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Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygene species

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Abstract

Cadmium poses a serious environmental threat in aquatic ecosystems but the mechanisms of its toxicity remain unclear. The purpose of this work was first to determine whether cadmium induced apoptosis in trout hepatocytes, second to determine whether or not reactive oxygen species (ROS) were involved in cadmium-induced apoptosis and genotoxicity. Hepatocytes exposed to increasing cadmium concentrations (in the range of 1–10 μM) showed a molecular hallmark of apoptosis which is the fragmentation of the nuclear DNA into oligonucleosomal-length fragments, resulting from an activation of endogenous endonucleases and recognized as a 'DNA ladder' on conventional agarose gel electrophoresis. Exposure of hepatocytes to cadmium led clearly to the DEVD-dependent protease activation, acting upstream from the endonucleases and considered as central mediators of apoptosis. DNA strand breaks in cadmium-treated trout hepatocytes was assessed using the comet assay, a rapid and sensitive single-cell gel electrophoresis technique used to detect DNA primary damage in individual cells. Simultaneous treatment of trout hepatocytes with cadmium and the nitroxide radical TEMPO used as a ROS scavenger, reduced significantly DNA fragmentation, DEVD-related protease activity and DNA strand breaks formation. These results lead to a working hypothesis that cadmium-induced apoptosis and DNA strand breaks in trout hepatocytes are partially triggered by the generation of ROS. Additional studies are required for proposing a mechanistic model of cadmium-induced apoptosis and genotoxicity in trout liver cells, in underlying the balance between DNA damage and cellular defence systems in fish.

Keywords: Fish; Hepatocytes; Cadmium; TEMPO; 3-MC; B(*a*)P; Reactive oxygen species; Apoptosis; Genotoxicity; Comet assay

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1. Introduction

Understanding the toxic actions of contaminants in aquatic ecosystems remains fundamental for predicting important sublethal effects (Stripped and Trombetta, 1994). Among the most toxic compounds cadmium poses a serious environmental threat, although the mechanisms of its toxicity are still poorly understood (El-Azzouzi et al., 1994).

This heavy metal is already known to be mutagenic (Amacher and Paillet 1980; Ochi and Ohsawa 1983; Mandel and Ryser, 1984), clastogenic (Shiraishi et al., 1972; Röhr and Bauchinger, 1976; Ochi et al., 1984), teratogenic and carcinogenic (Degrave, 1981). In earlier studies with cultured mammalian cells, cadmium has been shown to induce lipid peroxidation, DNA single-strand breaks (Ochi and Ohsawa, 1983) and chromosomal aberrations (Ochi et al., 1984).

Both the interaction with DNA repair processes and the induction of oxidative DNA damage may account for cadmium genotoxicity (Hartwig, 1995). Many studies have highlighted the potential role of active oxygen species and free radicals as mediators in the induction of genotoxicity by cadmium (Hartwig, 1994; Goering et al., 1995; Beyersmann and Hechtenberg, 1997). The fact that reactive oxygen species induce apoptosis (programmed cell death) suggests that oxidative stress may be also involved in cadmium-induced apoptosis (Buttke and Sandström, 1994; Forrest et al., 1994; McConkey and Orrenius, 1994).

Toxic insult of cadmium can result in the injured cells dying by necrosis or apoptosis. Cells normally undergo apoptosis in response to mildly adverse conditions while exposure to severe conditions (higher doses of the toxin) will result in necrosis. Both processes are truly distinct and have important implications. Apoptosis has been observed in skin epithelial cells of carp (Iger et al., 1994) and trout (Lyons-Alcantara et al., 1998) exposed to cadmium. Cadmium has been reported to cause cell death through apoptosis in a mammalian cell line and a possible mechanism has been proposed (El-Azzouzi et al., 1994). Apoptosis is an active cellular death process characterized by distinctive morphological changes that include condensation of nuclear chromatin, cell shrinkage, nuclear disintegration, plasma membrane blebbing, and formation of membrane-bound apoptotic bodies. A molecular hallmark of apoptosis is degradation of nuclear DNA into oligonucleosomal-length fragments as the result of activation of endogenous endonucleases, recognized as a 'DNA ladder' on conventional agarose gel electrophoresis (Wyllie, 1981). Recent developments strongly implicate members of the caspase family of proteases as central mediators of apoptosis, acting upstream from the endonucleases (Cohen, 1997; Wyllie, 1997). The caspase family consists of at least ten related cysteine proteases. All the caspases contain a conserved QACXG (where X is R, Q or G) pentapeptide active-site motif. Activation of caspases during apoptosis results preferentially in a cleavage at aspartate residues. The substrates of this reaction are widely dispersed in the nucleus, cytoplasm and cytoskeleton. Recently, by mapping the cleavage site of poly-(ADP-ribose)polymerase (PARP), an enzyme involved in DNA repair, Nicholson et al. (1995) have identified the tetrapeptide DEVD as the consensus cleavage site for caspase-3. Conjugation of a fluorometric moiety to DEVD provides a potential substrate for analysing DEVD-dependent protease activity (Gurtu et al., 1997).

Recent works have demonstrated the interest of using the comet assay (or single cell gel electrophoresis assay) for assessing the genotoxicity of chemical compounds to be found in aquatic ecosystems (Belpaeme et al., 1998; Devaux et al., 1998; Mitchelmore and Chipman, 1998). The comet assay is expanding in application because of its rapidity and its ability to discriminate cell types regarding the degree of DNA damage and DNA repair level (Fairbairn et al., 1995). In the comet assay, cells are embedded in agarose on microscopic slides, lysed with high concentrations of salts under alkaline conditions and electrophoresed. During electrophoresis, DNA from nuclei containing strand breaks is pulled out of the nucleus. This appears as a 'comet' when stained with DNA-binding dye and viewed under

the microscope. DNA migration from the nucleus and intensity of fluorescence are used to evaluate the extent of DNA damage. The running of electrophoresis in alkaline conditions enables single and double strand breaks to be detected, in particular after triggering apoptosis (Gopalakrisna and Khar, 1995; Olive and Banath, 1995).

Fish cell cultures have been broadly used to study the fate and effects of xenobiotics at the cellular level (Babich and Borenfreund, 1991). Special attention has been paid to primary cultures of hepatocytes since this cell type is involved in several critical functions including xenobiotic metabolism (Baksi and Frazier, 1990; Monod et al., 1998). Moreover fish hepatocytes and in particular trout hepatocytes appear to be well adapted to the measure of DNA strand breaks due to fish exposure to xenobiotic acting through oxidative damage (Belpaeme et al., 1996; Devaux et al., 1997).

