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DNA damage induced by organotins on trout-nucleated erythrocytes

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The effect of tributyltin-chloride (TBTC), dibutyltin-chloride (DBTC) and monobutyltin-chloride (MBTC) on rainbow trout (*Salmo irideus*) nuclear DNA, was investigated by means of single cell gel electrophoresis ('comet' assay). Our data show that TBTC presents a marked genotoxic effect, whereas the genotoxic effect is less pronounced for DBTC and it is completely absent for MBTC. These results could be important in evaluating the environmental risks deriving from the use of these molecules as a antifouling agents in marine paints and as agricultural biocides. Copyright © 2001 John Wiley & Sons, Ltd.

Abbreviations: TBTC, tributyltin-chloride; DBTC, dibutyltin-chloride; MBTC, monobutyltin-chloride; Hb, hemoglobin.

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1. INTRODUCTION

Organotin compounds are pollutants of anthropogenic origin.[1] Their presence in the environment is due to their uses in many industrial applications[2] and also as agricultural biocides. In particular, in water it principally depends on their use in marine antifouling paint formulations and especially as stabilizers of PVC.[3] As a consequence, organotins are ubiquitous contaminants in aquatic ecosystems. The toxicity of organotins has been the object of several studies in recent years, resulting in the demonstration that it occurs at several biological levels (i.e. cellular energy production,[4–6] cell membrane functionality[7,8] and protein conformation[9–11]). In general, the toxicity of organotin[12,13] is determined by the number and nature of the organic substituents on tin(IV).

In the last few years we have investigated in considerable detail the effect of different organotins on trout-nucleated erythrocytes.[14] The effects were studied by following the hemolytic process, measuring steady-state fluorescence anisotropy of different probes on isolated membranes and evaluating the stability of trout hemoglobins. The results obtained[15,16] indicated a plasma membrane perturbation when the process was followed in the presence of triorganotins (tributyltin chloride (TBTC) and triphenyltin chloride (TPTC)); however, the presence of dibutyltins or monobutyltins produced a slight protecting effect against hemolysis. It is known that trout erythrocytes contain four hemoglobin (Hb) components (denoted HbI HbII HbIII and HbIV according to their anionic mobilities) which have been extensively characterized and whose functional role is largely understood.[17] Studies on trout hemoglobins stability[18] suggest that TBTC and TPTC protect HbI most efficiently from oxidation. On the other hand, the same compounds accelerated the precipitation process in HbIV to a great extent.

Now, by using the alkaline comet assay or single cell microgel electrophoresis, we have reinvestigated the system to explore if several organotin compounds (TBTC, DBTC and monobutyltin chloride (MBTC)) influence the DNA status in these nucleated cells. This technique is an increasingly popular tool for the measurement of DNA damage in individual cells.[19] By using this technique we examined DNA damage in whole and in density-separated trout erythrocytes (older cells are characterized by an increased density) and the results obtained reflected different degrees of DNA damage.[20]

