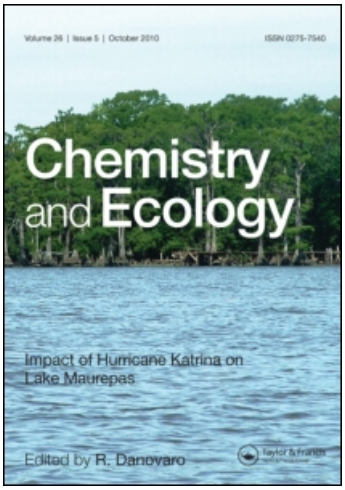

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Effects of 2,4,6-trinitrotoluene (TNT) on neurosteroidogenesis in the European eel (*Anguilla anguilla*; Linnaeus 1758)

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The aim of the present study is to investigate the endocrine disrupting potential of the explosive 2,4,6-trinitrotoluene (TNT) on marine organisms using the European eel (*Anguilla anguilla* Linnaeus, 1758) as model fish species. Crucial brain steroidogenic parameters such as the estrogen receptors (ERs), P450 aromatase (CYP19), steroidogenic acute regulatory (StAR) protein and P450-mediated cholesterol side-chain cleavage (P450scc) enzyme were selected as potential biomarkers for the assessment of TNT endocrine disrupting potential. Juvenile European eels were exposed for 6 and 24 h to 0.5, 1 and 2.5 mg/l nominal concentration of TNT dissolved in dimethyl sulfoxide (DMSO). ERα, CYP19, StAR protein and P450scc genes expression were quantified using real-time PCR. Exposure to TNT altered the expression of ER gene at the lowest TNT concentration after 6 h of exposure, while after 24 h gene expression levels increased respect to controls only in eels at the maximum TNT dose (2.5 mg/l). A significant increase of CYP19 gene expression occurred after 6 h in all eels exposed to TNT compared to controls. On the opposite at 24 h, CYP19 expression seems reduced in eels exposed to 0.5 and 1mg TNT/l compared to 6 h, while no changes were observed at the highest dose (2.5 mg/l). Both StAR and P450scc gene expression were decreased at 6 h in eels exposed to 0.5 and 1mg TNT/l, while at 2.5 mg TNT/l, the gene expression levels were restored to background level. No further modulation was evident after 24 h for both genes. The overall results indicate the ability of TNT to affect neurosteroidogenic pathways.

Keywords: 2,4,6-trinitrotoluene; estrogen receptor; aromatase; StAR; P450scc; European eel

1. Introduction

Disposal at sea of obsolete and useless ordnance has been commonly performed worldwide to such an extent that nowadays the huge amounts of bombs lying on the seabed represent an actual danger for benthic ecosystems. 2,4,6-trinitrotoluene is one of the principal components of conventional ordnance. The high persistency at sea and well-known toxic properties makes the compounds potentially noxious for marine organisms [1]. Reproductive effects of TNT have been reported for fish species [2] but the molecular pathways that determine these effects are still unknown. The present research focuses on the effects of TNT on the expression of genes involved in the estrogen responses and in the steroidogenesis process of a marine fish species, the European eel *Anguilla anguilla* (Linneus, 1758) in order to evaluate the estrogenic potential of the compound. The biological effects of estrogens are mediated by their binding to nuclear proteins defined as

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estrogen receptors (ERs) [3]. The ERs are ligand activated transcription factors that modulate specific genes by interacting with estrogen and estrogen-like molecules [4,5]. The ERs can also bind to estrogen mimics (xenoestrogens) and interfere with growth and developmental processes of the organism. Among mechanisms involved in the control of reproduction mechanisms, steroid hormones synthesis and biotransformation processes hold a crucial role in the regulation of several physiological functions such as growth, sexual differentiation and stress responsiveness [6].

In fish, as in other vertebrates, the brain is a steroidogenic organ. Neurosteroids are involved both in the development of the central nervous system and represent a group of fundamental neuromodulators through the GABA receptors [7]. In the brain, as in other tissues, steroid hormones are synthesised from the precursor cholesterol. The delivery of cholesterol from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein and its subsequent conversion to pregnenolone by CYP450 side-chain cleavage (P450scc) represent rate-limiting steps in steroid hormone synthesis (including neurosteroidogenesis). Several xenobiotic compounds are able to affect steroid synthesis by targeting this initial phase of steroidogenic pathway and may produce a cascade of adverse effects [6, 8–10]. In vertebrates, another crucial enzyme involved in reproductive steroid synthesis is the CYP aromatase (CYP19) that catalyzes the conversion of androgens to estrogens. In teleost, there are two different CYP19 isoforms, namely CYP19A and CYP19B [11]. CYP19A is mainly expressed in ovaries where it plays a key role in sexual differentiation and oocytes growth. CYP19B is preferentially expressed in neural tissues and involved in regulating the development of the central nervous system [11]. The European eel possess only one CYP19 form which shares more similarities with others vertebrates than teleosts, presumably in relation with its peculiar life cycle [12]. The expression of CYP19 is considered a sensitive marker of effects for endocrine disrupting chemicals [13–16] and is currently used to screen endocrine disrupting potential of pollutants [11].