Because of intensive research on oxidative damage, the role played by antioxidants in diminishing the free radical-induced damage has become of prime interest (Wang et al., 1996; Villarini et al., 1998). In this present study, the nitroxide radical TEMPO is used as a reactive oxygen species (ROS) antioxidant scavenger.

The purpose of this work was first to determine whether cadmium induced apoptosis in trout hepatocytes, second to determine by using TEMPO whether or not ROS were involved in cadmium-induced apoptosis and genotoxicity.

2. Materials and methods

2.1. Fish

Immature rainbow trout (*Oncorhynchus mykiss*) with an average weight of 400–500 g were obtained from a local hatchery (Auribeau-sur-Siagne, France). Fish were kept in tanks with aerated, charcoal-filtered circulating tap water at a temperature of 15°C. Fish were fed on commercial fish food, and acclimatized to laboratory conditions for a minimum of two weeks before use in the experiments.

2.2. Isolation of hepatocytes and culture conditions

After killing a fish, the liver was rapidly perfused with saline buffer containing 10 mM HEPES and 0.63 mM EGTA, pH 7.4 at 25°C, to eliminate Ca^{2+} from the extracellular matrix: liver was then perfused at 25°C with a simple HEPES solution followed by a collagenase solution in HEPES (0.5 mg/ml) containing 5 mM $CaCl_2$. After perfusion, the liver was placed in a Petri dish, rinsed with Leibovitz's (L-15) culture medium supplemented with glutamine and 5% foetal calf serum (FCS), 50 units penicillin/streptomycin, 0.1 unit insulin and then minced with a stainless steel forceps in order to disperse the cells in the medium. The resulting cell suspension was repeatedly pipetted and filtered through sterile nylon, then centrifuged three times at $50 \times g$ for 5 min at room temperature. The cell pellet was suspended in previous L-15 culture medium but containing 10% FCS. Cells were counted and viability determined using the Erythrosin B (0.36%) exclusion test. Each perfusion yielded about 2×10^8 cells, with viability over 90%. Isolated trout hepatocytes were directly mixed with a collagen solution composed mainly of type I collagen and prepared from rat tails. Cells were maintained in culture in this collagen gel as previously described (Risso-de Faverney et al., 1999). Cells were seeded in wells (2 and 4×10^6 cells per well of 35 and 60 mm diameter, respectively) and cultured under atmospheric air at 15°C. Culture medium was changed 24 h after cell seeding. All liquids and glassware were sterilized by filtration or autoclaving before use.

2.3. Exposure of cells

Stock solution of TEMPO (2,2,6,6-tetramethylpiperidinyl-1-oxyl) were prepared in DMSO, and Cd(II) in distilled water. TEMPO or Cd(II) stock solutions were diluted in L-15 culture medium without serum, but supplemented with 2% bovine serum albumin (BSA, v/v). The following increasing Cd(II) concentrations were used: 1, 2, and 10 μM. After medium removal from the wells, hepatocytes in gel were exposed at 15°C to

the various compounds for different time exposure according to the experiments. For genotoxicity studies cells were exposed for 24 and 48 h to increasing Cd(II) concentrations (1 to 10 μM) and/or 100 μM TEMPO. For all other experiments, trout hepatocytes were treated with Cd(II) and/or TEMPO for 3, 6, 24 and 48 h.

2.4. Neutral red cytotoxicity test

For assessing Cd(II) cytotoxicity, cultures of trout hepatocytes were exposed for 24 h to increasing Cd(II) concentrations in the range of 15–75 μM. Moreover, cells were exposed for 72 h to different TEMPO concentrations in the range of 5–100 μM to check if this compound was or not cytotoxic per se. Cells in culture were loaded with 300 μl of Neutral Red solution (50 μg/ml) for 3 h at 15°C. Cells in collagen gel were then recovered and centrifuged for 2 min at $1000 \times g$. The pellet was washed with PBS before addition of an alcohol (50%)–acetic acid (1%) solution and homogenised with a teflon PotterElvehjem type homogeniser. After a brief centrifugation, the supernatant was discarded and the optical density measured at 550 nm.

2.5. DNA gel electrophoresis

The genomic DNA was extracted using Gibco BRL DNAzol™ Reagent (Darfler and Karaszkiewicz, 1996) from 4×10^6 cells exposed to different treatments. It was quantified by using PicoGreen dsDNA Quantitation Reagent and kits (Molecular probes). Quantification was determined spectrophotometrically ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 530$ nm) and DNA concentration of the samples was determined from standard curve obtained from the lambda DNA standard. One microgram of each DNA sample was electrophoresed at 100 V for 2–3 h in 1% agarose gel prepared in a $0.5 \times$ TAE buffer (20 mM Tris, 0.5 mM acetic acid, 10 mM EDTA, pH 8.3). DNA was stained with ethidium bromide and transilluminated with UV light for photography. The 1 kb Plus DNA Ladder from Gibco BRL is suitable for sizing linear double-stranded DNA fragments from 100 bp to 12 kbp. The ladder contains a total of 20 bands: 12 bands ranging in size from 1000 to 12 000 bp in 1000-bp increments and eight bands ranging in size from 100 to 1650 bp.

2.6. Analysis of DEVD-dependent protease activity

This DEVD-dependent protease activity was assayed fluorometrically using a synthetic tetrapeptide, Asp-Glu–Val–Asp (DEVD), labeled with 7-amino-4-trifluoromethylcoumarin (AFC) as a substrate (Alexis Corporation). The fluorochrome AFC was released upon cleavage by DEVD-dependent protease. Following exposure to different treatments, 2×10^6 cells were lysed by two successive freezing and thawing cycles, after removing the culture medium. Cells were homogenised in a buffer A (25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A), then 40 μl homogenate were incubated with 2.5 mM DEVD-AFC as a substrate in a reaction buffer B (312 mM HEPES pH 7.5, 31% sucrose, 0.3% CHAPS) at 20°C as described in the manufacturer's instructions (PROMEGA, 1998, CaspACETM Assay System fluorometric, Technical Bulletin, Madison WI). The fluorometric assay was carried out using a multiwell fluorescence plate reader (Dynatech fluorolite 1000) at an excitation/emission wavelength of 390/530 nm. The DEVD-dependent protease activity was measured in the samples by following the kinetics of the appearance of free AFC after cleavage from DEVD-AFC every 3 min for 45 min. The amount of fluorescence in these samples was proportional to the amount of the DEVD-dependent proteases activity. This activity was expressed in pmol AFC liberated/min/2×10^6 cells.

