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Ecotoxicological evaluation of sodium fluoroacetate on aquatic organisms and investigation of the effects on two fish cell lines

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Abstract

Sodium monofluoroacetate (compound 1080) is one of the most potent pesticides. It is also a metabolite of many other fluorinated compounds, including anticancer agents, narcotic analgesics, pesticides or industrial chemicals. Other sources of water contamination are the atmospheric degradation of hydrofluorocarbons and hydrochlorofluorocarbons. However, there is little information available about the adverse effects of sodium fluoroacetate in aquatic organisms. Firstly, the bacterium *Vibrio fischeri* (decomposer), the alga *Chlorella vulgaris* (1st producer) and the cladoceran *Daphnia magna* (1st consumer) were used for the ecotoxicological evaluation of SMFA. The most sensitive models were *C. vulgaris* and *D. magna*, with a NOAEL of 0.1 and an EC_{50} of 0.5 mM at 72 h, respectively. According to the results after the acute exposure and due to its high biodegradation rate and low bioaccumulation potential, sodium fluoroacetate is most unlikely to produce deleterious effects to aquatic organisms. Secondly, two fish cell lines were employed to investigate the effects and mechanisms of toxicity in tissues from 2nd consumers. The hepatoma fish cell line PLHC-1 was more sensitive to SMFA than the fibroblast-like fish cell line RTG-2, being the uptake of neutral red the most sensitive bioindicator. Lysosomal function, succinate dehydrogenase and acetylcholinesterase activities were inhibited, glucose-6-phosphate dehydrogenase activity was particularly stimulated, and metallothionein and ethoxyresorufin-O-deethylase levels were not modified. Intense hydropic degeneration, macrovesicular steatosis and death mainly by necrosis but also by apoptosis were observed. Moreover, sulphydryl groups and oxidative stress could be involved in PLHC-1 cell death induced by SMFA more than changes in calcium homeostasis.

Keywords: Fluoroacetate; Ecotoxicity; Cytotoxicity; Alternative methods; Aquatic environment

1. Introduction

Sodium fluoroacetate (SMFA, compound 1080) is mainly used as a rodenticide and as a vertebrate pest control agent. It is applied at bait stations or aerially, as well as a predacide against coyotes which prey on sheep and goats in livestock protection collars. At least since the 1940s, SMFA is known as one of the most toxic substances around the world, retaining the restricted use classification imposed by the USEPA in 1978. It may be used only by trained and certified applicators. In the United Kingdom and the

Abbreviations: AChE, acetylcholinesterase; EC_{50}, mean effective concentration; EROD, ethoxyresorufin-O-deethylase; G6PDH, glucose-6-phosphate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; NOAEL, non-observed adverse effect level; SDH, succinate dehydrogenase; SMFA, sodium monofluoroacetate.

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United States of America, it is only used to control rodents in sewers, ships and warehouse with restricted access. In Australia and New Zealand, vertebrate pest, such as rabbits, wallabies, goats, wild pigs and opossums are controlled with the use of baits (WHO, 1975; ACGIH, 1991).

Fluoroacetate is also a metabolite of many other fluorinated pesticides, industrial chemicals, anticancer agents and narcotic analgesics (Goncharov et al., 2005). The popular anaesthetic agent halothane and the anticancer drug 5-fluoruracil are biotransformed into fluoroacetate (Arellano et al., 1998). Fluoroacetate is also formed and concentrated to high levels in certain plants, after fluoride uptake from soil, water or air (Twigg, 1993). Fluoroacetate has been also found in water after contamination of the atmosphere with hydrofluorocarbons and hydrochlorofluorocarbons (Berends et al., 1999). As consequence of the degradation of the mentioned compounds and through elutriation, fluoroacetate enters aquatic ecosystems.

SMFA is a white powder, odourless and tasteless, soluble in water but practically insoluble in non-polar solvents (Hayes and Laws, 1991). It is rapidly absorbed by the gastrointestinal tract and by inhalation, but it is not well absorbed by intact skin (Brockmann et al., 1955). Plasma levels of SMFA are roughly double that of other organs, but it is also distributed to lipid-rich organs, such as the liver, brain and kidneys (Eason et al., 1993).

Following ingestion of fluoroacetate there is a latent period before the onset of symptoms, during which the compound is activated to fluorocitrate (Sherley, 2004). SMFA produces its toxic action by blocking the Krebs cycle. The compound is incorporated into fluoroacetyl coenzyme A, which is condensed with oxalacetate to form fluorocitrate, a process dubbed as ''lethal synthesis'' (Buffa and Peters, 1950). Fluorocitrate inhibits competitively the enzyme aconitase and thereby blocks conversion of the citrate to isocitrate (Hardman et al., 2001). Only a minor fraction of fluorocitrate can be eliminated through the urine.

SMFA has caused human poisonings (Such et al., 1970; LaGoy et al., 1992) and many serious cases of livestock poisoning with high stock losses (Oelrichs and McEwan, 1962). The toxicity of SMFA varies markedly between species. It has been found that dogs and other mammals (warm-blooded species) are highly sensitive, but reptiles and amphibians (cold-blooded species) are relatively insensitive to this pesticide. This large difference of sensitivity to SMFA may be due to the characteristic metabolic activities of the different species (Twigg et al., 2003). A variety of factors could influence sensitivity to SMFA, including the level of exposure, the age of individuals, their breeding condition, inherited tolerance, body size and metabolic rate.

Despite its widespread use, the toxicological studies have been mainly focussed on vertebrates or recently in soil organisms (O'Halloran et al., 2005), but little information is known about the aquatic ecotoxicity of SMFA. Therefore, in order to study the hazard of this pesticide in aquatic organisms, a representative and cost-effective test battery comprising organisms from four trophic levels of the aquatic ecosystem has been applied. The use of *in vitro* methods in environmental testing, particularly those employing fish cell cultures, is an area of expanding possibilities in the ecotoxicological evaluation of mixtures, for controlling chemicals, emissions, effluents and hazardous wastes (Castaño et al., 2003; Repetto et al., 2003).

Five ecological model systems with several endpoints were employed for the investigation of the effects of SMFA at different exposure time periods. Firstly, three systems were used for the ecotoxicological evaluation of SMFA, including the inhibition of the bioluminescence of the bacterium *V. fischeri* (decomposer), the inhibition of the growth of the alga *Chlorella vulgaris* (1st producer) and the immobilization of the cladoceran *Daphnia magna* (1st consumer). The immobilization of *Daphnia* and the inhibition of the growth of algae are included in most environmental legislations, including Guidelines for testing of chemicals of the Organization for the Economic Cooperation and Development. However, the bioluminescence inhibition in *V. fischeri* has only been adopted by some regulations for the characterization of effluents.