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2. MATERIALS AND METHODS

Organotin compounds were obtained from Aldrich. All reagents were of analytical grade. The cells used in this study were obtained from *Salmo irideus*, an inbred strain of rainbow trout. The fish were kept in tanks and fed with commercial fish food. Fresh water was pumped into the tanks from the Scarsito River, a tributary of the Potenza, Italy. Experiments were done using fish of the same age (2 years old) weighing between 200 and 300 g. Blood was withdrawn with a syringe from the lateral tail vein into an isotonic medium (0.1 M phosphate buffer, 0.1 M NaCl, 0.2% citrate, 1 mM EDTA, pH 7.8) and further treated within 2 h at 4 °C. After removal of the plasma and buffy coat by centrifugation, the erythrocytes were washed three times with isotonic phosphate buffer. After washing, the erythrocyte suspension was adjusted to an Hb concentration of 60 mg ml^{-1} and divided into different aliquots (the concentration of Hb was determined spectrophotometrically using the absorbance A (1%, 1 cm) = 8.5 at 541 nm for the oxygenated derivative. Organotin compounds dissolved in ethanol (100%) were added to the erythrocytes (10 µl ml^{-1} of erythrocyte suspension) to a final concentration of 10 µM. The choice of this organotin concentration derives from the fact that, in our experimental conditions, both hemolysis and met-Hb formation are nearly absent. Furthermore, the TBTC concentration used by us is of the same order of magnitude of other papers.[1,21] Control experiments were performed by adding an equal volume of ethanol. The erythrocytes were tested immediately after addition of organotin (incubation time of zero min) and after incubation at 27 °C for 30 min (incubation time 30 min). To evaluate DNA damage in the erythrocytic suspension we performed alkaline single-cell microgel electrophoresis ('comet' assay). The test was performed basically according to Singh *et al.*,[19] with minor modifications.[20] Briefly, the comet assay consists of embedding cells in agarose, followed by lysis, electrophoresis and staining to visualize DNA damage using fluorescence microscopy. Breaks in the duplex DNA molecule release its complex supercoiling and the liberated DNA migrates toward the anode so that the cells resembles a comet, with a brightly fluorescent head and a tail streaming away from it. Cells with increased DNA damage display an increased migration of genetic material in the direction of the electrophoresis. Experiments involving CO–hemoglobin were carried out after exposure of the oxygenated erythrocyte suspension to a weak vacuum, by using a rotary vane pump, and then to pure CO gas. The vacuum was operated for a few seconds and this permits removal of a part of the oxygen, making easier the formation of CO–hemoglobin (Hb affinity for CO is greater than that for oxygen by about 250-fold).

3. RESULTS

The extent of DNA damage was quantified by measuring the displacement of the genetic material between the cell nucleus (comet 'head') and the resulting 'tail'. The parameters used as an index of DNA damage are tail length, tail intensity, and tail moment; the latter is one of the best indices of induced DNA damage among the various parameters calculated by computerized image analysis. It considers both the length of DNA migration in the comet tail (tail length) and the percentage of nuclear material migrated out from the comet head into the comet tail (tail intensity).

The comet assay was performed on trout erythrocyte suspensions incubated in the presence of organotin compounds (10 µM at 27 °C and pH 7.8 for 30 min). Under these experimental conditions both hemolysis and met-Hb formation are nearly absent.

In Table 1, the tail length and mean values of the different samples are reported. The values of these parameter remained nearly the same for all the samples.

The results referring to tail intensity (percentage

Table 1 Observed distributions of comet parameter tail length (mean ± SEM) in trout erythrocyte suspension after incubation in phosphate buffer, pH 7.8, at 27 °C. Data (at least 150 scores/sample) are mean values of three replicated experiments. Organotin compounds were dissolved at a final concentration of 10 µM[a]

	Tail length (µm)	
Sample	t = 0 min	t = 30 min
Control	16.47 ± 0.32	15.94 ± 0.44
TBTC	15.62 ± 0.36	17.50 ± 0.33*
DBTC	16.20 ± 0.22	16.89 ± 0.35
MBTC	15.46 ± 0.29	15.18 ± 0.29

* $p < 0.01$.
[a] For discussion see text.

Table 2 Observed distributions of comet parameter tail intensity (mean ± SEM) in trout erythrocyte suspension after incubation in phosphate buffer, pH 7.8, at 27 °C. Data (at least 150 scores/sample) are mean values of three replicated experiments. Organotin compounds were dissolved at a final concentration of 10 μM

	Tail intensity (%)	
Sample	$t = 0$ min	$t = 30$ min
Control	8.02 ± 0.38	9.38 ± 0.44
TBTC	8.59 ± 0.53	13.54 ± 0.57**
DBTC	7.55 ± 0.33	11.27 ± 0.48*
MBTC	8.42 ± 0.38	9.49 ± 0.54

* $p < 0.01$.
** $p < 0.001$.

of DNA in the tail) are given in Table 2. Considering this parameter, a different pattern with respect to the tail length was observed. In fact, the percentage of DNA in the tail was significantly increased under the same treatment time by the presence of TBTC ($p < 0.001$) and to a minor extent by DBTC ($p < 0.01$). On the contrary, the presence of MBTC does not change this parameter with respect to the control. (Note: p is the probability of this *not* happening, i.e. the probability that TBTC- or DBTC-treated samples and the control would *not* be significantly different.)

