IN VIVO AND *IN VITRO* INHIBITION OF CYP1A ENZYMES ACTIVITY BY 2,4,6-TRINITROTOLUENE (TNT) IN EUROPEAN EEL *ANGUILLA ANGUILLA*

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RUNNING TITLE CYP1A ENZYMES INHIBITION BY TNT IN *A. ANGUILLA*

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ABSTRACT

The present research was carried out in order to investigate the interaction of 2,4,6-trinitrotoluene (TNT) with CYP1A catalytic enzymes in specimens of European eel *(Anguilla anguilla* L.*).* An *in vivo* experiment was performed on juveniles exposed for 6 h and 24 h to 0.5, 1 and 2.5 mg/L nominal concentrations of TNT. Catalytic activity of CYP1A enzymes such as 7-ethoxyresorufin-*O*-deethylase (EROD) and methoxyresorufin-*O*- deethylase (MROD) was investigated in liver microsomal fraction of *in vivo* exposed eels; an *in vitro* study was also performed with the same TNT concentrations by using control eels. Significant inhibition of EROD activity was observed *in vivo* after 6h (36%) at the lowest concentration of 0.5 mg/L but not increasing at higher concentrations. A more evident significant inhibition of EROD activity was observed at 24h (55%) with the same trend starting from 0.5 mg/L. On the opposite, MROD activity was not affected after 6h of exposure while a significant inhibition was observed at 24h starting from the lowest exposure concentrations (64%). No further inhibition was evident at the highest concentrations (1 and 2.5 mg/L). The *in vitro* exposure confirmed the inhibition of EROD and MROD catalytic activities by TNT: both the enzymes activity resulted significantly inhibited (26%) but only at the highest concentration of 2.5mg/L. An irreversible inhibition at both enzyme level (EROD and MROD) by TNT might be hypothesised in European eels on the base of the absence of recovering of catalytic activities despite further adding NADPH during *in vitro* kinetic assay.

Keywords: 2,4,6-trinitrotoluene, EROD, MROD, CYP1A, European eel

INTRODUCTION

2,4,6-Trinitrotoluene (TNT) is the most widely used nitroaromatic explosive since the first World War. An extensive release of this chemical in the marine environment has been associated not only with military activities but also with ammunition manufacturing (1). In the Italian seas dumped ordnances are in such a quantity to represent an actual concern for marine species living close to dumping sites thus highlighting the need to investigate any adverse effects on aquatic organisms.

TNT is persistent in deep sea water (1, 2). It's log K_{ow} (1.6÷2.7) indicates low tendency both to bioaccumulation in aquatic organisms and biomagnification in the food chain (1). Once absorbed by aquatic organisms, TNT is readily metabolized mostly to aminodinitrotoluene (ADNTs) whose behaviour in aquatic organisms is still not well known (2, 3).

The acute and chronic toxicity of TNT and its degradation products have been reported for several aquatic species (2). However only few studies focused on the involvement of cytochrome P450 system in the metabolism/detoxification and in particular no studies have been performed to date on marine fish species (4-7).

The aim of the present study is to investigate the mechanism by which TNT interacts with CYP1A enzymes catalytic activity such as EROD and MROD.

MATERIALS AND METHODS

40 specimens of European eels (TL 34.64 ± 2.69) were exposed for 6 h and 24 to 0.5, 1 and 2.5 mg/L nominal concentrations of TNT using DMSO at 0.1‰ as a carrier in tank (40 l) with artificial sea water (Instant Ocean) at 35 ‰ and at 18°C.

The eel livers were homogenized in a 1:4 (w/v) ratio with sucrose buffer (50 mM K_2HPO_4, 0.75 M sucrose, 1 mM EDTA, 0.5 mM DTT, 400 µM PMSF, pH 7.5) using a Potter-Elvenjem glass/Teflon homogeniser at 2000 rpm. Microsomes were obtained by differential centrifugation in a Sorvall RC28S Ultracentrifuge. Homogenates were first centrifuged at 9000 g x 20 minutes to remove nuclei, mitochondria, lysosomes and cell debris while the resulting supernatants (S9 fractions) were transferred and centrifuged at 100,000 g x 1h. The resulting microsomal pellets were subsequently transferred and resunspended in a 1:2.6 (w/v) solution with Tris-(base) buffer (10 mM Tris), 20% p/v glycerol, 0.5 mM DTT, 400 µM PMSF, pH 7.5). All the procedures were carried out at 4 °C as previously described (8). EROD and MROD activities were assayed by spectrofluorimetry (9). Total protein content was measured spectrophotometrically (10).