Secondly, two fish cell lines were employed as *in vitro* systems to investigate the effects and mechanisms of toxicity of the pesticide in tissues from 2nd consumers. Cell morphology, total protein content, neutral red uptake, methylthiazol (MTS) metabolization, lysosomal function, succinate dehydrogenase (SDH) activity and glucose-6-phosphate dehydrogenase (G6PDH) leakage and activity were studied in two different fish cell lines: PLHC-1 derived from a hepatocellular carcinoma of the topminnow *Poeciliopsis lucida* and RTG-2 derived from the gonad of the rainbow trout *Oncorhynchus mykiss*. In addition, acetylcholinesterase (AChE) activity, metallothionein levels and ethoxyresorufin-O-deethylase (EROD) activity were studied in PLHC-1 cells.

2. Materials and methods

2.1. Toxicant exposure

A range of different concentrations of exposure solutions of SMFA (trade name: compound 1080) (Riedel-de Haën®) was prepared before use directly in the different complete culture media, according to the appropriate assay, and sterilized by filtration through a 0.22 μm Millipore® filter. After replacing the medium with the exposure solutions, the systems were incubated for the adequate exposure time period.

2.2. Model systems

2.2.1. Vibrio fischeri

Bioluminescence inhibition in the marine bacterium *V. fischeri* was evaluated at 5 and 15 min according to Cordina et al. (1993) by using freeze-dried bacteria incubated at 15 °C from Microtox® test (Microbics Corp. Carlsbad, USA).

2.2.2. Chlorella vulgaris

Growth inhibition of the alga *C. vulgaris var viridis*, kindly provided by Dr. Muñoz-Reoyo (CISA, Spain) was evaluated up to 72 h in 96-well culture plates seeded with 200 µl/well of a 1 000 000 cells/ml algae culture in exponential growth phase in Bold's Basal Medium, using constant agitation and a temperature of 22 °C, under a water-saturated sterile atmosphere containing 5% CO_2 and a cold light source of 8000 lux. Absorbance at 450 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland). As quality criteria, the control cultures had to grow at least 10-fold in 48 h (Ramos et al., 1996).

2.2.3. Daphnia magna

D. magna, kindly provided by Dr. Muñoz-Reoyo (CISA, Spain), was maintained at 20 °C and fed with *C. vulgaris*. Acute toxicity immobilization tests were performed up to 72 h in standard reference water according to OECD Guideline 202 (2004) in replicate groups of 10 neonates per 25 ml, in 70 ml polystyrene flasks (Costar, Cambridge, MA, USA).

2.2.4. PLHC-1 cells

The hepatoma PLHC-1 cell line was derived from a hepatocellular carcinoma induced with 7,12-dimethylbenz(*a*)anthracene in an adult female *P. lucida*, a topminnow from the Sonoran desert (ATCC® # CRL-2406). The cells retain some of the characteristic morphology of primary liver hepatocytes, are epithelial, present an average population doubling time of 39.4 h, express aryl hydrocarbon receptors and basal and inducible P450IA activity (Ryan and Hightower, 1994; Fent, 2001). PLHC-1 cells (passages 90–105) were grown at 30 °C in a humidified incubator containing 5% CO_2 and propagated in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum (Gibco™), L-glutamine (BioWhittaker), sodium pyruvate (BioWhitaker) and non-essential amino acids (BioWhittaker). PLHC-1 cells in exponential growth phase were plated applying 0.2 ml of 450 000 cells/ml in each well of 96-well tissue-culture plates (Costar). After 24 h at 30 °C, the cultures received 0.2 ml medium containing the test chemical and were incubated for a further 24, 48 or 72 h. For the morphological study, PLHC-1 cells were seeded in Lab-Tek® tissue culture 8 well chamber slides applying 0.5 ml of 500 000 cells/ml (Nunc, Inc., Naperville, IL) previously coated with MatrigelTM (BD Biosciences). They were then exposed to SMFA for 24, 48 and 72 h, fixed in 70% methanol and stained with Mayer's hematoxylin and eosin or subjected to *in situ* hybridization (TUNEL) to detect induction of apoptosis (Enzo, Diagnostics, Farmingdale, US).

2.2.5. RTG-2 cells

The RTG-2 salmonid fish cell line, derived from the gonad of rainbow trout (*O. mykiss*) was kindly provided by Dr. Castaño (ISCIII, Spain). The cells present long spindle-like and fibroblast-like morphology, an average population doubling time of 72 h and basal and inducible P450IA activity (Araujo et al., 2000). The cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum (Biochrom), L-glutamine (BioWhittaker) and non-essential amino acids (BioWhittaker). RTG-2 cells in exponential growth phase (passages 107–120) were plated in 0.2 ml of 40 000 cells/ml in 96-well tissue-culture plates (Costar). After 48 h at 20 °C, the culture medium was replaced with 0.2 ml test medium and then incubated for a further 24 or 48 h (Castaño et al., 2003). For the morphological study, RTG-2 cells were seeded in Lab-Tek® tissue-culture chamber slides applying 0.5 ml of 50 000 cells/ml (Nunc, Inc., Naperville,

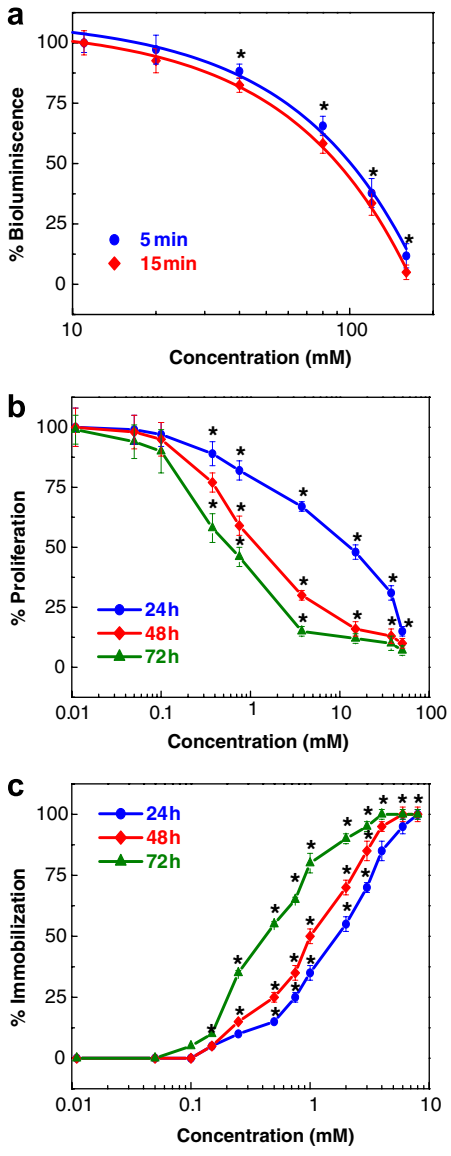

Fig. 1. Effects of exposure to different concentrations of sodium fluoroacetate studied as (a) bioluminescence inhibition of the bacterium *Vibrio fischeri* at 5 (●) and 15 min (♦); (b) proliferation of the alga *Chlorella vulgaris* and (c) immobilization of the cladoceran *Daphnia magna* at 24 (●), 48 (♦) and 72 h (▲). Data expressed in % of unexposed controls (mean ± SEM). * Indicates significant difference from control value ($p < 0.05$).