2.7. Comet assay procedure

The comet assay was performed on trout hepatocytes according to the initial procedure described by Singh et al. (1988) and modified as follows. Microscope slides were first immersed in melted (37°C) normal agarose prepared in phosphate buffer saline (PBS, 0.8% agarose final con-

centration) and let them dry overnight at room temperature. Then 75 μl of 1% low melting point agarose in PBS mixed with an equal volume of cells were added in order to have a final density of about $5x10^4$ cells per slide. After application of a coverslip, the slides were allowed to gel at 0°C on a cooled metal tray. Coverslips were removed and a third layer of 90 μl of 0.5% low melting point agarose in PBS without cells was gently applied as before. After removal of coverslips the slides were placed in a freshly prepared lysing solution for 1 h (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, pH set to 10 with NaOH). Three ml of Triton X-100 and 30 ml of DMSO were added to 270 ml of the lysing solution before use. This volume was sufficient for 20 slides. The lysing and the next steps were performed in red dim light at 20°C. After the lysis the slides were gently transferred into a horizontal electrophoresis tank filled with a freshly prepared buffer (0.3 M NaOH, 1 mM EDTA). The DNA was allowed to unwind for 20 min before electrophoresis. Electrophoresis was carried out at by adjusting the voltage to 20 V and the current to 300 mA and allowed to run for 24 min. After the electrophoresis, the slides were neutralized two times with a fresh neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 5 min. Slides were drained, dried in absolute ethanol for 10 min and stored in a dry place until image analysis. Before analysis slides were stained with 50 μl of a 0.05 mM ethidium bromide solution. Two slides were prepared from each cell culture plate. Analyses of 50 cells were made using an Axioskop epi-fluorescent microscope, equipped with an excitation filter of 515–560 nm, a barrier filter of 590 nm. DNA damage was expressed as a tail extent moment value (product of the tail length by the tail DNA content) using an image analysis computerized method (Komet 3.1, Kinetic Imaging Ltd).

2.8. Statistical analysis

Our experiments were carried out with five trout. Statistical analysis was performed using the nonparametric Mann–Whitney *U*-test. Each result of DEVD-dependent protease activity represented the arithmetic mean of enzyme activities for five trout ($n = 5$) and was expressed as the percentage of enzyme activity measured in control cultures.

3. Results

Before measuring the effects of Cd(II) on trout hepatocyte DNA, the cells were exposed to increasing Cd(II) concentrations (15, 25, 40, 50, 60 and 75 μM) for 24 h in order to determine by using the Neutral Red test the midpoint cytotoxicity value (24 h IC_{50}). IC_{50} was found to be 45 μM of Cd(II).

To determine whether Cd(II) induced apoptosis in trout hepatocytes, DNA fragmentation was measured in cells treated with Cd(II). Apoptosis was identified on the basis of the occurrence of internucleosomal DNA cleavage on agarose gel electrophoresis. Gel electrophoretic patterns of the DNA of Cd(II)-treated or untreated cells were shown in Fig. 1a. No significant internucleosomal DNA cleavage was detectable in the control cells after 3, 6 and 24 h exposure. Cells treated with 1 and 2 μM Cd(II) showed a DNA ladder pattern characteristic of apoptosis after 6 h exposure, while a weak ladder staining was observed after an exposure to 10 μM Cd(II). When the treatment was prolonged to 24 and 48 h, Cd(II) promoted also DNA fragmentation, but the ladder staining decreased slightly under these conditions compared to 6 h exposure.

In parallel experiments, quantification of DEVD-dependent protease activity was done to provide information concerning the effects of Cd(II) on this enzyme activity as well as its role in apoptosis. Thus, by comparing the fluorescence of Cd(II)-treated cells with that of untreated hepatocytes, we could determine the level of protease activity following induction of apoptosis. Fig. 2 shows that treatment of cells with 1, 2 and 10 μM Cd(II) significantly increased DEVD-dependent protease activity. After 24 h exposure, this enzyme activity increased in a dose-dependent manner to reach at 10 μM Cd(II) more than 55% above the control level ($P < 0.01$). When the treatment was prolonged to 48 h, it was induced significantly to more than 71, 88 and 113% above the control

level in cells treated respectively with 1, 2 and 10 μM Cd(II). As shown in Fig. 2, the levels of protease activity decreased when the time-exposure of cells is prolonged (3–48 h).

To investigate the possible protective effect of TEMPO against Cd(II)-mediated apoptosis (this compound is able to scavenge free oxiradicals and to form stable compounds), we performed two different series of experiments. Trout hepatocytes in culture were exposed simultaneously to Cd(II) (1, 2 and 10 μM) and 100 μM TEMPO, concentration shown to be not cytotoxic using the Neutral Red test (data not shown). Fig. 1b shows that the presence of TEMPO had a protective effect against Cd(II)-mediated DNA fragmentation. Indeed, the exposure of cells to increasing Cd(II) concentrations and 100 μM TEMPO reduced significantly Cd(II)-induced DNA cleavage detected after 6–48 h of treatment.

We next examined whether the presence of TEMPO would have also a suppressive effect on the activation of DEVD-related proteases. Cas-

