Food and Chemical Toxicology 55 (2013) 526–532

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

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

γ-Oryzanol protects against acute cadmium-induced oxidative damage in mice testes

Cristiano C. Spiazzi [a], Vanusa Manfredini [b], Fabiana E. Barcellos da Silva [c], Érico M.M. Flores [d], Aryele P. Izaguirry [a], Laura M. Vargas [a], Melina B. Soares [a], Francielli W. Santos [a,*]

[a] Laboratório de Biotecnologia da Reprodução (Biotech), Campus Uruguaiana, Universidade Federal do Pampa (UNIPAMPA), CEP 97500-970 Uruguaiana, RS, Brazil
[b] Laboratório de Hematologia e Citologia Clínica, Campus Uruguaiana, Universidade Federal do Pampa, CEP 97500-970 Uruguaiana, RS, Brazil
[c] Núcleo de Pesquisa em Fármacos e Medicamentos, Campus Uruguaiana, Universidade Federal do Pampa, CEP 97500-970 Uruguaiana, RS, Brazil
[d] Departamento de Química, Universidade Federal de Santa Maria, CEP 97105-900 Santa Maria, RS, Brazil

ARTICLE INFO

Article history:
Received 14 September 2012
Accepted 20 January 2013
Available online 8 February 2013

Keywords:
Cadmium
γ-Oryzanol
Testes
Oxidative stress
Antioxidant

ABSTRACT

Cadmium is a non-essential heavy metal that is present at low levels mainly in food and water and also in cigar smoke. The present study evaluated the testicular damage caused by acute cadmium exposure and verified the protective role of γ-oryzanol (ORY). Mice were administrated with a single dose of 2.5 mg/kg of $CdCl_2$, and then treated with ORY (50 mM in canola oil, 5 mL/kg). Testes were removed after 24 h and tested for lipid peroxidation (TBARS), protein carbonylation, DNA breakage, ascorbic acid, cadmium and non-proteic thiols contents, and for the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and δ-aminolevulic acid dehydratase (δ-ALA-D). Cadmium presented a significant alteration in all parameters, except GPx and CAT activities. Therapy reduced in a slight degree cadmium concentration in testes (around 23%). ORY restored SOD and GST activities as well as TBARS production to the control levels. Furthermore, ORY partially recovered δ-ALA-D activity inhibited by cadmium. This study provides the first evidence on the therapeutic properties of ORY in protecting against cadmium-induced testicular toxicity.

1. Introduction

Cadmium is a non-essential heavy metal present in the environment at low levels, however anthropogenic activities has greatly increased its concentration in the atmosphere (ATSDR, 2008; WHO, 2010). Its emission in the atmosphere has been a health concern due to its long biological half-life in many living beings, including humans (10–35 years) (WHO, 2011). It may accumulate in many organs, such as liver and kidneys (Jihen et al., 2008), lungs (Klimisch, 1993; Luchese et al., 2009) and testes (Haouem et al., 2008). Contamination occurs mainly through food intake, but cigar smoke is also one of the major sources of exposures, bearing in mind the fact that lung absorption of cadmium is almost 10-fold higher than gastrointestinal absorption (Goering et al., 1994; Waalkes, 2003). Thus, an average person absorbs roughly 1 μg Cd/day via food, while an additional 1–3 μg Cd/day is absorbed by smoking one pack of cigarettes per day (ATSDR, 2008), therefore heavy smokers have more than twice the Cd body burden (Lewis et al., 1972).

This heavy metal has the potential to affect reproduction and development in many different ways, and at every stage of reproductive process (Thompson and Bannigan, 2008). Testes seem to be greatly affected by cadmium (Santos et al., 2004a,b). Cadmium can cause significant pathologies, such as haemorrhage, oedema, atrophy and calcification (Santos et al., 2006; Brandão et al., 2009). Many authors have related these tissue damages to oxidative stress, either by formation of free radicals or inhibition of cellular defences (Santos et al., 2004a; Acharya et al., 2008; Ognjanović et al., 2010). The mechanisms for such interactions are not fully understood, since cadmium cannot participate directly in Fenton reactions (Moriwaki et al., 2008). Consequently, the use of antioxidant compounds could help to prevent or reduce the damages caused by cadmium.