IL). They were then exposed to SMFA for 24 and 48 h, fixed in 70% methanol and stained with Mayer's hematoxylin and eosin or subjected to *in situ* hybridization (TUNEL) to detect induction of apoptosis (Enzo, Diagnostics, Farmingdale, US).

2.2.6. Fish cell bioindicators

Total cellular protein content was quantified *in situ*, using Coomassie brilliant blue G-250 (Repetto and Sanz, 1993) in the same 96-well tissue-culture plates in which exposure originally took place (Zurita et al., 2005a). Absorbance at 620 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland). Neutral red uptake was evaluated according to Babich and Borenfreund (1987) and lysosomal function in relative form to protein content (Repetto and Sanz, 1993). The MTS tetrazolium reduction assay was performed according to the procedure of Baltrop et al. (1991). The MTS tetrazolium compound is bioreduced by cells into a coloured formazan product soluble in the culture medium. G6PDH activity, in cells and in culture medium, was determined as described by García-Alfonso et al. (1998). AChE activity on intact cells was measured by adapting the method of Repetto et al. (1994). Metallothionein induction in cells was determined by atomic absorption spectrophotometry using the Cadmium/Haemoglobin affinity assay (Eaton and Cherian, 1991). EROD activity, a catalytic measurement of cytochrome P4501A induction, was determined by a direct fluorometric method described by Hahn et al. (1996).

2.2.7. Modulation studies

Before the exposure to 150 mM SMFA, PLHC-1 cell cultures were pre-treated for 30 min with 25 μM α-tocopherol succinate (Sigma), 20 mM mannitol (Sigma), 1 μM deferoxiamine mesylate (Sigma), 250 μM 1,4-dithiotreitol (Merck), 5 μM BAPTA-AM (Sigma) or 500 μM EGTA (Sigma), and for 24 h with 90 μM malic acid diethyl ester (Sigma) or 10 mM 2-oxothiazolidine-4-carboxylic acid (Sigma). After 24 h exposure to SMFA, the leakage of G6PDH was quantified as previously described.

2.3. Calculations and statistical analysis

All experiments were performed at least three times and at least in triplicate per concentration. Values for enzyme activities, lysosomal function and metallothionein levels were corrected for cell culture total protein content, as previously stated, to avoid misinterpretation due to the influence of the chemical tested on cell proliferation and cell detachment. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. Mean effective concentration (EC_{50}) values were determined by probit analysis. The estimated non-observed adverse effect levels (NOAEL) was identified as the highest concentration of a substance, which causes

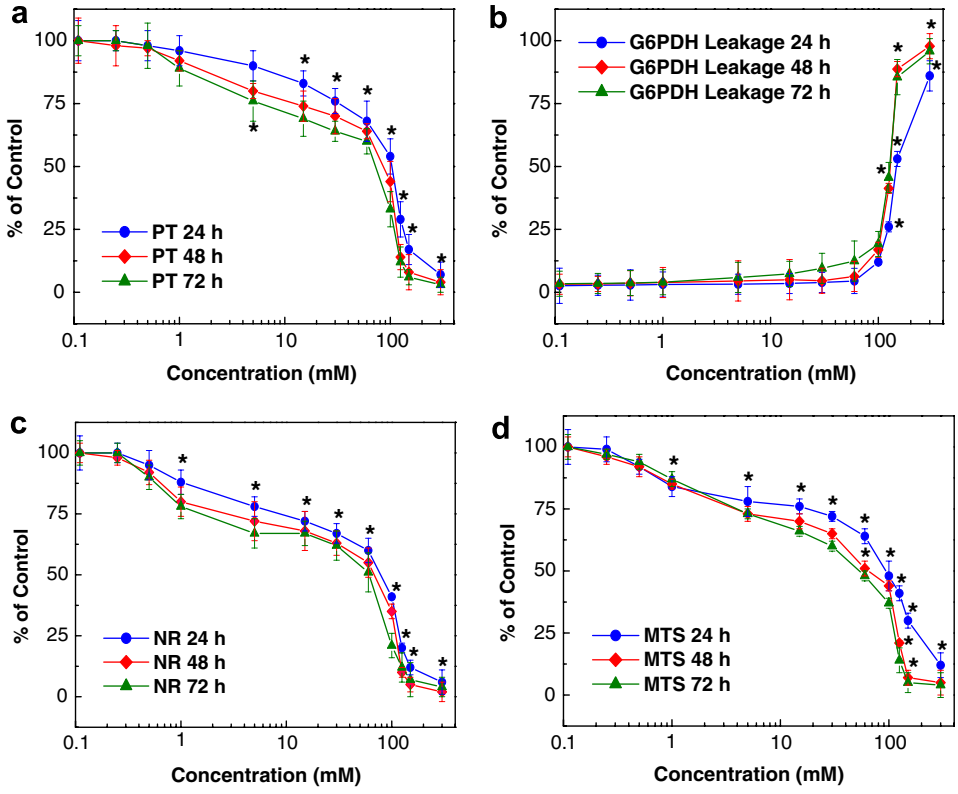

Fig. 2. Effects of sodium fluoroacetate on PLHC-1 fish cell cultures (a) total protein content, (b) glucose-6P dehydrogenase leakage, (c) neutral red uptake and (d) methylthiazol (MTS) metabolization after exposure to different concentrations for 24 (●), 48 (♦) and 72 h (▲). Data expressed in % of unexposed controls (mean ± SEM). * Indicates significant difference from control value ($p < 0.05$).

no detectable adverse effect on the target organism under defined conditions of exposure.

3. Results

3.1. Effects on Vibrio fischeri, Chlorella vulgaris and Daphnia magna

The results of this study demonstrated that the marine bacterium *V. fischeri* presented a low sensitivity to SMFA in comparison with other studied models. The inhibition of bioluminescence in the bacterium *V. fischeri* showed a dose-dependent curve, with EC_{50} values of 93 mM at 5 min and 67 at 15 min of exposure (Fig. 1a). On the contrary, both the green alga *C. vulgaris* and the cladoceran *D. magna* were very sensitive to SMFA. The inhibition of the proliferation of the fresh-water alga *C. vulgaris* presented the EC_{50} values of 12, 1.2 and 0.6 mM after 24, 48 and 72 h of exposure, respectively (Fig. 1b). A similar range of effects was observed for the immobilization of the crustacean *D. magna* with an EC_{50} value of 1 mM at 48 h (Fig. 1c).