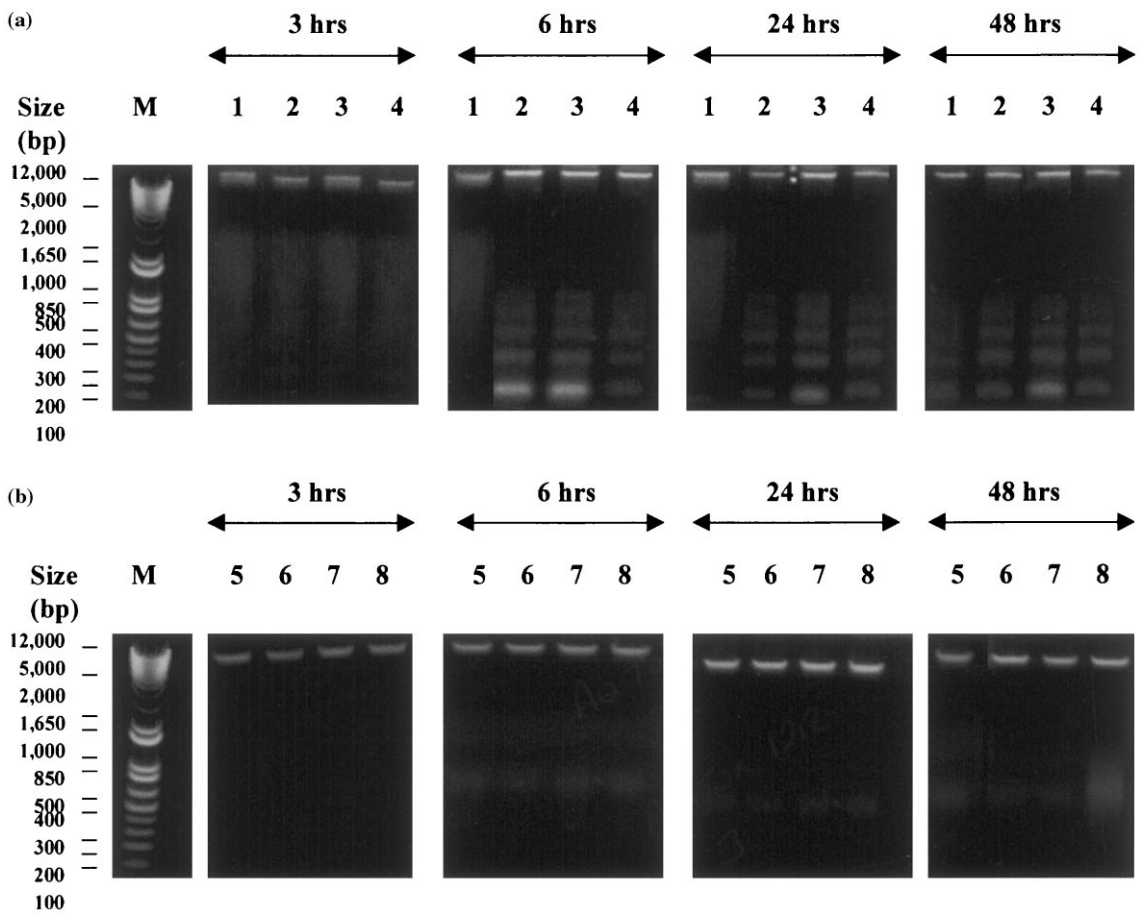

Fig. 1. Dose- and time-dependent responses of internucleosomal DNA degradation of trout hepatocytes exposed to increasing cadmium concentrations in the absence and presence of TEMPO. DNA fragmentation was visualized as oligonucleosome-size fragments stained with ethidium bromide in agarose gels and transilluminated with UV light for photography. DNA was prepared from 4×10^6 cells incubated with 1, 2 and 10 μM Cd(II) (lanes 2, 3 and 4 respectively, (a)), and simultaneously with 100 μM TEMPO and 1, 2 and 10 μM Cd(II) (lanes 6, 7 and 8 respectively, (b)), for 3, 6, 24 and 48 h and analysed as described in Section 2. One microgram of each DNA sample was electrophoresed on each lane. Lane M shows the molecular weight markers (12 kb–100 bp) (1 kb Plus DNA Ladder, Gibco BRL Life Technologies, Cergy Pontoise, France). Lane 1 (Fig. 1a): control cells. Lane 5 (Fig. 1b): cells exposed to 100 μM TEMPO.

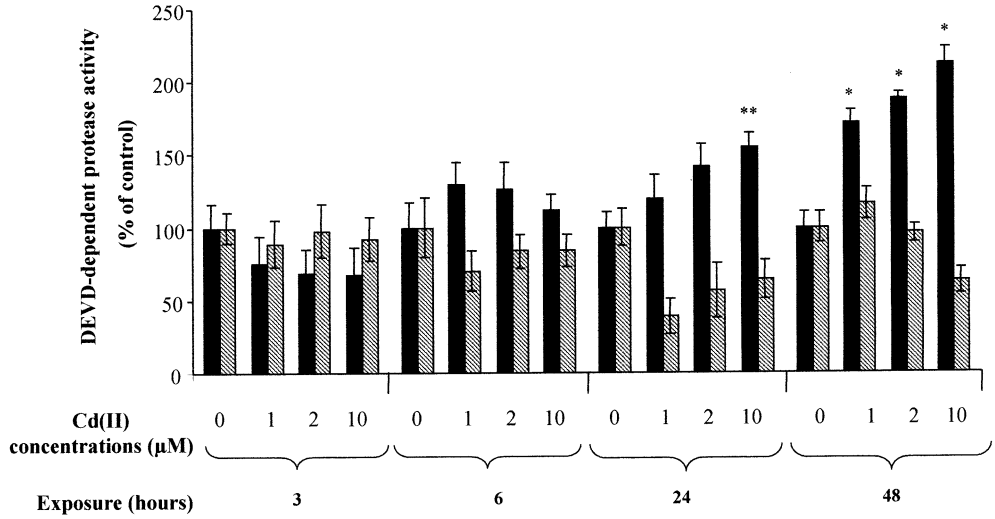

Fig. 2. Dose- and time-dependent effects of increasing cadmium concentrations on DEVD-dependent protease activity in the absence and presence of TEMPO in trout hepatocytes. Cells were incubated for 3, 6, 24 and 48 h with 1, 2 and 10 μM Cd(II) alone (■) and with both 100 μM TEMPO and 1, 2 and 10 μM Cd(II) (▨). Each bar represents the mean of DEVD-dependent protease activities for five different experiments expressed as the percentage of enzyme activity found in control cultures. Asterisks indicate significant difference from control (*$P < 0.05$; **$P < 0.01$; Mann–Whitney U-test).

pases are involved in early phases of apoptosis, they cleave proteins. As shown in Fig. 2, TEMPO significantly reduced the Cd(II)-mediated activation of this enzyme activity after 6 h exposure ($P < 0.05$). Indeed, there was no significant change between the enzyme activity levels of hepatocytes exposed to both TEMPO and Cd(II) and those of cells incubated with TEMPO alone. Furthermore our results emphasized that exposure to TEMPO and 1, 2 and 10 μM Cd(II) for 24 h decreased significantly the levels of the protease activity compared with those found with Cd(II) alone ($P < 0.05$).