The present study investigated the potential endocrine effects of TNT on ER and by considering new and potential endpoints such as neurosteroidogenic key proteins in order to provide relevant information on the endocrine disrupting potential of TNT on marine organisms. In addition, we tested the responsiveness of these parameters to the compound in order to select sensitive biomarkers of TNT exposure that could be applied in biomonitoring studies in dumping areas. Plasma cortisol levels were also measured in order to reveal a physiological effect correlated to altered neurosteroidogenesis.

2. Materials and methods

2.1. *Chemicals and reagents*

2,4,6-Trinitrotoluene was kindly supplied by NBC Joint Logistic Technical Centre. Dimethylsulphoxide (DMSO) was purchased from Sigma (St Louis, MO, USA). Chloroform, isopropanol and ethanol were also obtained from Baker (Phillipsburg, NJ, USA). Deoxynucleotide triphosphates (dNTPs) were obtained from Fermentas (GmbH, Germany); Tripure reagent was purchased from Sigma (St Louis, MO, USA). IScript cDNA Synthesis Kit, iTAQ^{TM} SYBR® Green Supermix with ROX, agarose were purchased from Bio-rad (Hercules, CA, USA). Plasma cortisol Delfia® kit was purchased from Perkin Elmer.

2.2. *In vivo study*

Forty specimens of yellow European eels were collected in January 2005 (TL 34.64 ± 2.69 cm) using fish traps placed in various sites throughout Orbetello lagoon (Tuscany, NW Mediterranean). Eels were transported to the laboratory in oxygenated water tanks and kept for three days in a

401 aquarium containing 35‰ artificial sea water (Instant Ocean) at 18 ± 1 °C with 12:12 h photoperiod, at the Department of Environmental Sciences fish facility of the University of Siena. Three groups of fish ($n = 4$) were exposed to waterborne 2,4,6,-TNT dissolved in DMSO (0.1‰) at concentrations of 0.5, 1 and 2.5 mg/l. In addition, one group (as control) was exposed to 0.1‰ DMSO and a blank in clean water were maintained during the entire experiment. The TNT concentrations chosen were below or in the range of the 96-h LD50 calculated for fish (0.8–3.7 mg/l) [2]. Eels were sacrificed after 6 and 24 h. Brains were excised and stored at −80 °C. Blood was collected and centrifuged for 5 min at 12000 rpm for plasma isolation.

2.3. *Total RNA isolation and quantitative (real-time) polymerase chain reaction (PCR)*

Total RNA from brain tissue was extracted in Tripure reagent according to the manufacturer's protocol. Total RNA concentrations were measured using NanoDrop spectrophotometry (NanoDrop Technologies Inc. Wilmington DE, USA). Quantitative (real-time) PCR was used to evaluate gene expression profiles. Primers with an annealing temperature of 55–60 °C were designed based on eel species sequences by selecting conserved regions of the ERα, CYP19B, StAR and P450scc genes using PCR designer software PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer pair sequences and their amplicon size are shown in Table 1. All primer pairs gave a single band pattern for the expected amplicon size in all reactions, and no amplification occurred in reverse transcriptase (RT) reactions without enzymes. cDNA for the RT-PCR was generated with 1 μg total RNA from all samples in 20 μl reaction volume using poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad). Each DNA amplification reaction contained 12.5 μl 2X SYBR Green mix, 0.75 μl 1 mM ROX (Reference dye) and 1–3 μL cDNA. The RT-PCR program included an enzyme activation step at 95 °C (5 min) and 40 cycles at 95°C (30 sec), 55–60 °C (30 sec) and 72 °C (30 sec). Control samples without cDNA template were included to determine the specificity of target cDNA amplification. The cycle threshold (Ct) values obtained were converted into mRNA copy numbers using standard plots of Ct versus log copy number as previously described by [17]. The criterion for using the standard curve is based on equal amplification efficiency (usually >90%) with unknown samples and this is usually checked prior to extrapolating unknown samples on the standard curve. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest (see below). Data was expressed in nanograms per microliter of initial total RNA used for the reverse transcriptase (cDNA) reaction.

2.4. *RT-PCR cloning*

PCR products of all four genes were carefully excised from agarose gel and purified with a QUIAEX II Gel Extraction Kit (Quiagen Inc., Valencia, CA, USA). PCR products were ligated and subcloned into a PCR 2.1 vector (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. Clones were selected by blue-white screening. Positive clones were grown overnight and harvested for plasmid purification using a Quiagen kit. Plasmids containing the gene products were used to obtain a standard curve for quantitative RT-PCR (see above).

2.5. *Plasma cortisol*

Plasma cortisol content was measured by a solid-phase time resolved fluoroimmunoassay Delfia® following the manufacturer protocol. The assay is based on the competitive reaction between europium-labeled cortisol and sample cortisol for a limited amount of binding sites on cortisol specific, biotinylated monoclonal antibodies derived from mice. Victor3 1420 Multilabel Counter (Wallak) was used for quantification.