3.2. Effects on the hepatoma fish cell line PLHC-1

The effects of different concentrations of SMFA were also investigated using PLHC-1 cells. The results obtained

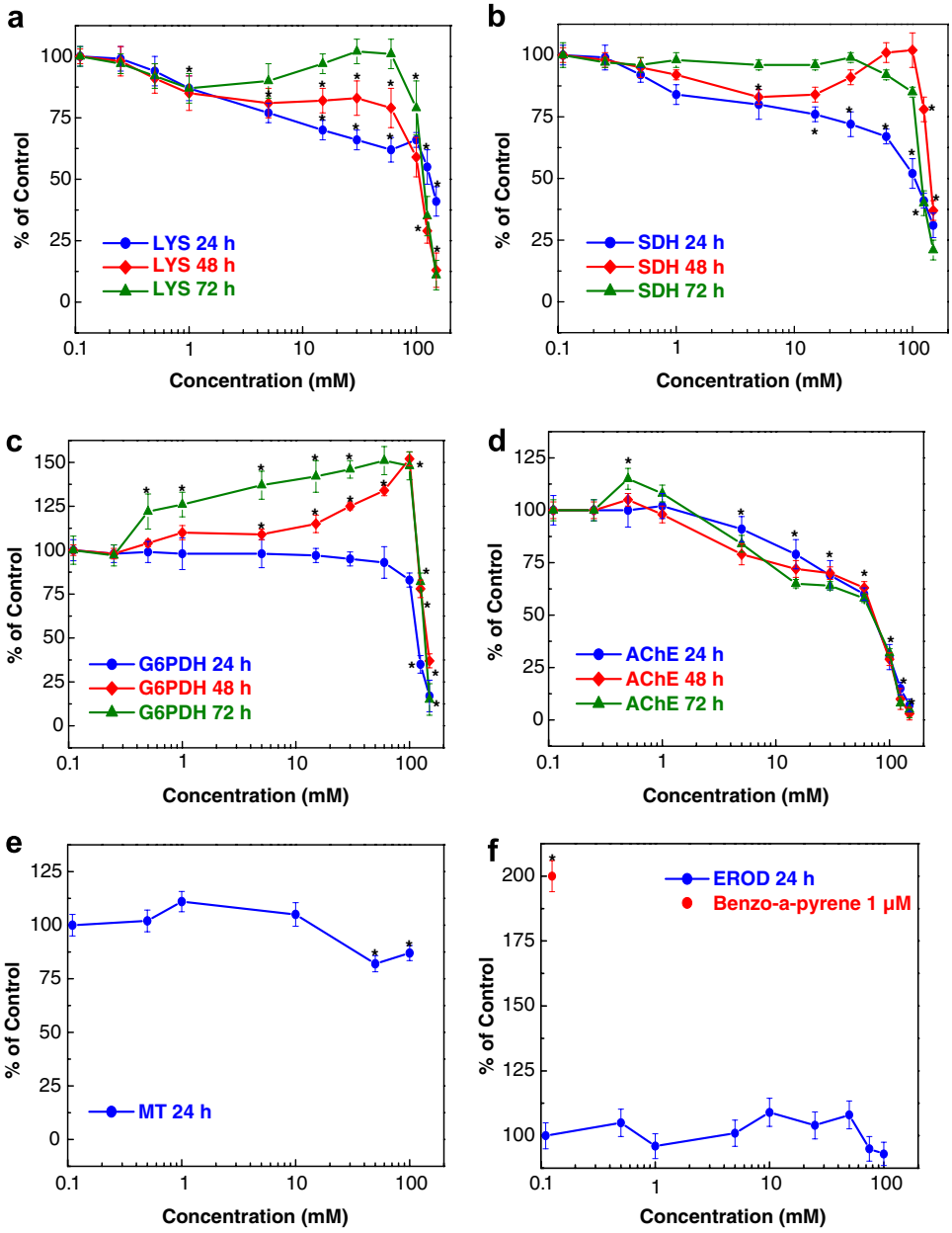

Fig. 3. Effects of sodium fluoroacetate on PLHC-1 fish cell cultures (a) lysosomal function, (b) succinate dehydrogenase activity, (c) glucose-6P dehydrogenase activity and (d) acetylcholinesterase activity at 24 (●), 48 (♦) and 72 h (▲); (e) metallothionein levels and (f) ethoxyresorufin-O-deethylase activity after 24 h (●). Data expressed in % of unexposed controls (mean ± SEM). * Indicates significant difference from control value ($p < 0.05$).

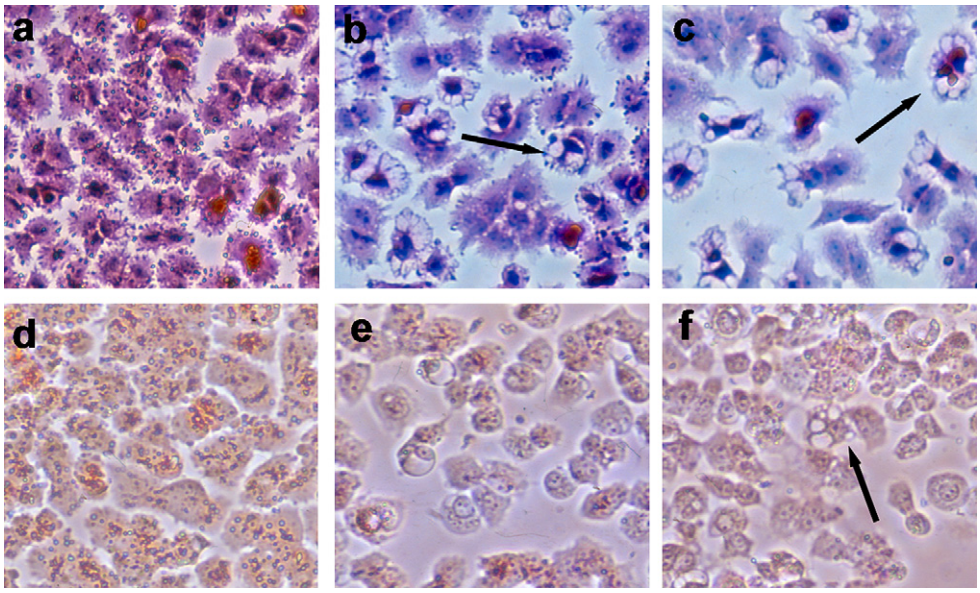

Fig. 4. Morphology of PLHC-1 cell cultures (×1600) stained with Mayer's Hematoxylin and Eosin (a–c) or neutral red (d–f): (a) control culture of PLHC-1 cells presents polygonal form, sinuous borders, with secretion vesicles around the cellular surface. (b) Culture treated with 1 mM sodium fluoroacetate for 24 h showing cytoplasmic vesicles and hydropic degeneration of the cytoplasm (cellular swelling) (→). (c) Multiples changes were observed in the cultures treated with 25 mM sodium fluoroacetate after 48 h, including decrease of the number of cells and hydropic degeneration (→). (d) Control culture of PLHC1 cells stained with neutral red. (e) Culture of cells exposed for 24 h to 1 mM sodium fluoroacetate showing a general decrease of the uptake of neutral red. (f) Culture exposed for 24 h to 25 mM sodium fluoroacetate with more evident damage, reduction in cell number, rounded cells and reduced uptake, and the presence of hydropic degeneration (→).