Typical photomicrographs of comets due to DNA strand breaks observed after alkaline gel electrophoresis assay can be seen in Fig. 3. Fig. 3b shows a cell which had undergone apoptosis after 48 h exposure to 2 μM Cd(II). The extensive DNA fragmentation present in this apoptotic cell allowed most of the DNA to migrate, leaving only a small amount of DNA in the head of the comet figure. A lower DNA migration was observed in cells treated for 24 h with 0.5 μM 3-MC (Fig. 3c) and 1 μM B(a)P (Fig. 3d) and in both cases the shape of the comet figures appeared to be similar, exhibiting a larger head size than that observed in Cd(II) treated cells.

In order to evaluate the relative contribution of the oxidative pathway to the global genotoxicity expression of cadmium measured as DNA strand break formation, hepatocytes were exposed both to Cd(II) and to TEMPO. Fig. 4 shows the results of a 24 and 48 h exposure. DNA damage clearly increases according to the Cd(II) concentrations, reaching a threefold value compared to the control after 24 h exposure to 10 μM Cd(II). Exposure for 24 h to both 1 or 2 μM Cd(II) and 100 μM TEMPO cancels the DNA damaging effect of Cd(II). Simultaneous exposure of cells to 10 μM Cd(II) and 100 μM TEMPO leads to a DNA damage level close to that observed after exposure to 10 μM Cd(II) alone. Genotoxic effect of Cd(II) appeared to be more pronounced after 48 h compared with 24 h exposure, since nuclei of hepatocytes exposed to 2 μM Cd(II) for 48 h exhibited a tail extent moment value threefold higher than the control one (versus 1.5-fold after 24 h exposure). A sharp decrease in tail extent moment is noted after exposure of liver cells to 10 μM Cd(II), in contrast to the plateau observed in DNA damage

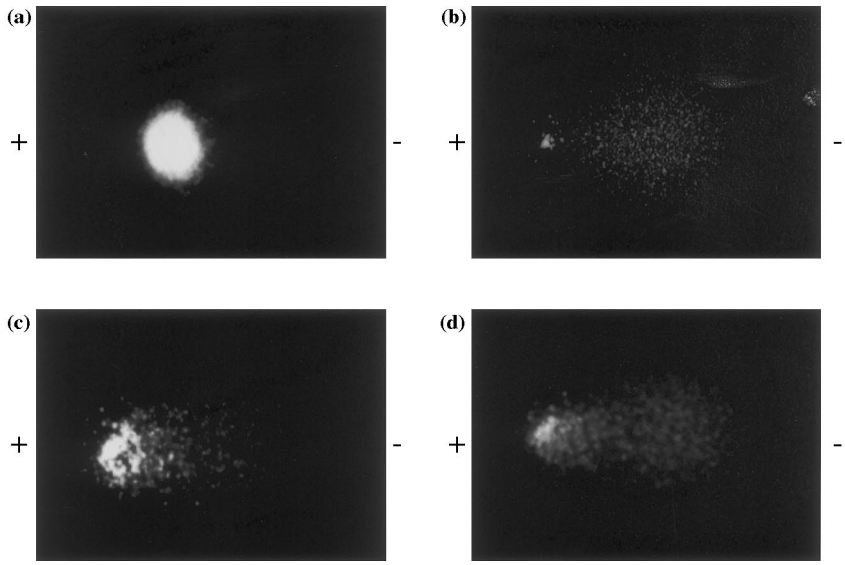

Fig. 3. Fluorescence photomicrographs of trout hepatocytes (× 400). The plus and minus signs indicate the anode and cathode during electrophoresis (negatively charged DNA migrates towards the anode). (a) Comet figure of control cell; (b) comet figure of cells treated for 48 h with 2 μM Cd(II); (c) comet figure of cells treated for 24 h with 0.5 μM 3-MC; (d) comet figure of cells treated for 24 h with 1 μM B(a)P.

level of cells exposed simultaneously to TEMPO. Addition of TEMPO for 48 h contributes significantly to decrease the DNA damage level after cell exposure to 1 and 2 μM Cd(II), but to a less extent than after 24 h exposure.

4. Discussion

During the last decade, interest in apoptosis has increased significantly, in great part due to the fact that its deregulation may have important implications on immune system disorders and carcinogenesis. However, apoptosis in the liver has not been extensively studied in general and in particular few attention has been devoted to apoptosis studies in fish cells. Lyons-Alcantara et al. (1998) have previously observed apoptosis in the skin epithelial cells of rainbow trout, and Reader et al. (1999) have reported that tributyltin triggered apoptosis in trout hepatocytes.

The present study indicates that Cd(II) can induce cell death by apoptosis in rainbow trout hepatocytes. The activation of DEVD-dependent proteases is a key event in Cd(II)-induced apoptosis and our results demonstrate the utility of the assay for assessing the role of caspase-family proteases in apoptotic cell progression. However, the precise identification of these proteases impli-

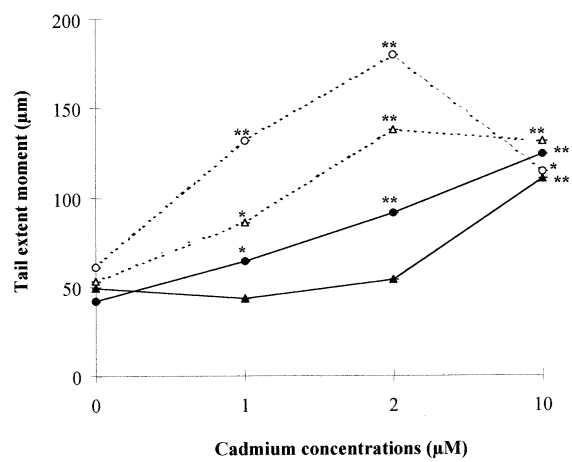

Fig. 4. Effect of the oxyradical scavenger TEMPO on DNA strand breaks formation in trout hepatocytes exposed to cadmium. —●— 24 h Cd exposure; – –○– – 48 h Cd exposure; —▲— 24 h Cd + TEMPO exposure; – –△– – 48 h Cd + TEMPO exposure. Asterisks mean significantly different from the corresponding control (*$P < 0.05$; **$P < 0.01$).

cated in the pro-apoptotic process induced by Cd(II) is as yet unresolved. Moreover, DNA fragmentation was evidenced in the present study by DNA gel electrophoresis in Cd(II)-treated trout hepatocytes. The internucleosomal DNA cleavage with the production of oligonucleosomal fragments was the first biochemical event identified in apoptosis (Wyllie, 1981) and it occurs almost in all instances of apoptosis (Martin et al., 1994).

