Ecotoxicology and Environmental Safety 73 (2010) 1279–1285

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

# *In vivo* genotoxicity and stress defences in three flatfish species exposed to $CuSO_4$

H. Chairi [a], C. Fernández-Diaz [b], J.I Navas [c], M. Manchado [b], L. Rebordinos [a,*], J. Blasco [d]

[a] Laboratorio de Genética, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono Río San Pedro, s/n, 11510, Puerto Real, Cádiz, Spain
[b] IFAPA Centro 'El Toruño', Junta de Andalucía, Ctra Tiro Pichon s/n, 11500 El Puerto de Santa María, Cádiz, Spain
[c] IFAPA, Centro Agua del Pino, Junta de Andalucía, Ctra, Punta Umbría-Cartaya s/n, 21459, Cartaya, Huelva, Spain
[d] Instituto de Ciencias Marinas de Andalucia (CSIC), Polígono Río San Pedro, s/n11510, Puerto Real, Cádiz, Spain

ARTICLE INFO

*Article history:*
Received 14 April 2010
Received in revised form
14 July 2010
Accepted 17 July 2010
Available online 3 August 2010

*Keywords:*
Genotoxicity
Flatfish
$CuSO_4$
Antioxidant enzymes
Stress protein
Comet assay

ABSTRACT

We have used the comet assay to analyse, after 3 h, 24 h and 6 days, the genotoxic effect *in vivo* of applying a single intraperitoneal injection of $CuSO_4$, at a concentration of 2 mg/kg, to adult specimens of *Solea senegalensis*, *Dicologlossa cuneata* and *Scophthalmus rhombus*. Metals content (Cu, Zn and Cd) in liver was also measured. The activity of key stress defences was evaluated by analysing antioxidant enzyme activity (catalase (CAT), superoxide dismutase (SOD), total glutathione peroxidase (t-GPX), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH)), metallothionein (MT) and heat shock proteins (HSP70 and HSP60). The results show that $CuSO_4$ intake generates high and cumulative levels of genotoxicity throughout the 6 days in all 3 species. After 6 days, metals content detected in specimens showed significant differences from controls. Inter-species differences were detected in enzyme activity ($P < 0.05$). A clear response to $CuSO_4$ was detected only in *S. rhombus*, with an increase of MT and a decrease of HSPs. Variations in antioxidant defence levels and their comparative responses to the stress-inducing agent are discussed.

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## 1. Introduction

Pleuronectiformes (flatfish) constitute a broad taxonomic group comprising 11 families and about 500 species worldwide (Froese and Pauly, 2007; Helfman et al., 1997). All these species share in common an asymmetrical body development and a bottom-dwelling mode of life. Some of these species, such as Atlantic halibut (Pleuronectidae: *Hippoglossus hippoglossus*), turbot (Scophthalmidae: *Psetta maxima*), Dover sole (Soleidae: *Solea solea*) and Japanese flounder (Paralichthyidae: *Paralichthys olivaceus*) are of significant commercial interest in fisheries and aquaculture. Moreover, additional flatfish species are being studied for aquaculture diversification in southern Europe including wedge sole (Soleidae: *Dicologlossa cuneata*), brill (Scophthalmidae: *Scophthalmus rhombus*) and Senegalese sole (Soleidae: *Solea senegalensis*).

Copper sulphate ($CuSO_4$) is a compound commonly used as an algaecide, antifungal and antiparasite agent in aquaculture, especially in extensive production systems such as ponds. By other hand, copper is a ubiquitous metal which is present in estuary and coastal ecosystems as consequence of inputs from agricultural, mining, industrial activities and urban sewage. This metal is bioaccumulated by marine organisms and it can exert toxic effects. To produce these effects the reactive cation $Cu^{2+}$ seems to bind non-specifically to proteins and nucleic acids in the cell, as well as increasing the rate of free radical formation, to promote cytotoxicity. However, copper sulphate could have collateral effects in fish by inducing DNA strand breakages, and genotoxicity in a tissue- and species-specific way (Arkhipchuk and Garanko, 2005; Cavas et al., 2005; Gabbianelli et al., 2003; Gravato et al., 2006; Oliveira et al., 2008). The comet assay is a toxicogenetic technique considered to be a good indicator of genotoxicity. It was originally developed and applied in medical research (Neri et al., 2006; Kopjar and Garaj-Vrhovac, 2005), but it is now widely used for environmental biomonitoring (AbouChakra et al., 2007; Pruski and Dixon, 2007). This assay has proved to be useful for measuring DNA strand breaks in marine and freshwater species in the presence of genotoxic compounds (Nogueira et al., 2006; Cabrita et al., 2005).