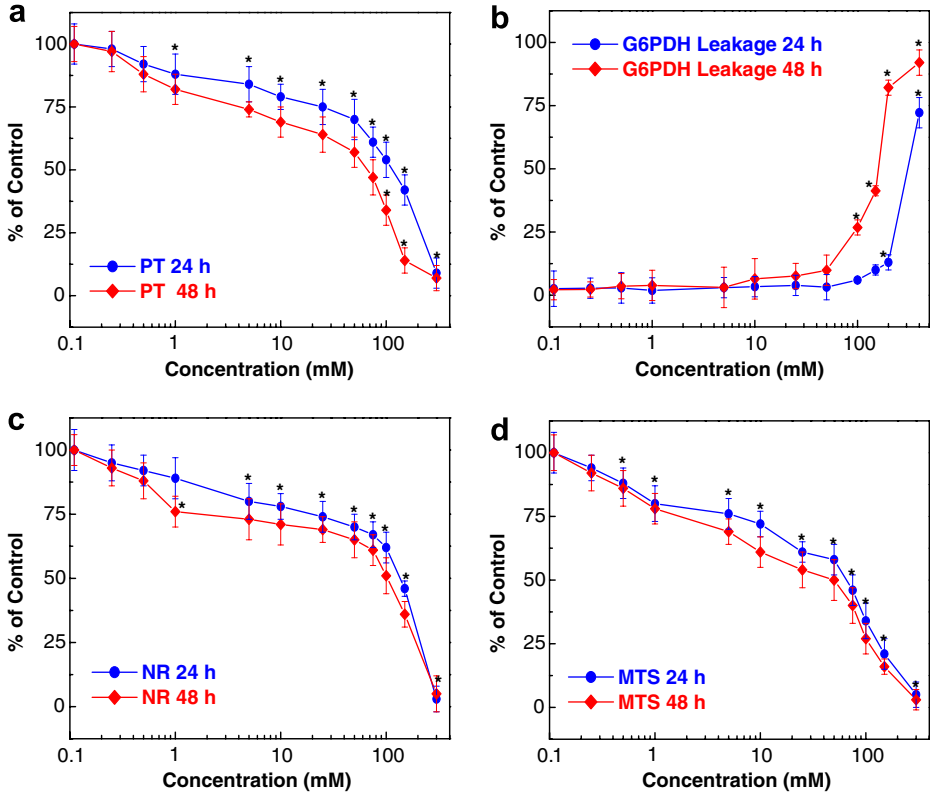

Fig. 5. Effects of sodium fluoroacetate in rainbow trout RTG-2 cells (a) total protein content, (b) glucose-6P dehydrogenase leakage, (c) neutral red uptake and (d) methylthiazol metabolization after exposure for 24 (●) and 48 h (♦). Data are expressed in % of each respective control treatment (mean ± SEM). * Indicates significant difference from control value ($p < 0.05$).

for the different endpoints evaluated on PLHC-1 cells are shown in Figs. 2 and 3. Cell proliferation, evaluated by quantification of total protein content, was inhibited with EC_{50} values from 81 to 108 mM (Fig. 2a). The leakage of

G6PDH, a marker of cell death, was the least sensitive bioindicator to SMFA, with levels over 120 mM needed to increase the leakage by 50% (Fig. 2b), while the uptake of neutral red was the most sensitive bioindicator in the fish cell line PLHC-1 with an EC_{50} value below 80 mM (Fig. 2c). A similar result to cellular growth was obtained with the metabolization of MTS (Fig. 2d). A marked slope change was detected around 50 mM SMFA in the dose–response curves of the four biomarkers of basal cytotoxicity, with a steeper slope at high concentrations.

The lysosomal function was reduced with less potency than SDH, which activity decreased with the increase of the exposure time period (Fig. 3a and b). However, G6PDH activity was not inhibited at 24 h except at high concentrations, being increased at 48 and 72 h of exposure (Fig. 3c). AChE activity was a sensitive endpoint with an EC_{50} value of 73 mM after 24 h of exposure (Fig. 3d). It was inhibited, except for a small increase at 72 h at low concentrations. No significant increases were observed in metallothionein levels after 24 h of exposure (Fig. 3e). As also expected, EROD activity was not induced in PLHC-1 cells exposed to different concentrations of SMFA (Fig. 3f).

Morphological changes, induced by SMFA, were investigated in the hepatoma fish cell line PLHC-1 (Fig. 4). As described by Ryan and Hightower (1994), the control cultures retain some of the characteristic morphology of hepatocytes. PLHC-1 cells present polygonal form, sinuous borders, with secretion vesicles around the cellular surface and are disposed in a uniform monolayer. They have abundant deposits of glycogen, tight junctions near the apical surface and basolateral interdigitations. Morphological changes were detected after 24 h exposure to 1 mM, and were more marked from 10 mM. The most prominent morphological changes were the induction of hydropic degeneration and of macrovesicular steatosis, increasing cellular swelling with high SMFA concentrations. Other morphological alterations included loss of cells and decrease in secretion vesicles. PLHC-1 cells treated with SMFA and stained with the cationic dye neutral red presented clear differences with regard to the corresponding untreated PLHC-1 controls. A reduction of the lysosomal function was observed. Cell death was evident after 24 h of exposure from 25 mM, mainly by necrosis but also by apoptosis, confirmed by *in situ* hybridization (TUNEL). The increase of effects was clear from 24 to 48 h.

3.3. Effects on the fibroblastic fish cell line RTG-2

The last model system studied was the salmonid fish cell line RTG-2. The results for the different endpoints evaluated are shown in Figs. 5 and 6. The most sensitive endpoint was the metabolization of MTS, followed closely by the reduction of SDH activity. The activity of G6PDH was mainly stimulated from 100 to 200 mM, with a 40% rise. Nevertheless, no significant increase was detected in lysosomal function. A progressive concentration-dependent increase in G6PDH leakage, a marker of cell death, was observed from 75 mM. The results obtained with RTG-2 cells were very similar to those found in PLHC-1 cells, being the total protein content, the uptake of neutral red and MTS metabolization also reduced of the same way, showing dose–response curves with two differentiated levels.