DEVD-dependent protease activity and extent of internucleosomal DNA degradation remained unchanged after Cd(II) treatment for 3 h, but then they increased progressively up to 48 h exposure compared to the control. The present results demonstrate clearly that treatment with Cd(II) induces a delayed response of DEVD-dependent protease activity and internucleosomal DNA degradation. This time-delayed effect suggests that induction of enzyme activity and internucleosomal DNA degradation are not due to only a direct interaction of Cd(II) with DNA. It seems that Cd(II) triggers a series of events leading to the activation of proteases and to DNA fragmentation. A time delay in internucleosomal DNA degradation has also been described in other carcinogenic metals such as arsenite and chromium (Manning et al., 1994; Wang et al., 1996).

The induction of DEVD-dependent protease activity was not parallel to the internucleosomal DNA degradation. For example, 24 h treatment of hepatocytes with 10 μM Cd(II) induced an higher DEVD-dependent protease activity than with 1 and 2 μM Cd(II), but the amount of internucleosomal DNA degradation induced by 10 μM Cd(II) did not follow this trend. In the same way, a prolonged exposure of cells to Cd(II) for 48 h induced a dose-dependent increase of DEVD-dependent protease activity, whereas the amount of internucleosomal DNA degradation obtained from these cells remained unchanged. Thus, these results emphasize the fact that the induction of DEVD-dependent protease activity occurs upstream in the apoptotic pathway as an earlier step than the internucleosomal DNA fragmentation event considered as an ultimate consequence of apoptosis. A 24 h exposure of cells to 10 μM Cd(II) and a 48 h treatment with increasing Cd(II) concentrations in the range of 1–10 μM might induce DNA degradation at sites other than internucleosomal junction. As revealed by alkaline comet assay, a significant induction of DNA strand breaks was noted after 24 h exposure of cells to 10 μM Cd(II). In spite of the insufficient results in the present study, it can be hypothesized that single-strand breaks could play a role in apoptosis, acting as signals to induce this process, even if such a role is still controversial (Yoshida et al., 1993; Martin et al., 1994).

Recent studies in different systems suggest that oxidative stress with the consequent generation of reactive oxygen species may serve as a common mediator in apoptosis (Kane et al., 1993; Buttke and Sandström, 1994; Hartwig, 1994; Beyersmann and Hechtenberg, 1997; Lyons-Alcantara et al., 1998). Many chemical and physical treatments capable of inducing apoptosis are also known to cause oxidative stress, and are either oxidants or stimulators of cellular oxidative metabolism. In some cells the occurrence of apoptosis will be determined by the ability of a cell to maintain an appropriate oxidant–antioxidant balance (Buttke and Sandström, 1994). A marked reduction of apoptosis was obtained by treating cells simultaneously with Cd(II) plus the spin probe TEMPO: the induction of DEVD-dependent protease activity and the internucleosomal DNA degradation were effectively reduced by the presence of TEMPO. Results of the present study indicate that reactive oxygen species may be involved in Cd(II)-induced apoptosis in trout hepatocytes. This hypothesis is further supported by the fact that Cd(II) is known to induce oxidative stress by depleting intracellular stores of glutathione and protein-bound sulphydryl groups resulting in the production of reactive oxygen species (Stohs and Baghi, 1995). Results from studies using cultured cells have demonstrated Cd(II)-induced formation of superoxide anion radicals (Amoruso et al., 1982) and implicated superoxide anions in Cd(II)-induced DNA single-strand scission (Ochi et al., 1983). Cd(II) inhibits superoxide dismutase in vivo, resulting in elevated superoxide levels (Shukla et al., 1987). Cd(II) has been shown to increase peroxidation of lipids in isolated rat hepatocytes (Stacey and Kappus, 1982a,b) and in other target tissues in vivo and in vitro (Gabor et

al., 1978; Wahba and Waalkes, 1990). Thus, increased levels of lipid peroxides following exposure to Cd(II) could constitute a source of active oxygen species (Goering et al., 1995).

Cadmium exposure of trout hepatocytes clearly elicits DNA damage leading to DNA strand breaks measurable through the comet assay. It has first to be pointed out that DNA strand breaks can accumulate inside the cells, since hepatocytes exposed for 48 h exhibit a higher DNA strand break amount than those exposed for 24 h, up to 2 μM Cd(II). Surprisingly the level of DNA strand breaks observed in cells exposed to 10 μM Cd(II) for 48 h remains significantly lower than that measured at 2 μM and close to the value obtained after 24 h exposure to 10 μM Cd(II). This trend was not noted after 24 h exposure suggesting a threshold requirement. This decrease in DNA strand breaks with increasing exposure time and higher toxicant concentration could be due to a high degree of DNA fragmentation, thus leading to a possible loss of these fragments during electrophoresis (Olive et al., 1993; Devaux et al., 1997).

TEMPO addition to hepatocytes exposed to 1 and 2 μM Cd(II) for 24 h and to a less extent for 48 h cancels the genotoxic effect of the metal. There is a balance between both cadmium and TEMPO effects since beyond a certain DNA damage level, TEMPO can not totally inhibit cadmium effect. Toxic effects of cadmium in living organisms have been extensively studied and even if conflicting results have been published in particular about its mutagenic and clastogenic properties, some underlying modes of action have been proposed depending on the organism and the metal concentration. Both direct and indirect effects of cadmium have been described (Hartwig, 1994). Among them, two modes of action accounting for the genotoxicity potential of cadmium seem to be predominant: the induction of oxidative DNA damage and the interaction with DNA repair processes (Hartwig, 1994; Beyersmann and Hechtenberg, 1997).

TEMPO is capable of protecting cells from free-radical-induced damage, apparently due to the reaction with carbon-centered radicals (Chateauneuf et al., 1988), and capability of oxidizing Fe^{2+} to Fe^{3+} (Voest et al., 1993). This nitroxide, being hydrophobic, could penetrates and cross cell membranes, thus conferring a certain degree of protection at the site at which DNA damage occurs. The mechanism by which the nitroxide seems to protect DNA is probably due to their free radical scavenging abilities because they react with carbon- and oxygen-centered radicals, including superoxide anion, thereby preventing these radicals from reaching and reacting with DNA. The reactivity of these different reactive species and the protection by various nitroxide radicals have already been studied in model systems (Grinberg and Samuni, 1994; Beyersmann and Hechtenberg, 1997; Villarini et al., 1998). Since TEMPO is known to effectively scavenge reactive oxygen species by forming stable free radical, our results corroborate the fact that cadmium genotoxicity observed in trout hepatocytes could arise at least partially from the oxidative DNA damage pathway.