Reactive oxygen species (ROS) and Cu interact in the redox cycling Haber–Weiss reaction to form highly reactive radicals, which in turn cause membrane lipid peroxidation and membrane disruption (Luza and Speisky, 1996). In order to maintain integrity and homoeostasis, cells possess diverse protective and antioxidant defences. The antioxidant enzymes catalase and superoxide

* Corresponding author. Fax: +34956016448.
*E-mail address:* laureana.rebordinos@uca.es (L. Rebordinos).

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doi:10.1016/j.ecoenv.2010.07.028

dismutase represent an important defensive barrier that is activated in stressful situations (Livingstone, 2001). Metallothionein (MT) is a protein involved in metal homoeostasis that also plays an essential role in the detoxification of non-essential metals within the cell. MT levels can be enhanced by exposure to metals and by oxidants (Viarengo et al., 1999); this makes it a potential biomarker for evaluating the effects of Cu in fish. Finally, heat shock proteins (HSPs) are chaperones that play a key role in the transport, folding and assembly of proteins. They can be induced under abnormal conditions by various causal agents including metals and oxidative stress (Basu et al., 2002). The HSP70 and HSP60 families in particular are involved in protein homoeostasis as they stabilise and refold damaged proteins, and target mis-folded proteins in specific degradative pathways (Daugaard et al., 2007).

The aim of this work is to evaluate the effect of copper sulphate in three flatfish species. Genotoxicity was evaluated using the comet assay and for assessing the inter-species differences in defence mechanisms, additional biomarkers involved in the defensive cellular responses against oxidative stress (antioxidant enzymes, MT and HSPs) were also studied. The information obtained will be useful for understanding the action mechanisms of copper in fish and to determine the effect on the health and welfare status of these species in aquaculture and in wild fish.

## 2. Materials and methods

### 2.1. Animals

Juvenile individuals of Senegalese sole, wedge sole and brill (Table 1) were obtained from the IFAPA Centro *Agua del Pino* facilities (Cartaya, Huelva, Spain). Fish rearing conditions are reported in Jiménez-Cantizano et al. (2008). Animals were dosed via a single intraperitoneal injection with 2 mg $kg^{-1}$ of copper sulphate ($CuSO_4$ Sigma-Aldrich) dissolved in phosphate buffered saline (PBS). Fish controls were injected with PBS. The copper dose was chosen as it has been seen to induce oxidative stress into the sea bass (*Dicentrachus labrax*) at the times used in this experiment (Roméo et al., 2000). Prior to manipulation, fish were anaesthetized with phenoxyethanol (100 mL/$m^3$). After injection, animals recovered normally without any signs of stress. Fish specimens ($n=30$) from the treated and control groups were sampled at 3 h, 24 h and 6 days post injection (p.i.). For the comet assay, livers were extracted (Table 1) and kept on ice until analysis. For the other analyses, livers were frozen in liquid nitrogen and stored at −80 °C until use. All animals were treated humanely with regard for alleviation of suffering, and all laboratory procedures involving animals were carried out in compliance with the Guidelines of the European Union Council (*86/609/EU*) and Spanish laws at the time of the experiment.

### 2.2. Biochemical determinations

Livers (pooled wet mass of three individuals per treatment) from each sampling point were separated into six groups for the assessment of the different variables. Liver aliquots were homogenised in 50 mM Tris–Cl pH 7.8 buffer containing 5 mM EDTA, 1 mM DTT and Cocktail inhibitor proteases (Sigma P8340). Samples were cooled in an ice bath and centrifuged (100,0000*g*; 1 h; 4 °C). Upper layer of fat was eliminated and aliquots were finally frozen at −80 °C.

Catalase (CAT) and superoxide dismutase (SOD) enzyme activities were measured using a Lambda 25 spectrophotometer (Perkin–Elmer) at 25 °C. Assays were run at least in duplicate. CAT activity was determined by measuring the decrease in hydrogen peroxide concentration at 240 nm (Aebi, 1974). SOD was measured by the xanthine–xanthine oxidase system as described by McCord and Fridovich (1969). One unit of SOD activity is defined as the amount of sample causing a 50% inhibition of cytochrome c under these conditions.