The *in vitro* study was performed using microsomes from control eels: samples were pre-incubated with same TNT concentrations of *in vivo* experiment (0.5, 1 and 2.5 mg/L) using again DMSO and then EROD and MROD assays were performed according to standard method mentioned above (9). Furthermore TNT and its main metabolite, the 4-ADNT, were pre-incubated together to evaluate potential additive effect. A further experiment was also performed by adding NADPH every 5 min and let EROD and MROD activities run for 30 min.

Comparison among TNT doses and time of exposure were evaluated by the Mann-Whitney-Wilcoxon rank sum non-parametric test. Correlations were determined with the Pearson correlation coefficient (*r*). Statistical analyses were performed with Statistica 5.1 (StatSoft, USA).

RESULTS

Significant inhibition of EROD activity was observed *in vivo* after 6h (36%) at the lowest concentration of 0.5 mg/L but not increasing at higher exposure concentrations of 1 and 2.5 mg/L. A more evident significant inhibition of EROD activity was observed at 24h (55%) with the same trend starting from 0.5 mg/L (Figure 1). On the opposite, MROD activity was not affected after 6h of exposure while a significant inhibition was observed at 24h starting from the lowest exposure concentrations of 0.5mg/L (64%). No further inhibition was evident at highest concentrations (1 and 2.5 mg/L) (Figure 2).

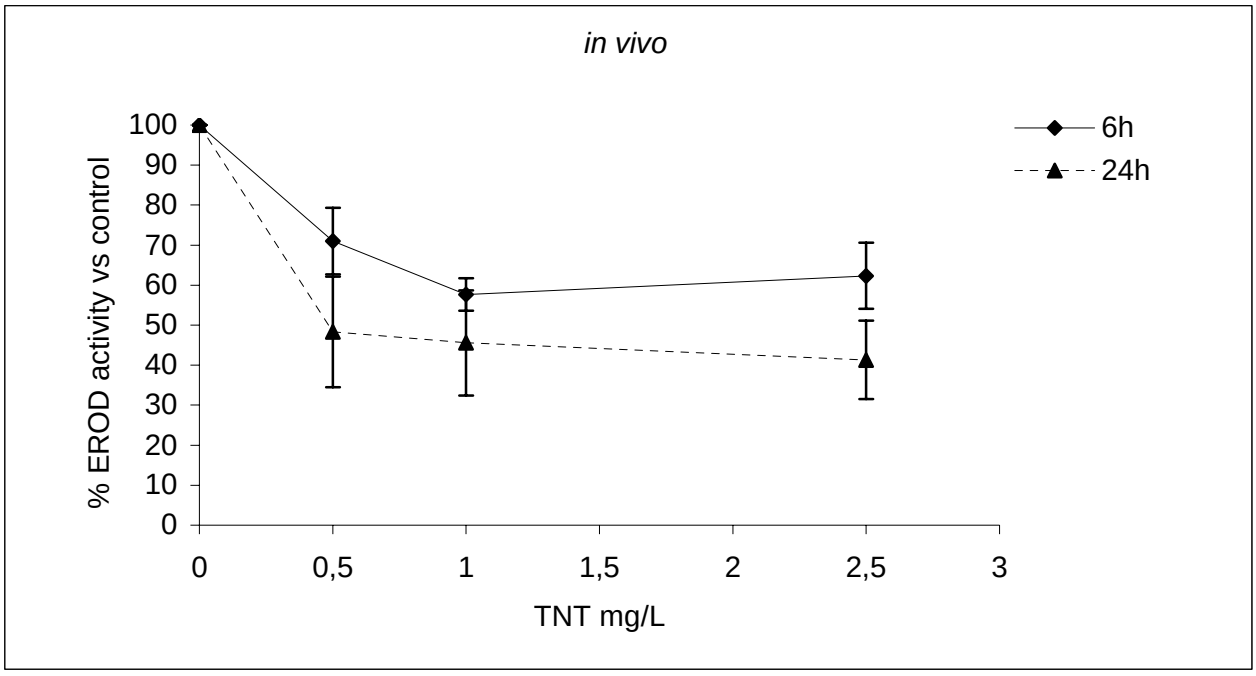

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Fig 1 *In vivo* study: inhibition of EROD activity in European eel incubated with 0.5, 1 and 2.5 mg/L TNT after 6h and 24h