In conclusion, our results which demonstrate activation of caspase 3 activity, cleavage of DNA to nucleosomal size fragments and induction of DNA strand breakage (COMET assay) in trout hepatocytes exposed to acute doses of Cd(II) show that this metal induces cellular apoptosis and that this is reduced by loading the cells with the radical scavenger TEMPO. We therefore hypothesise that the acute toxicity of Cd(II) can be attributed to generation of reactive oxygen species which induces apoptosis. Additional studies are required for proposing a mechanistic model of Cd(II)-induced apoptosis and genotoxicity in trout liver cells, in underlying the balance between DNA damage and fish defence systems in terms of DNA repair, detoxification enzymes and heavy metal binding with specific scavengers, such as metallothioneins.

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References

Amacher, D.E., Paillet, S.C., 1980. Induction of trifluorothymidine-resistant mutant by metal ions in L5178Y/TK + / − cells. Mutat. Res. 78, 279–288.

Amoruso, M.A., Witz, G., Goldstein, B.D., 1982. Enhancement of rat and human phagocyte superoxide anion radical production by cadmium in vitro. Toxicol. Lett. 10, 133–138.

Babich, H., Borenfreund, E., 1991. Cytotoxicity and genotoxicity assays with cultured fish cells: a review. Toxicol. In Vitro 5, 91–100.

Baksi, S.M., Frazier, J.M., 1990. Isolated fish hepatocytes-model systems for toxicology research. Aquat. Toxicol. 16, 229–256.

Belpaeme, K., Delbeke, K., Zhu, L., Kirsch-Volders, M., 1996. Cytogenetic studies of PCB77 on brown trout (*Salmo trutta fario*) using the micronucleus test and the alkaline comet assay. Mutagenesis 11, 485–492.

Belpaeme, K., Cooreman, K., Kirsch-Volders, M., 1998. Develpoment and validation of the in vivo alkaline comet assay for detecting genomic damage in marine flatfish. Mutat. Res. 415, 167–184.

Beyersmann, D., Hechtenberg, S., 1997. Cadmium, gene regulation, and cellular signalling in mammalian cells. Toxicol. Appl. Pharm. 144, 247–261.

Buttke, T., Sandström, P.A., 1994. Oxidative stress as a mediator of apoptosis. Immunol. Today 15, 7–10.

Chateauneuf, J., Lusztyz, J., Ingold, K.U., 1988. Absolute rate constants for the reactions of some carbon-centered radicals with 2,2,6,6-tetramethylpiperidine-*N*-oxyl. J. Org. Chem. 53, 1629–1632.

Cohen, G.M., 1997. Caspases: the executioners of apoptosis. Biochem. J. 326, 1–16.

Darfler, M.M., Karaszkiewicz, J.W., 1996. In situ localisation of apoptosis using terminal deoxynucleotidyl transferase. Focus 17 (3), 81–83.

Degrave, N., 1981. Carcinogenic, teratogenic and mutagenic effects of cadmium. Mutat. Res. 86, 115–135.

Devaux, A., Pesonen, M., Monod, G., 1997. Alkaline comet assay in rainbow trout hepatocytes. Toxicol. In Vitro 11, 71–79.

Devaux, A., Flammarion, P., Bernardon, V., Garric, J., Monod, G., 1998. Monitoring of the chemical pollution of the river Rhone through measurement of DNA damage and cytochrome P4501A induction in chub (*Leuciscus cephalus*). Mar. Environ. Res. 46, 257–262.

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, 127–139.

Fairbairn, D.W., Olive, P.L., O'Neill, K.L., 1995. The comet assay: a comprehensive review. Mutat. Res. 339, 37–59.

Forrest, V.J., Kang, Y.H., McClain, D.E., Robinson, D.H., Ramakrishnan, N., 1994. Oxidative stress-induced apoptosis prevented by Trolox. Free Radic. Biol. Med. 16, 675–684.

Gabor, S., Anca, Z., Bordas, E., 1978. Cadmium-induced lipid peroxidation in kidney and testes: effect of zinc and copper. Rev. Roum. Biochim. 15, 113–117.

Goering, P.L., Waalkes, M.P., Klaassen, C.D., 1995. Toxicology of cadmium. In: Goyer, R.A., Cherias, M. (Eds.), Toxicology of Metals: Biochemical Aspects, Handbook of Experimental Pharmacology, vol. 15(9), pp. 189–214.

Gopalakrisna, P., Khar, A., 1995. Comet assay to measure DNA damage in apoptotic cells. J. Biochem. Biophys. Methods 30, 69–73.

Grinberg, L.N., Samuni, A., 1994. Nitroxide stable radical prevents primaquine-induced lysis of red blood cells. Biochim. Biophys. Acta 1201, 284–288.

Gurtu, V., Kain, S.R., Zhang, G., 1997. Fluorometric and colorimetric detection of caspase activity associated with apoptosis. Anal. Biochem. 251, 98–102.

Hartwig, A., 1994. Role of DNA repair inhibition in lead- and cadmium-induced genotoxicity: a review. Environ. Health Perspect. 102, 45–50.

Hartwig, A., 1995. Current aspects in metal genotoxicity. Biometals 8, 3–11.

Iger, Y., Lock, R.A.C., Van der Meij, J.C.A., Wenderlaar Bonga, S.E., 1994. Effects of water-borne cadmium on the skin of the common carp (*Cyprinus carpio*). Arch. Environ. Contam. Toxicol. 26, 342–350.

Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Butler Gralla, E., Selvertone Valentine, J., Ord, T., Bredesen, D.E., 1993. Bcl-2 inhibition of neural cell death: decreased generation of reactive oxygen species. Science 262, 1275–1277.

Lyons-Alcantara, M., Mooney, R., Lyng, F., Cottell, D., Mothersill, C., 1998. The effect of cadmium exposure on the cytology and function of primary cultures from rainbow trout. Cell Biochem. Funct. 16, 1–13.