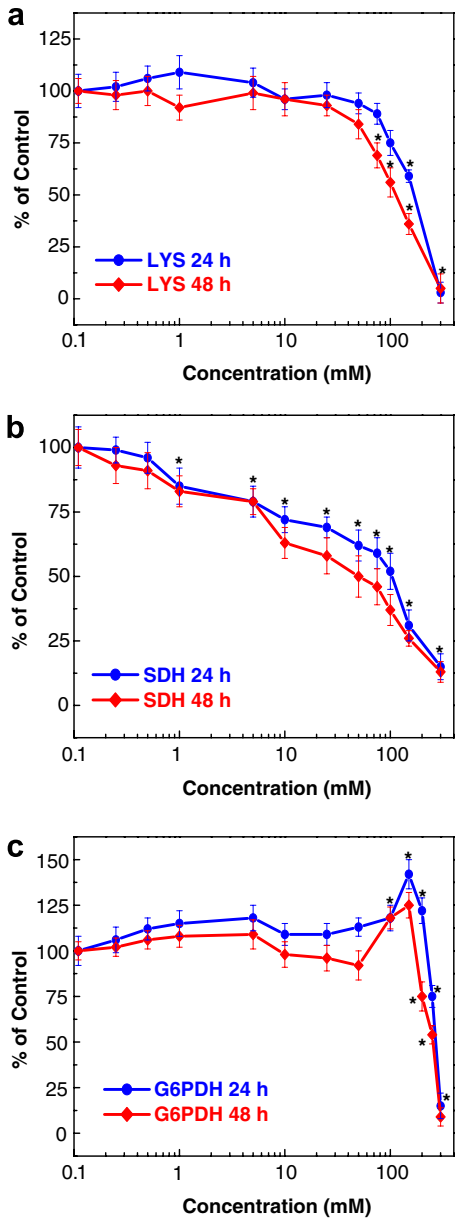

Fig. 6. *In vitro* effects of sodium fluoroacetate on RTG-2 cell culture (a) lysosomal function, (b) succinate dehydrogenase activity and (c) glucose-6P dehydrogenase activity for 24 (●) and 48 h (♦). Data are expressed in % of each respective control treatment (mean ± SEM). * Indicates significant difference from control value ($p < 0.05$).

Morphological changes, induced by SMFA, were also investigated in RTG-2 cells (Fig. 7). The control culture showed fusiform cells, arranged in plaques and disposed in parallel. They have well-defined borders, eosinophilic cytoplasm and central nuclei. The morphological alterations were detected from 1 mM at 24 h and were more

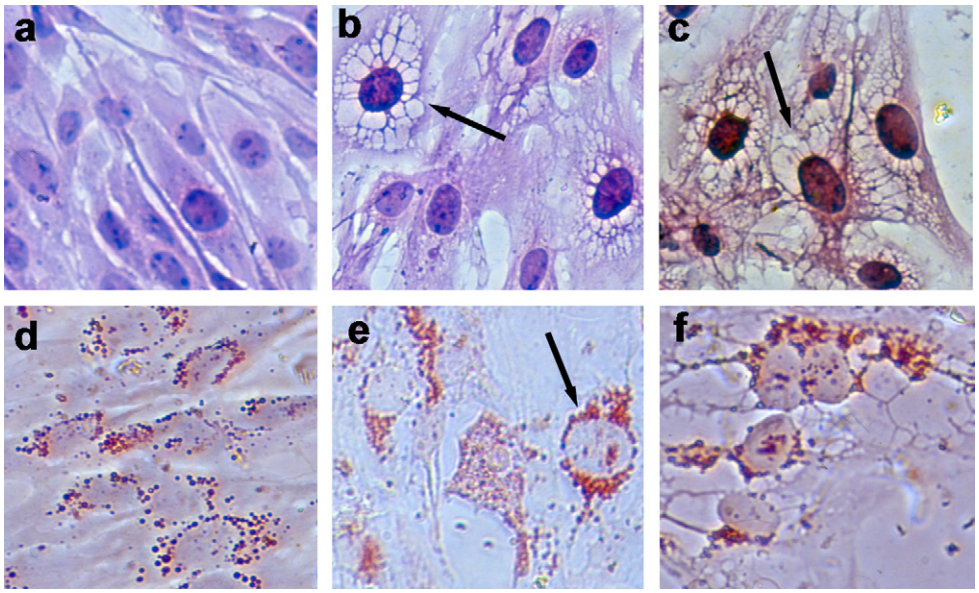

Fig. 7. Morphology of RTG-2 cell cultures (×1600) stained with Mayer's Hematoxylin and Eosin (a–c) or neutral red (d–f): (a) control culture of RTG-2 cells showing fusiform cells, with eosinophilic cytoplasm and central nucleus, arranged in plaques in parallel. (b) Cell culture exposed to 1 mM sodium fluoroacetate for 24 h showing loss of cells, induction of cellular pleomorphism, and a very evident hydropic degeneration of the cytoplasm (→). (c) Culture of cells exposed for 24 h to 25 mM of sodium fluoroacetate, with more generation of vacuoles in the cytoplasm (→). (d) Control culture stained with neutral red. (e) The exposure to 1 mM sodium fluoroacetate for 24 h induced a general loss of lysosomes but with a remarked perinuclear pattern of accumulation (→). (f) After exposure to 1 mM sodium fluoroacetate for 48 h only a few cells remained alive presenting more damage.

evident from 25 mM, including a marked reduction in cell number, a prominent hydropic degeneration of the cytoplasm and death by necrosis and apoptosis. When cells were stained with neutral red, a general loss of lysosomes and of their perinuclear pattern of distribution was detected.

Table 1 includes the EC_{50} values for the different systems and biomarkers studied in the proposed ecotoxicological test battery. Considering all the data obtained, the sensitivity of the model systems to SMFA decreased as follows: *D. magna* > *C. vulgaris* > *V. fischeri* > PLHC-1 cells ⩾ RTG-2 cells. The estimated non-observed adverse effect levels

Table 1
Toxic effects of sodium fluoroacetate on the selected models and biomarkers of the proposed ecotoxicological battery

Model system	Origin	Indicator	24 h	48 h	72 h
Vibrio fischeri	Bacteria (Decomposer)	Bioluminescence	93[a]	67[b]	–
Chlorella vulgaris	Unicel. Alga (1st Producer)	Growth	12	1.2	0.6
Daphnia magna	Cladoceran (1st Consumer)	Immobilization	1.7	1.0	0.5
PLHC-1 cell line	Topminnow (2nd Consumer)	Protein content	108	83	81
		G6PDH leakage	147	135	126
		Neutral red uptake	78	67	59
		MTS metabolization	93	63	50
		Lysosomal function	136	105	114
		SDH activity	104	143	119
		G6PDH activity	112	74	140
		AChE activity	73	66	65
		Metallothionein	–[c]	–	–
		EROD activity	–[c]	–	–
RTG-2 cell line	Rainbow Trout (2nd Consumer)	Protein content	110	66	–
		G6PDH leakage	212	157	–
		Neutral red uptake	133	97	–
		MTS metabolization	67	48	–
		Lysosomal function	168	115	–
		SDH activity	102	51	–
		G6PDH activity	271	252	–

EC_{50} values (mM).

Ec_{50} (mM), concentration of test chemical that modified each biomarker by 50% (positive or negative) in comparison with appropriate untreated controls.

[a,b] Values referred to 5 and 15 min exposure times, respectively.

[c] Not modified at the highest concentration tested.