Total glutathione peroxidase (t-GPX), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) measurements were carried out using a microplate reader (Genios, Tecan). Assays for these four enzymes were run at least in triplicate in 96-well format (McFarland et al., 1999). t-GPX activity was measured according to Flohe and Gunzler (1984) using cumune hydroperoxide as substrate, and GR activity according to Cohen and Duvel (1988), measuring the rate of NADPH oxidation. G6PDH activity was determined according to Lörh and Waller (1974) and 6PGDH according to Bonamusa et al. (1992), measuring in both cases the appearance of NADPH.

**Table 1**
Some details of the sampling (wet weight, with mean ± SE, liver weight, $n=30$).

| Species | Age | Total weight (g) | Liver weight (g) |
|---|---|---|---|
| Senegalese sole | 2 years | 63.3 ± 2.8 | 1.10 |
| Wedge sole | 2 years | 43.0 ± 3.1 | 0.60 |
| Brill | 1 year | 39.3 ± 4.1 | 0.25 |

### 2.3. Comet assay

The comet assay was performed essentially according to the procedure of Tice (1996). To prepare the cell suspension, a small piece of liver was placed in 1 mL of cold Hank's buffered solution (HBSS) containing 20 mM EDTA and 10% DMSO. Then, it was minced into fine pieces, and after it had settled, the cell suspension was removed (Tice, 1996). To evaluate the rate of DNA damage, slides were stained by adding 40 μL of ethidium bromide (2 μg/mL). For each individual, images of 300 randomly selected cells (100 cells −50 per replicate—from each of three individuals) from the inoculated and control groups were analysed using an Axioskope 2 plus microscope equipped with a Photometrics CoolSNAP camera. Imaging was performed using specialized analysis Comet Assay Software Project (CASP) (University of Wroclaw, Poland) (Konca et al., 2003; Garcia et al., 2007). To quantify the DNA damage, the tail length and the fraction of DNA in the comet tail were estimated. Tail moment was calculated according to the following formula: Tail moment = (% DNA in tail * tail length)/100.

### 2.4. Chemical analysis

Metals were analysed in digested samples according the procedure described by Martín-Díaz et al. (2005). The results were checked using reference material (DOLT1 of NRC Canada). The recovery ranged between 97.25 ± 8.95 for Cu and 103.2 ± 14.4 for Cd. Certified values and our values are very close for all analysed metals. The detection limits were: 0.9 μg/L for Cu; 0.6 μg/L for Cd and 0.2 μg/L for Zn. Metal concentration was analysed by ICP-OES (Perkin–Elmer 2000DV). The results are expressed as μg $g^{-1}$ dry weight.

### 2.5. Protein determination

Total protein content (TPC) for normalisation of biochemical determinations was determined in a multiwell plate according to the Bradford (1976) method using bovine serum albumin (BSA) as standard. Metallothionein (MT) levels were determined according to the procedure described by Olafson and Olsson (1987) on the cytosol fraction (100,000*g* × 60 min) after denaturation at 95 °C for 4 min. The results are expressed as μg MT $mg^{-1}$ protein. Stress proteins HSP70 and HSP60 were measured according to Solé et al. (2004). Bands were visualised using the alkaline phosphatase revealing mixture using NBT (p-nitroblue tetrazolium) and the same amount of BCIP (5-bromo-4chloro-3-indolyl phosphate) and semi-quantified by scanning with a Laser Densitometer (Epson GT-8000). Absorbance of the samples was expressed as arbitrary units/mg protein.

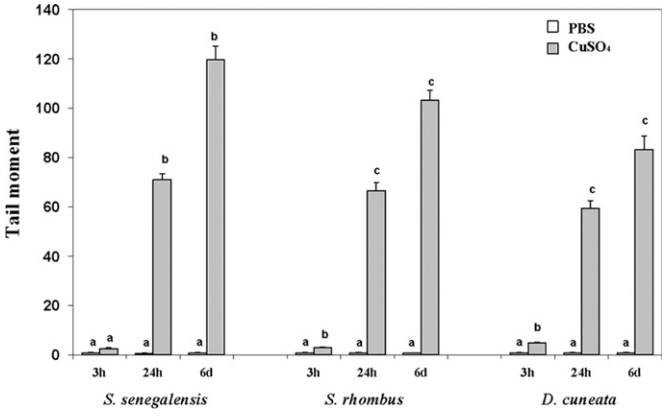


**Fig. 1.** Genotoxic effect of $CuSO_4$ in the three flatfish species, Senegalese sole (*S. senegalensis*), wedge sole (*D. cuneata*) and brill (*S. rhombus*). Tail moments of treated and control groups at 3 h, 24 h and 6 days p.i. are represented. Values with the same superscript are not significantly different ($P<0.05$).

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