Mandel, R., Ryser, H.J.P., 1984. Mutagenicity of cadmium in *Salmonella typhimurium* and its synergism with two nitrosamines. Mutat. Res. 138, 9–16.

Manning, F.C., Blankenship, L.J., Wise, J.P., Xu, J., Bridgewater, L.C., Patierno, S.R., 1994. Induction of internucleosomal DNA fragmentation by carcinogenetic chromate: relationship to DNA damage, genotoxicity, and inhibition of macromolecular synthesis. Environ. Health Perspect. 102, 159–167.

Martin, S.J., Green, D.R., Cotter, T., 1994. Dicing with death: dissecting the components of the apoptosis machinery. TIBs Rev. 19, 26–30.

McConkey, D.J., Orrenius, S., 1994. Signal transduction pathways to apoptosis. Trends Cell Biol. 4, 370–374.

Mitchelmore, C.L., Chipman, J.K., 1998. Detection of DNA strand breabs in brown trout (*Salmo trutta*) hepatocytes and blood cells using the single cell gel electrophoresis (comet) assay. Aquat. Toxicol. 41, 161–182.

Monod, G., Devaux, A., Valotaire, Y., Cravedi, J.P., 1998. Primary cell cultures from fish in ecotoxicology. In: Braunbeck, T., Hinton, D.E., Streit, B. (Eds.), Fish Ecotoxicology. Birkhäuser Verlag, Basel, pp. 39–60.

Nicholson, D.W., Ambereen, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Garreau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y., Munday, N.A., Raju, S.M., Smulson, M.E., Yaurin, T.T., Yu, V.L., Miller, D.K., 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376, 37–43.

Ochi, T., Ohsawa, M., 1983. Induction of 6-thioguanine-resistant mutants and single strand scission of DNA by cadmium chloride in cultured Chinese hamster cells. Mutat. Res. 111, 69–78.

Ochi, T., Ishiguro, T., Ohsawa, M., 1983. Participation of active oxygen species in the induction of DNA single-strand scission by cadmium chloride in cultured Chinese hamster cells. Mutat. Res. 122, 169–175.

Ochi, T., Mogi, M., Watanabe, M., Ohsawa, M., 1984. Induction of chromosomal abberations in cultured Chinese hamster cells by short-term treatment with cadmium chloride. Mutat. Res. 137, 103–109.

Olive, P., Frazer, G., Banath, J.P., 1993. Radiation-induced apoptosis measured in TK6 human B lymphoblast cells using the comet assay. Radiat. Res. 136, 130–136.

Olive, P., Banath, J.P., 1995. Sizing highly fragmented DNA in individual apoptotic cells using the comet assay and a DNA crosslinking agent. Exp. Cell Res. 221, 19–26.

PROMEGA, 1998. CaspACE™ Assay System fluorometric, Technical Bulletin, Madison WI, pp. 1–11.

Reader, S., Moutardier, V., Denizeau, F., 1999. Tributyltin triggers apoptosis in trout hepatocytes: the role of Ca^{2+}, protein kinase C and proteases. Biochem. Biophys. Acta 1448, 473–485.

Risso-de Faverney, C., de Sousa, G., Lafaurie, M., Rahmani, R., 1999. Expression and induction of CYP1A1 in black seabream (*Spondyliosoma cantharus*) hepatocyte cultures: effects of heavy metals. Polycyclic Arom. Comp. 13, 285–299.

Röhr, G., Bauchinger, M., 1976. Chromosome analysis in cell cultures of the Chinese hamster after application of cadmium sulphate. Mutat. Res. 40, 125–130.

Shiraishi, Y., Kurahashi, H., Yoshida, T.H., 1972. Chromosomal abberations in cultured human lymphocytes induced by cadmium sulphide. Proc. Jpn. Acad. 48, 133–137.

Shukla, G.S., Hussain, T., Chandra, S.V., 1987. Possible role of regional superoxide dismutase activity and lipid peroxide levels in cadmium neurotoxicity: in vivo and in vitro studies in growing rats. Life Sci. 41, 2215–2221.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

Stacey, N.H., Kappus, H., 1982a. Cellular toxicity and lipid peroxidation in response to mercury. Toxicol. Appl. Pharmacol. 63, 29–35.

Stacey, N.H., Kappus, H., 1982b. Comparison of methods of assessment of metal-induced lipid peroxidation in isolated rat hepatocytes. J. Toxicol. Environ. Health 9, 277–285.

Stohs, S.J., Baghi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med. 18, 321–336.

Stripped, R.A., Trombetta, L.D., 1994. Anti-oxidant enzyme activity as biomarkers of acid stress in natural aquatic systems. In: Muller, R., Lloyd, D. (Eds.), Sublethal and Chronic Effects of Pollutants on Freshwater Fish, Fishing New Books. Blackwell Science, Oxford, pp. 249–261.

Villarini, M., Moretti, M., Damiani, E., Greci, L., Santroni, A.M., Fedeli, D., Falcioni, G., 1998. Detection of DNA damage in stressed trout nucleated erythrocytes using the comet assay: protection by nitroxide radicals. Free Radic. Biol. Med. 24, 1310–1315.

Voest, E.E., Van Faassen, E., Marx, J.J., 1993. An electron paramagnetic resonance study of the antioxidant properties of the nitroxide free radical Tempo. Free Radic. Biol. Med. 15, 589–595.

Wahba, Z., Waalkes, M.P., 1990. Effect of in vivo low-dose cadmium pretreatment on the in vitro interactions of cadmium with isolated interstitial cells of the rat testes. Fundam. Appl. Toxicol. 15, 641–650.

Wang, T.S., Kuo, C.F., Jan, K.Y., Huang, H., 1996. Arsenite induces apoptosis in Chinese hamster ovary cells by generation of reactive oxygen species. J. Cell. Physiol. 169, 256–268.

Wyllie, A.H., 1981. Cell death: a new classification separating apoptosis from necrosis. In: Bowen, I.D., Lockskin, R.A. (Eds.), Cell Death in Biology and Pathology. Chapman Hall, London, pp. 9–34.

Wyllie, A.H., 1997. Apoptosis and carcinogenesis. Eur. J. Cell Biol. 73, 189–197.

Yoshida, A., Ueda, T., Wano, Y., Nakamura, T., 1993. Single strand breaks induce apoptosis in human lymphocytes. Jpn. J. Cancer Res. 84, 566–573.