Rice is the staple food for the largest number of people on Earth, being the most important economic activity in the world (Maclean et al., 2002). Through its milling process, rice bran is obtained when removed from the starchy endosperm of raw rice (Lakkakula et al., 2004). Rice bran oil (RBO) contains a significant number of the antioxidant compounds (or biological active compounds), including about 0.1–0.14% vitamin E components and 0.9–2.9% γ-oryzanol; the concentrations can diverge substantially according to the origin of the rice bran (Diack and Saska, 1994; Hu et al., 1996; Lloyd et al., 2000).

γ-Oryzanol is a fraction of RBO containing a mixture of steryl and other triterpenyl esters of ferulic acid (Rogers et al., 1993;

* Corresponding author. Tel./fax: +55 55 3413 4321.
E-mail address: francielliweber@yahoo.com.br (F.W. Santos).

0278-6915/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2013.01.048

Orthoefer, 2005), in which cycloartenyl ferulate, 24-methylenecycloartenyl ferulate, β-sitosterol ferulate and campesteryl ferulate are the major compounds (Lloyd et al., 2000; Xu and Godber, 1999). γ-Oryzanol is thought to be the main antioxidant component of RBO. Xu et al. (2001) compared the antioxidant activities of vitamin E components (α-tocopherol, α-tocotrienol, γ-tocopherol and γ-tocotrienol) against γ-oryzanol major constituents. It was demonstrated that the last had higher activities, being 24-methylenecycloartenyl ferulate the one with the highest.

Therefore, the aim of this study was to evaluate the possible protective role of the nutritional source ORY in testicular oxidative damage induced by acute cadmium exposure. Thus, we evaluated enzymatic (SOD, CAT, GPx, GST, δ-ALA-D) and non-enzymatic (ascorbic acid and non-proteic thiols) antioxidant defences as well as lipid peroxidation, proteins oxidation, DNA damage and cadmium accumulation in mice testes.

2. Methods and materials

2.1. Reagents

γ-Oryzanol was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Cadmium chloride, glutathione reductase from baker's yeast, β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH) and oxidised glutathione (GSSG) were purchased from Sigma (St. Louis, MO, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Aldrich Chemical Co. (USA). All the other reagents used in this study were of analytical grade and obtained from standard commercial suppliers.

2.2. Treatment

Male adult Swiss albino mice (25–30 g) were used for this experiment. The animals were kept in appropriate animal cabinet with forced air ventilation, in a 12 h light/dark cycle, at a controlled room temperature of 22 °C, with food (Puro Trato, RS, Brazil) and water *ad libitum*. The animals were separated in four groups: control; cadmium; ORY; cadmium/ORY. ORY treatment was given orally through gavage at a dose of 5 mL/kg b.w.; saline and cadmium (2.5 mg Cd/kg b.w.) were administrated intraperitoneally, according to our previous works (Santos et al., 2004a; Ardais et al., 2008). Canola oil was used as control and also as diluent for ORY (50 mM). ORY concentration was similar to the dose used by Nagasaka et al. (2011). Therapy was given just after the administration of cadmium solution. The experimental scheme can be better visualised at Table 1. The animals were euthanized 24 h after cadmium administration using sodium pentobarbital (100 mg/kg, i.p.). The testes were removed, homogenised in buffer and stored at negative 20 °C until analysis, except for δ-ALA-D activity, which was assessed shortly after testes removal. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil. This study was approved by the Ethics Committee on the Use of Animals of Federal University of Pampa (Protocol no. 004/2011).

2.3. Non-enzymatic assay

2.3.1. Lipid peroxidation (TBARS)

The testes were rapidly homogenised in 50 mM Tris-HCl, pH 7.5 (1/10, w/v) and centrifuged at 2400×*g* for 15 min. An aliquot (100 μL) of supernatant was incubated at 95 °C for 2 h. Thiobarbituric acid reactive species (TBARS) were determined as described (Ohkawa et al., 1979).

2.3.2. Determination of non-protein thiols (NPSH)

NPSH levels were determined by the method of Ellman (1959). To determine NPSH, an aliquot of homogenised sample was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation at 2400×*g* for 10 min, the protein pellet was discarded and free –SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB. The colour reaction was measured at 412 nm. NPSH levels were expressed as nmol NPSH/g tissue.

Table 1
Experimental model for cadmium-induced testes injury in mice ($n = 5$), using γ-oryzanol (ORY) as treatment.