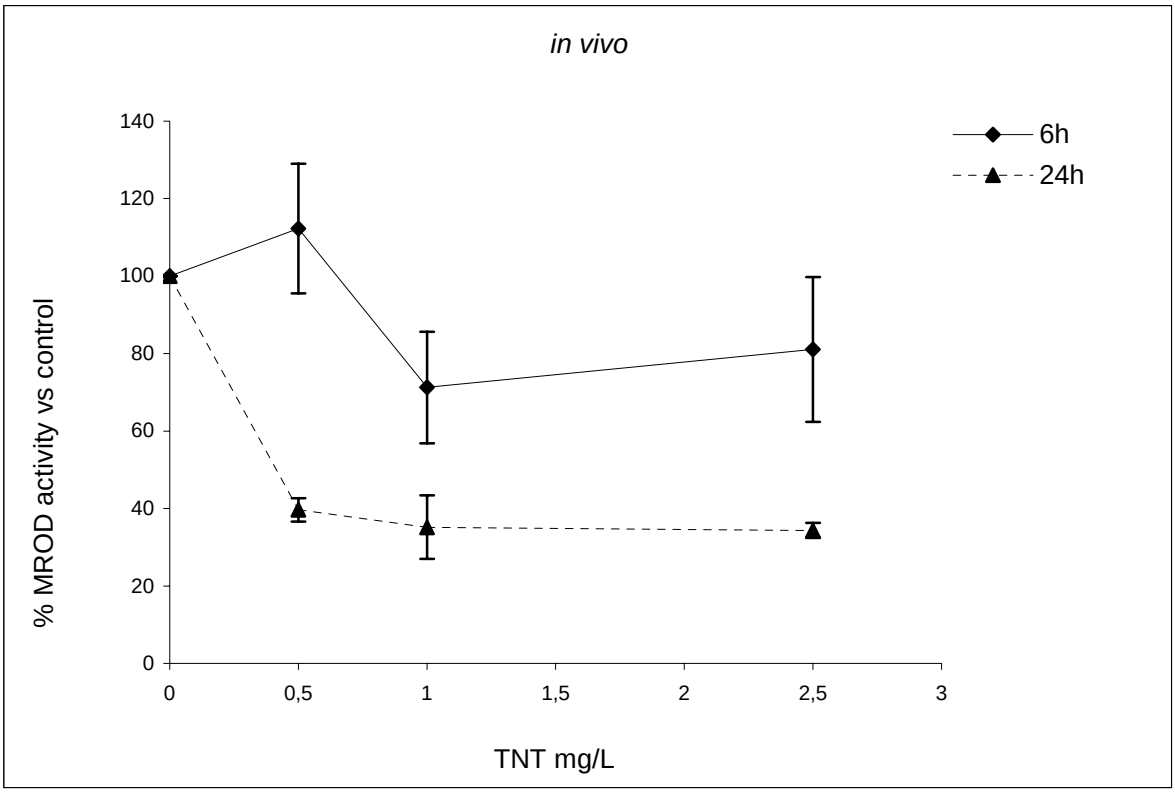

Fig 2 *In vivo* study: inhibition of MROD activity in European eel incubated with 0.5, 1 and 2.5 mg/L TNT after 6h and 24h.

A high correlation was observed between EROD and MROD inhibition at 24h of exposure (r=0.99). EROD and MROD activities are reported in Table 1.

The *in vitro* exposure confirmed the inhibition of EROD and MROD catalytic activities by TNT: EROD resulted significantly inhibited but only at the highest concentration (2.5mg/L) (28%); a similar trend was also observed for MROD (Figure 3; Table 2).

The pre-incubation with both TNT and 4-ADNT (metabolite) showed an additive effects with a significant inhibition of 42 % at the highest concentration of 2.5mg/L (Figure 4).

An irreversible inhibition at enzyme level of both CYP1A activities (EROD and MROD) by TNT in European eels might be hypothesised on the base of the absence of recovering of catalytic activities despite further adding of NADPH during *in vitro* kinetic assay.