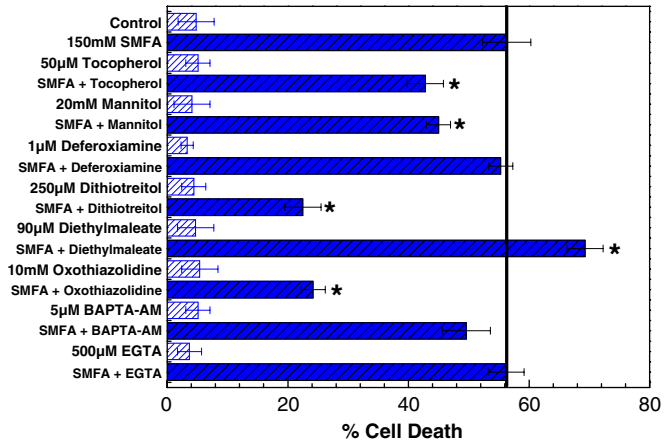

Fig. 8. Modulation of the toxic effects produced by 150 mM sodium fluoroacetate with the application of α-tocopherol succinate, mannitol, deferoxiamine mesylate, 1,4-dithiotreitol, malic acid diethyl ester, 2-oxothiazolidine-4-carboxylic acid, BAPTA-AM or EGTA. The effects were evaluated on PLHC-1 cells after 24 h of exposure by the quantification of the leakage of G6PDH. * Indicates significant difference from the effects induced by 150 mM sodium fluoracetate ($p < 0.05$).

(NOAELs) were 0.1, 0.15, 0.5, 0.5 and 20 mM SMFA for *C. vulgaris*, *D. magna*, PLHC-1 cells, RTG-2 cells and *V. fischeri*, respectively.

3.4. Modulation of cytotoxicity

The toxic effects of SMFA were studied under different circumstances in the fish cell line PLHC-1 measuring the leakage of G6PDH after 24 h of exposure to SMFA (Fig. 8).The cytotoxic effects of this pesticide produced 56% cell death with 150 mM. The toxicity of SMFA was modulated by the application of eight compounds. Deferoxiamine mesylate, BAPTA-AM and EGTA did not modify the toxic effects of SMFA, since the leakage of G6PDH was kept around 55%. Four of them, mannitol, α-tocopherol succinate, 2-oxothiazolidine-4-carboxylic acid and 1,4-dithiotreitol, prevented by 19%, 23%, 57% and 61% the toxicity of SMFA, respectively. Nevertheless, the application of malic acid diethyl ester increased by 23% the cell death induced by SMFA.

4. Discussion

The aim of the present study was to provide ecotoxicity data regarding the hazard of SMFA to aquatic organisms and to investigate its main effects in two fish cell line. Microorganisms are basic points in the breakdown and transformation of organic matter, contributing to the fertility and health of ecosystem. *V. fischeri* bacterium, a decomposer organism, presented a low sensitivity to SMFA in comparison with *D. magna* and *C. vulgaris*, and the highest NOAEL. The low sensitivity found for the marine bacterium may be explained because SMFA blocks the citric acid cycle, and does not interfere with the main energetic metabolism of *V. fischeri*, a facultative anaerobic bacterium. In another study, the presence of SMFA in soil (11.7 mmol/kg) did not reduce the ability of soil microorganisms to mineralize nitrogen (O'Halloran et al., 2005).

The green alga *C. vulgaris* and the cladoceran *D. magna* were very sensitive to SMFA. The crustacean *D. magna* was the most sensitive model system, with EC_{50} values of 1.7 and 0.5 mM at 24 and 72 h, respectively, and a NOAEL of 0.15 mM SMFA. Orme and Kegley (2004) showed comparable results. Adverse effects on the proliferation of the alga *C. vulgaris* were observed at low concentrations of SMFA. This system presented the lowest NOAEL, with EC_{50} values from 12 to 0.6 mM. These findings are supported by the results obtained by Berends et al. (1999) who reported, for the growth inhibition of the alga *C. vulgaris*, an EC_{50} value <0.9 mM after 72 h of exposure to SMFA. Leaf chlorosis, necrosis and suppression of seeding growth were reported in *Helianthus annus* exposed to 6.5 mM SMFA (Cooke, 1976).

The results obtained, showing EC_{50} values ranging from 0.5 to 93 mM, confirm the different sensitivity of the model systems used. Considering all the data obtained, the sensitivity of the model systems to SMFA decreased as follows: *D. magna* > *C. vulgaris* > *V. fischeri*. The large differences found in the evaluated models might be related to a variety of factors. In fact, the sensitivity of diverse species varies dramatically (Sherley, 2004).

Following the EU guideline for classification, packaging and labelling of dangerous substances (Commission Directive 2001/59/EC), and taking in consideration that the most sensitive model system was the cladoceran *D. magna*, with an EC_{50} of 0.5 mM and a NOAEL of 0.15 mM at 72 h, SMFA should be classified, as "R52 Harmful to aquatic organisms". In terms of risk assessment, SMFA is used as a pesticide in very low quantities. There are not reports quantifying the presence of SMFA in the aquatic environment. Neither, has been estimated the amount of fluoracetate formed from the human metabolization of halothane or 5-fluorouracil and from the environmental degradation of hydrochlorofluorocarbons. As SMFA presents a high biodegradation rate and a low bioaccumulation potential (Booth et al., 1999), according to our results after the acute exposure of three model systems, SMFA is most unlikely to produce deleterious effects to aquatic organisms, as toxic concentrations are unlikely to occur in streams as the result of baiting programmes, particularly by aerial application. In a recent ecotoxicological evaluation of SMFA on soil organisms realized by O'Halloran et al. (2005), SMFA was classified as a substance ecotoxic for the soil environment. However, it was showed that fluoroacetate-related effects on soil organisms occur at levels well above those that have been measured in soil. Furthermore, due to its toxicity in different species and according to Eason (2002), considerable care should be taken when using SMFA to ensure that the risks are outweighed by the ecological benefits achieved.

Although the reported LC_{50} at 96 h of SMFA are 0.54 mM for rainbow trout (*O. mykiss*) and 9.7 mM for

bluegill sunfish (*Lepomis macrochirus*) (Orme and Kegley, 2004), one or two orders of magnitude lower than those which affect the cell lines, we have applied the fish cell cultures as useful *in vitro* models in understanding the effects and mechanisms of toxicity of this pesticide.

The uptake of neutral red was the most sensitive cytotoxicity bioindicator in the hepatoma fish cell line PLHC-1. However, in RTG-2 cells, the most sensitive endpoint was the metabolization of MTS. The range of concentrations used did not produce an important cell death at concentrations lower than 100 mM, according to the leakage of G6PDH. Cell proliferation was inhibited in both fish cell lines and a time-dependent toxicity was found. From the basal cytotoxicity study carried out in both fish cell lines it is deduced that SMFA modifies the evaluated biomarkers in two stages. In the first step, the dose–response curve presents a soft slope, in which SMFA starts producing cellular injuries that reduce partially the activity of the cells. In the second phase, where the toxic effects of SMFA are more notable, it presents a more marked slope, when irreversible damage collapses the cellular physiology. The point of inflexion in the dose–response curve is located at a concentration of about 50 mM SMFA. This phenomenon, observed in both types of cells can be explained if we bear in mind that the SMFA does not exercise a lethal effect while the cells are able to maintain homeostasis.