The tail moment mean values calculated for the different samples are reported in Table 3. Statistical analysis of this data confirms that both TBTC and DBTC increase the extent of DNA observed in

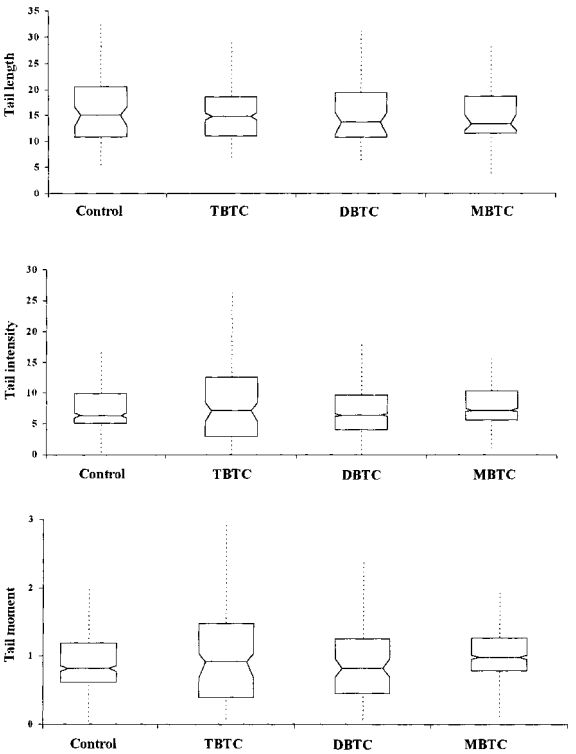

Figure 1 Box-plots representing distribution of all three comet parameters in trout erythrocyte suspension after incubation in phosphate buffer, pH 7.8, at 27 °C at time zero. The notched box shows the median, lower and upper quartiles, and confidence interval around the median. The dotted line connects the nearest observations within 1.5 inter-quartile ranges (IQRs) of the lower and upper quartiles.

Table 3 Observed distributions of comet parameters tail moment (mean ± SEM) in trout erythrocyte suspension after incubation in phosphate buffer, pH 7.8, at 27 °C. Data (at least 150 scores/sample) are mean values of three replicated experiments. Organotin compounds were dissolved at a final concentration of 10 μM

	Tail moment	
Sample	$t = 0$ min	$t = 30$ min
Control	0.97 ± 0.05	1.11 ± 0.05
TBTC	1.05 ± 0.07	1.67 ± 0.07**
DBTC	0.96 ± 0.04	1.38 ± 0.06**
MBTC	1.02 ± 0.04	1.11 ± 0.06

** $p < 0.001$.

control cells (TBTC is always more effective with respect to DBTC). The data distribution of all three comet parameters is reported as box-plots in Figs 1 and 2. Similar results were obtained when the experiments were carried out using erythrocytes saturated with CO, which binds to hemoglobin and stabilizes it. In Fig. 3, DNA damage is summarized as a measure of basal damage by reporting the ratio of tail parameters and control tail parameters. At time $t = 0$ samples incubated with TBTC in the presence and absence of CO present a slight increase in DNA damage in comparison with the control. This difference becomes much more relevant after 30 min of incubation: the tail intensity and tail moment increase twofold in TBTC-treated samples independently from CO saturation. On the contrary, tail length remains comparable to the control.