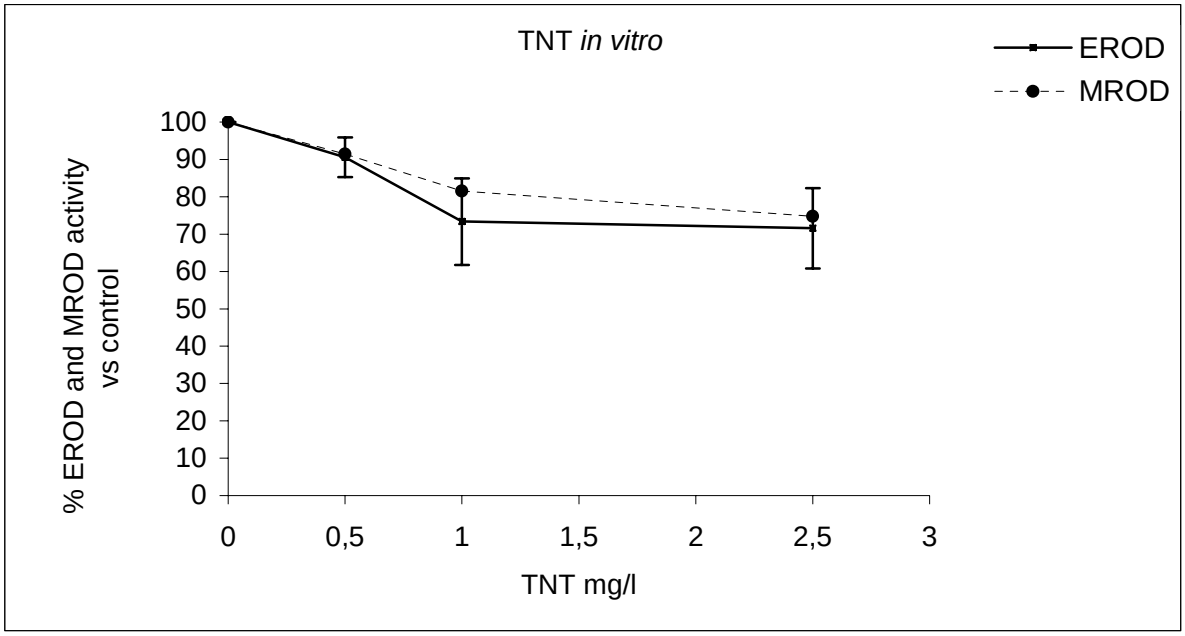

Fig 3. *In vitro* study: inhibition of EROD and MROD activities in European eel incubated with 0.5, 1 and 2.5 mg/L TNT.