At the biochemical level, it was found a reduction of lysosomal function and an inhibition of SDH activity in both cell lines. Inhibition of SDH activity was reported by Fanshier et al. (1964). Curiously, the clear reduction of the lysosomal function and SDH activity observed in PLHC-1 cells at 24 h of exposure was partially recovered at 72 h. In the case of G6PDH, the activity was increased from 24 h in RTG-2 cells and from 48 h in PLHC-1 cells. This stimulation may reflect the increase of the pentose phosphate pathway, an alternative metabolic route for glucose oxidation to supply reduction power (NADH), avoiding the blocked Krebs cycle. It has been shown that SMFA produces toxic effects by the metabolic conversion to fluorocitrate, which inhibits the aconitase activity blocking the energetic process (Elliott and Kalnitsky, 1996). It is thought to act as a suicide substrate of the enzyme's active site, causing a blockade at this point of the Krebs cycle (Clarke, 1991). Fluoroacetate is converted to fluoroacetyl-CoA, thereby gaining entry to the Krebs cycle. Citrate synthase then condenses fluoroacetyl-CoA with oxalacetate to form fluorocitrate. Fluorocitrate inhibits Krebs cycle and the formation of ATP. As consequence, energy supply is markedly reduced, developing cellular dysfunction or degeneration. During the process, citrate levels rise dramatically, causing chelation of divalent metal ions, especially Ca^{2+}, creating a general metabolic imbalance (acidosis) and blocking glucose metabolism by inhibiting phosphofructokinase activity (Sherley, 2004).

Comparing the pattern of modification of the other biochemical biomarkers in PLHC-1 cells, AChE activity was particularly inhibited, being a sensitive biomarker. This inhibition of AChE may contribute to the toxic effects of SMFA. Metallothionein levels and EROD activity did not show significant stimulations in the range of concentrations tested after 24 h of exposure in PLHC-1 cells.

The extent of variation of each cytotoxic and biochemical biomarker in both fish cell lines exposed to 50 mM SMFA for 24 h was compared in Fig. 9. Considering the global results obtained, the hepatoma fish cell line PLHC-1 was more sensitive to SMFA than the fibroblast-like fish cell line RTG-2, probably because of the higher metabolic activity of this cell line (Fent, 2001) may facilitate the metabolic activation of SMFA. All body cells are potentially affected by SMFA, although with different sensitivity (Chung, 1984). The heart, kidneys and the central nervous system are the tissues most altered by the inhibition of the oxidative energy metabolism.

Morphological changes were a sensitive indicator of the effects of SMFA in both fish cell lines from 1 mM SMFA. The most out-standing alterations were the development of hydropic degeneration of the cytoplasm as result of the energetic deficit produced by SMFA, loss of cells and death mainly by necrosis but also by apoptosis. PLHC-1 cells were more sensitive than RTG-2 cells, showing alterations at lower concentrations. Macrovesicular steatosis was also observed in the hepatoma cell cultures, a consequence of abnormal accumulations of fat inside vacuoles. The induction of a marked steatosis has been previously demonstrated in PLHC-1 cells exposed to diethanolamine (Zurita et al., 2005b) and microcystin (Pichardo et al., 2005).

In order to investigate the possible mechanism of toxic action of SMFA in the fish cell line PLHC-1, its toxic effects were studied under different circumstances. The toxicity of 150 mM SMFA was modulated by the application

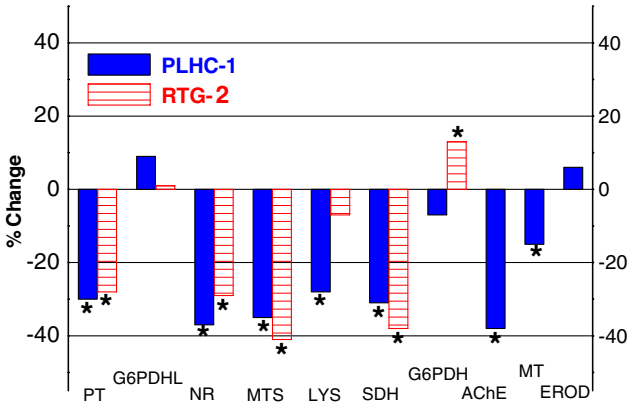

Fig. 9. Comparison of the extent of variation of each cytotoxicity and biochemical biomarker studied after 24 h exposure to 50 mM sodium fluoroacetate in PLHC-1 and RTG-2 cells. Toxicity indicators assessed in the *in vitro* test systems were: cell protein content (PT), glucose-6P dehydrogenase leakage (G6PDHL), neutral red uptake (NR), methylthiazol metabolization (MTS), lysosomal function (LYS), succinate dehydrogenase activity (SDH), glucose-6P dehydrogenase activity (G6PDH), acetylcholinesterase activity (AChE), metallothionein levels (MT) and ethoxyresorufin-O-deethylase activity (EROD). Data expressed in % of unexposed controls. * Indicates significant difference from control value ($p < 0.05$).

of eight compounds. The general antioxidants α-tocopherol succinate and mannitol decreased the toxic effects of SMFA, probably by the reduction of oxidative stress. The intracellular and extracellular calcium chelators, BAPTA-AM and EGTA, respectively, did not produce significant changes in the leakage of G6PDH. These results suggest that alterations in calcium homeostasis are not main mediators in cell death induced by SMFA.

In addition, we have found a 61% protection against SMFA toxicity by the membrane permeable sulphydryl-protecting agent 1,4-dithiotreitol. The influence of the modulation of the levels of glutathione was also evaluated by pre-treating the cells for 24 h with malic acid diethyl ester, an inhibitor of the synthesis of glutathione that depletes reduced glutathione without forming oxidized glutathione, and in a parallel experiment with 2-oxothiazolidine-4-carboxylic acid, a substrate for the glutathione synthesis that increases its cellular levels. Although cell death induced by SMFA was increased by 23% with malic acid diethyl ester, it was reduced by 57% with the pre-treatment of PLHC-1 cells with 2-oxothiazolidine-4-carboxylic acid. Therefore, taking into account the changes in SMFA toxicity observed in PLHC-1 cells pre-treated with the sulphydryl reagents, one of the possible mechanisms of toxicity of SMFA seems to be the binding to sulphydryl groups, including those of glutathione.

In conclusion, according to our results after the exposure of three model systems used for the aquatic evaluation, SMFA is most unlikely to produce acute deleterious effects to aquatic organisms. In addition, the hepatoma fish cell line PLHC-1 was more sensitive to SMFA than the fibroblast-like fish cell line RTG-2, being the uptake of neutral red the most sensitive bioindicator. Lysosomal function, SDH and AChE activities were inhibited, being G6PDH activity particularly stimulated. The cultures showed hydropic degeneration, reduction in cell number, macrovesicular steatosis and death mainly by necrosis but also by apoptosis. Sulphydryl groups and oxidative stress could be involved in PLHC-1 cell death induced by SMFA more than changes in calcium homeostasis.

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