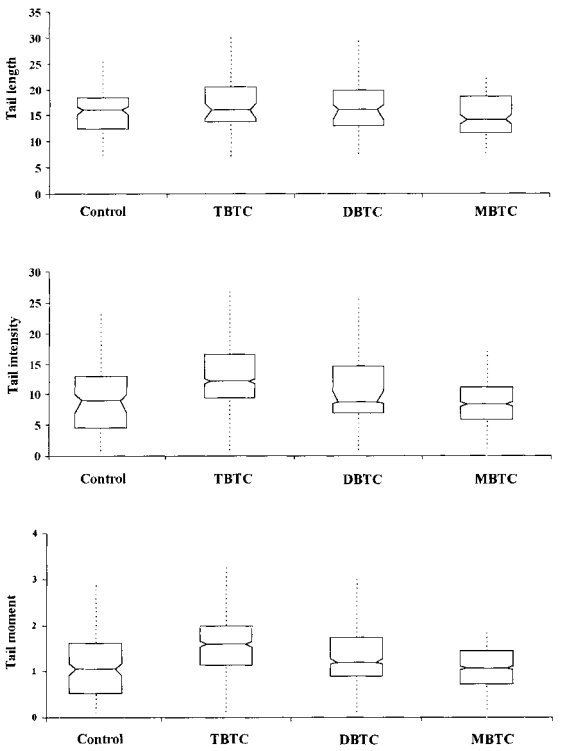

Figure 2 Box-plots representing distribution of all three comet parameters in trout erythrocyte suspension after incubation in phosphate buffer, pH 7.8, at 27 °C at time 30 min. The notched box shows the median, lower and upper quartiles, and confidence interval around the median. The dotted line connects the nearest observations within 1.5 IQRs of the lower and upper quartiles.

4. DISCUSSION

Evaluation of DNA damage by using the comet assay provides a direct assessment of the extent of DNA modification in individual cells. Comet assay represents a good technique to test the genotoxicity of different compounds, including pollutants. In the light of the observations reported here, a clear genotoxic effect of TBTC on trout erythrocytes is evinced. In fact the tail intensity and tail moment parameters increase significantly after 30 min of incubation in the presence of this compound. The tail length increases significantly, although the change is less marked with respect to the other parameters. The faint variation of tail length after incubation fits with the observation that, with increasing amount of damage, the tail intensity rather than length increases,[22] and tail length is determined primarily by the length of the relaxed loops forming the comet as reported by Cook *et al.*[23] The genotoxic effect instead is much blander for DBTC and totally absent for MBTC; the latter presents levels of DNA damage comparable to the control. This trend is in agreement with the general evidence that the toxicity of organotin is determined by the number and nature of the organic substituents on tin(IV); in general, the toxicity decreases from tri- to mono-alkyltins.[12,13] Experiments carried out using erythrocytes saturated with CO show that organotin-mediated DNA damage with respect to that in the presence of oxygen is of the same extent. Samples were incubated in the presence of CO because it combines readily and strongly with hemoglobin and stabilizes it, thus preventing hemoglobin autoxidation. As a consequence, met-Hb (i.e. product of hemoglobin oxidation or autoxidation) in CO-treated samples is reduced and, therefore, this may be considered as a reference value for the absence of met-Hb-mediated DNA damage. This implies that the DNA damage in our experimental conditions (isotonic phosphate buffer pH 7.8 and 30 min incubation at 27 °C) is not due to the formation of small amounts of met-Hb. In fact in a previous paper[24] we reported that *Salmo irideus* erythrocytes, suspended in isotonic buffer at pH 6.3 and incubated at 35 °C, presented DNA damage: under those experimental conditions, an endogenous oxidative stress was induced, due both to the formation of superoxide anion and the inactivation of gluthatione peroxidase, which are a consequence of Hb oxidation.[25]

Several studies have demonstrated immunotoxic and membrane perturbation of organotin in invertebrates and vertebrates, and the genotoxicity of these compounds has been a much discussed topic.