Group	Treatment	
	Intraperitoneal	Oral
Control	Saline solution	Canola oil (5 mL/kg b.w.)
ORY	Saline solution	ORY 50 mM (5 mL/kg b.w.)
Cd	$CdCl_2$ (2.5 mg/kg b.w.)	Canola oil (5 mL/kg b.w.)
Cd + ORY	$CdCl_2$ (2.5 mg/kg b.w.)	ORY 50 mM (5 mL/kg b.w.)

2.3.3. Cadmium analysis

Cadmium concentration was analysed by inductively coupled plasma mass spectrometry (ICP-MS) (Perkin–Elmer SCIEX, Elan DRC II model, Thornhill, Canada). The samples were digested with 4 mL of nitric acid for 30 min at 90 °C, followed by 90 min at 120 °C. Afterwards, 500 μL of hydrogen peroxide was added and the sample was incubated for 30 min at 120 °C.

2.3.4. Ascorbic acid

Ascorbic acid was determined according to method previously described (Jacques-Silva et al., 2001). Proteins were precipitated with 10 volumes of ice-cold 5% TCA solution, and then incubated at 37 °C for 3 h with a system containing dinitrophenylhydrazine (DNPH). The reaction was ended with sulphuric acid, and then assessed at 520 nm.

2.3.5. Carbonyl groups determination

The formation of carbonyl groups, a parameter of oxidative damage to proteins, was measured based on the reaction of these groups with dinitrophenylhidrazine (DNPH), as previously described (Levine et al., 1990). Samples were incubated at laboratory temperature in the dark for 60 min, stirring at 15-min intervals. After centrifugation, the samples were washed three times with 1 mL of ethanol-ethyl acetate (1:1; v/v) to remove the residual DNPH reagent. The final precipitates were dissolved in 6 N guanidine hydrochloride solution (1 mL). The absorption of the product of reaction was measured in a spectrophotometer at 370 nm. Results were expressed as nmol carbonyl/mg protein.

2.4. Single cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described (Singh et al., 1988) in accordance with general guidelines for use of the comet assay (Tice et al., 2000; Hartmann et al., 2003; Bajpayee et al., 2005). Homogenised testes were suspended in agarose and spread into a glass microscope slide pre-coated with agarose. Agarose was allowed to set at 4 °C for 5 min. Slides were incubated in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0, and 1% triton X-100 with 10% DMSO) to remove cell proteins, leaving DNA as 'nucleoids'. After the lysis procedure, slides were placed on a horizontal electrophoresis unit, covered with a fresh solution (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow DNA unwinding and the expression of alkali-labilesites. Electrophoresis was performed for 20 min (25 V; 300 mA; 0.9 V/cm). Slides were then neutralized, washed in bi-distilled water and stained using a silver staining protocol (Maluf and Erdtmann, 2000; Nadin et al., 2001). After drying at room temperature overnight, gels were analysed using an optical microscope. One hundred cells (50 cells from each of the two replicate slides) were selected and analysed. Cells were visually scored according to tail length and receive scores from 0 (no migration) to 4 (maximal migration) according to tail intensity (Tice et al., 2000). Therefore, the damage index (DI) for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The slides were analysed under blind conditions at least by two different individual. Median values of the scores were presented.

2.5. Enzymatic assay

2.5.1. Superoxide dismutase (SOD) activity

Superoxide dismutase activity was measured as described (Misra and Fridovich, 1972). This method is based on the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome. The colour reaction can be monitored at 480 nm. One enzymatic unit (1 UI) is defined as the amount of enzyme necessary to inhibit the auto-oxidation rate in 50% at 26 °C.

2.5.2. δ-Aminolevulic acid dehydratase activity

δ-Aminolevulic acid dehydratase (δ-ALA-D) activity was assessed by measuring the formation of porphobilinogen (PBG), according to Sassa (1982) method, except that 45 mM sodium phosphate buffer and 2.2 mM ALA were used. Samples were homogenised in 0.9% NaCl in the proportion (w/v) 1/5 and centrifuged at 2400×*g* for 15 min. An aliquot of 50 μL of homogenised tissue was incubated for 2 h at 37 °C. PBG formation was detected with the addition of modified Erlich's reagent at 555 nm.

2.5.3. Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity is measured in a system containing reduced glutathione (GSH), reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase (GR) according to Wendel (1981). GPx acts oxidising GSH into glutathione disulphide (GSSG). To complete the cycle, GR