations is based on the test of single metals at increasing concentrations that would reduce the viability of cells, followed by testing these concentrations in a binary mixture at a proportion of 1:10 (cadmium:lead). Statistical test of the difference between observed and calculated responses was used to evaluate the interactive effects ("additive", "antagonistic", or "synergistic"), using the statistical method of Ince et al. (1999). This method compares the observed toxicity of a binary mixture ($x + y$, where x and y are the concentrations of the first and the second metal, respectively), with the value of the null hypothesis at the level defined as "the sum of the toxicity indices of the two metals previously tested at x and y". In our study, the toxicity index was expressed as