Tributyltin (TBT) compounds have been investigated extensively in this respect, and generally negative results were obtained. As an example, Hamasaki and Nagase[26] reported that incubation of isolated lambda-DNA (double strand DNA) with *n*-butyltin compounds in the presence or the absence of hydrogen peroxide did not cause DNA breakage. Moreover, the lack of covalent binding to hepatic and thymic DNA was found *in vivo* and *in vitro* for dioctyltin dichloride,[27] an organotin compound largely used for the stabilization of PVC. Only recently has a mutagenic effect of organotins been reported. By a method that allowed estimation of the mutagenicity of bactericidal compounds, monobutyltin, dibutyltin, TBT, and dimethyltin were found to be mutagens on *Salmonella typhimurium*

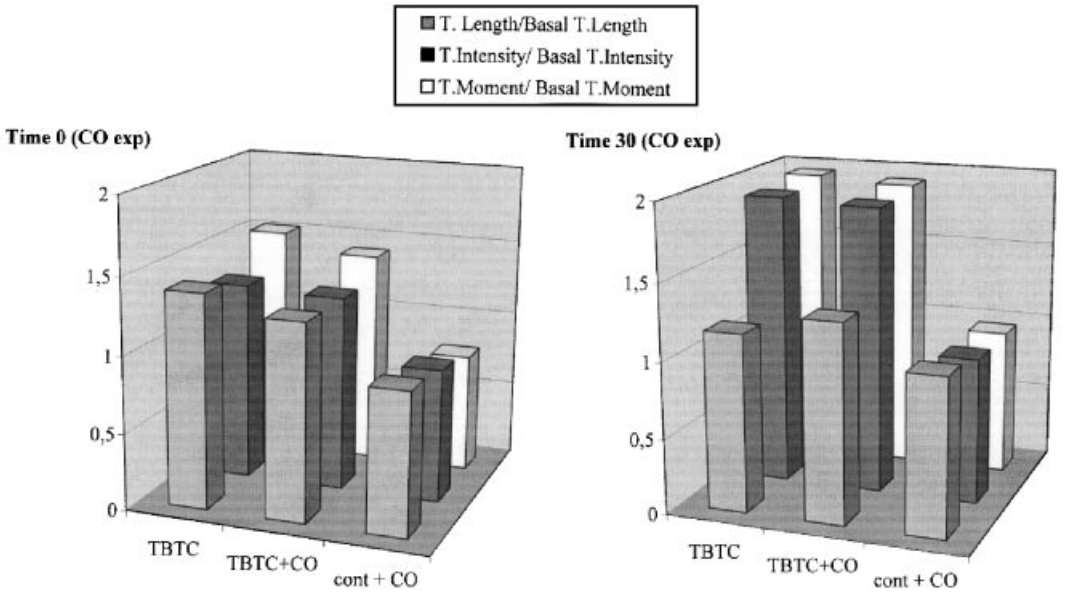

Figure 3 Tail parameters reported as a measure of basal tail parameters. Tail length, tail intensity and tail moment are reported for samples incubated in the presence of TBTC and control, with and without.

TA100.[28] Also, in eukaryotic cell models the organotin compounds were capable of inducing cytogenetic damage and apoptosis.[29–32]

In conclusion, the effect seen on trout erythrocytes, together with the literature evidence, suggests that genotoxicity exhibits species specificity. However, the molecular mechanism by which organotin compounds influence the DNA status in our cellular model is still unknown. Experiments performed with cells saturated with CO allow us to exclude the hypothesis of oxidative damage to DNA mediated by met-Hb formation. Further experiments will clarify if there is a direct attack to DNA, probably through charge neutralization of DNA phosphodiesters by organotin compounds, as reported for *in vitro* models,[33] or indirectly by means of other cellular effectors such as by perturbation of Ca^{2+} homeostasis. It has been reported that TBT at concentrations of 1 to 10 μM causes a rapid and sustained increase in cytosolic free Ca^{2+} concentration by enhancing influx and release from intracellular stores. This enhancement might be able to induce internucleosomal DNA cleavage typical of apoptotic death in thymocytes and mammalian cell lines.[31]

The data reported here could be important in evaluating the environmental risks deriving from the use of TBTC and its degradation compounds in marine paint formulations.

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