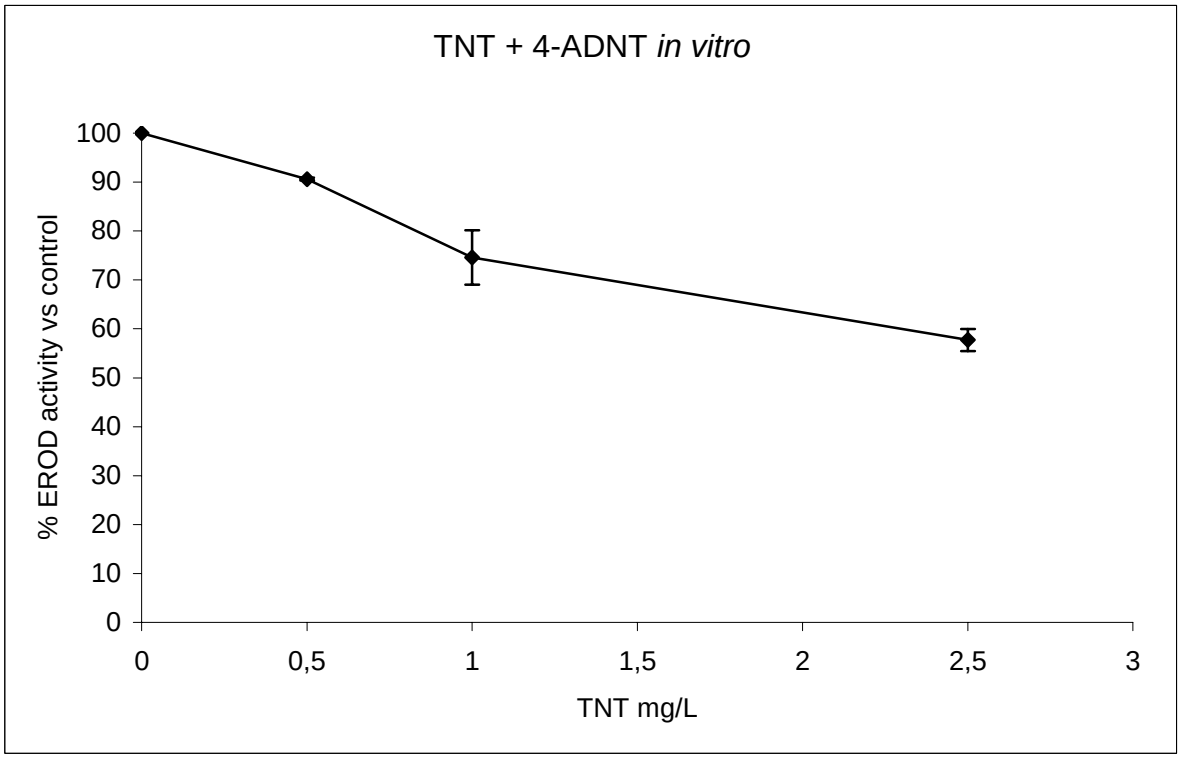

Fig 4. *In vitro* study: inhibition of EROD activity in European eel co-incubated with 0.5, 1 and 2.5 mg/L TNT and 4-A-DNT

Table 1 EROD and MROD activity in European eels from the *in vivo* study (0.5, 2 and 2.5 mg/L TNT) after 6 and 24 hours of exposure (N=4)

	Time	Control	0.5 mg/L	1 mg/L	2.5 mg/L
EROD pmol/min/mg prot	6h	110.17 ± 10.35	78.24 ± 9.12*	63.52 ± 4.47*	66.97 ± 11.87*
	24h	97.27 ± 14.34	47.04 ± 13.43*	44.33 ± 12.77*	40.21 ± 9.55*
MROD pmol/min/mg prot	6h	4.52 ± 1.50	5.78 ± 1.33	5.34 ± 2.59	3.66 ± 0.84
	24h	7.13 ± 3.39	2.83 ± 0.22	2.09 ± 0.97*	2.45 ± 0.14*

Values are expressed as mean ± s.d.
* means $p < 0.05$

Table 2 EROD and MROD activity in samples *in vitro* exposed to TNT (0.5, 2 and 2.5 mg/L TNT) Reference samples were incubated using 10µl of DMSO; TNT was dissolved and incubated in DMSO.

	Control	0.5 mg/L	1 mg/L	2.5 mg/L
EROD pmol/min/mg prot	82.42 ± 23.42	7207 ± 20.52	58.29 ± 8.21	53.99 ± 8.03*
MROD pmol/min/mg prot	5.74 ± 0.47	5.23 ± 0.04	4.70 ± 0.73	4.31 ± 0.68*

Values are expressed as mean ± s.d.
* means $p < 0.05$

DISCUSSION

The results of the *in vivo* experiment showed a significant inhibition of CYP1A enzymes catalytic activity in agreement with previous findings on freshwater fish species (4). EROD and MROD activities resulted strongly inhibited both *in vivo* and *in vitro* at concentrations in the range of LC_{50} values reported for TNT in fish species (0.8 – 3.7 mg/L) (2). Comparing the two CYP1A catalytic activities, EROD enzyme seems more time-responsive to TNT than MROD as shown in the *in vivo* study by the onset of EROD inhibition already evident after 6 h of exposure. However, MROD enzyme seems more sensitive to TNT than EROD at 24h based on the highest inhibition of its catalytic activity (64% vs 55% for EROD and MROD respectively). This might be explained by the presence of two forms of CYP1A genes in eels that might display different sensitivity towards substrates and inhibitors as reported in other studies (11) (12).

From the *in vivo* exposure, the strong inhibition observed for both EROD and MROD activities might have suggest a modulation at gene level if the *in vitro* exposure would have not confirmed the extent of inhibition observed (around 30%). An inhibition at enzyme level which seems also irreversible can then be hypothesised; moreover an additive effect seems evident from the co-incubation of both TNT and 4-ADNT.

These findings clarify the interaction between TNT and CYP1A catalytic enzymes, but further study at gene level need to be performed in order to understand higher level of regulation.

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