2.6. *Statistical analysis*

Data were reported as mean and standard error (SE). Comparison among TNT concentrations and time of exposure were also evaluated by the Mann-Whitney-Wilcoxon rank sum non-parametric test; $p = 0.05$ was assumed as maximum significant value. Statistical analyses were performed with Statistica 5.1 (StatSoft, USA).

3. Results

Exposure to TNT altered the expression of ER gene at the lowest TNT concentration at 6 h while at 24 h the gene expression levels increased only in eels exposed to the highest TNT concentration (2.5 mg/l) compared to controls (Figure 1). An increase of CYP19B mRNA expression occurred after 6 h (significantly so in the group receiving 2.5 mg/l, $p = 0.03$) of exposure to all TNT concentrations compared to control (Figure 2). At 24 h, CYP19B expression was reduced (albeit not significantly) in the 0.5 and 1mg TNT/l compared to 6 h, while no changes were observed with 2.5 mg/l at 24 h, compared to 6 h (Figure 2).

P450scc and StAR displayed a similar response pattern to TNT exposure (Figures 3 and 4, respectively). Gene expression was significantly ($p = 0.03$ and $p = 0.05$ respectively) decreased in a concentration-specific manner at 6 h with 0.5 and 1 mg TNT/l, while at 2.5 mg TNT/l, the

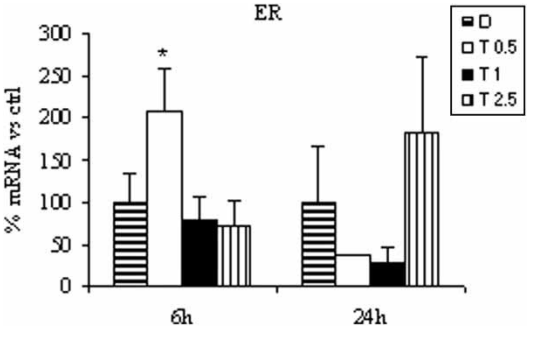

Figure 1. ERα gene expression in brain of European eels exposed to 0.5, 1 and 2.5 mg/l TNT for 6 h and 24 h. ERα mRNA expression was measured by quantitative real-time PCR with specific primer pairs. Values are mean ($n = 4$) $\pm$ standard error. *Significant difference with respect to DMSO group ($p < 0.05$).

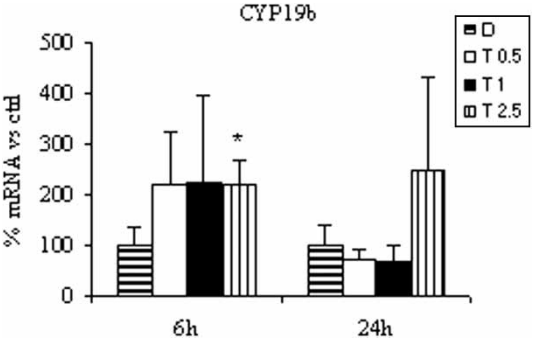

Figure 2. CYP19B gene expression in brain of European eels exposed to 0.5, 1 and 2.5 mg/l TNT for 6 h and 24 h. CYP19B mRNA expression was measured by quantitative real-time PCR with specific primer pairs. Values are mean ($n = 4$) $\pm$ standard error. *Significant difference with respect to DMSO group ($p < 0.05$).

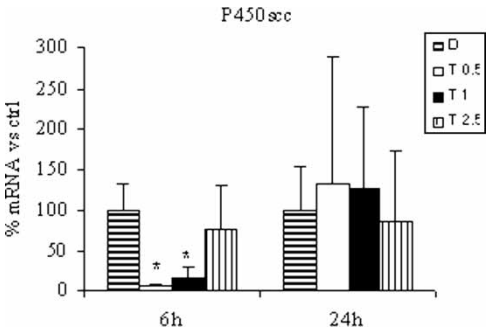

Figure 3. P450scc gene expression in brain of European eels exposed to 0.5, 1 and 2.5 mg/l TNT for 6 h and 24 h. P450scc mRNA expression was measured by quantitative real-time PCR with specific primer pairs. Values are mean ($n = 4$) ± standard error. *Significant difference with respect to DMSO group ($p < 0.05$).

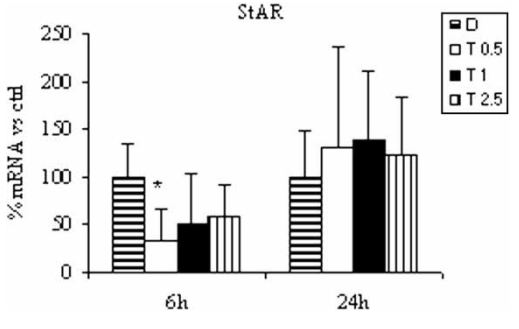

Figure 4. StAR gene expression in brain of European eels exposed to 0.5, 1 and 2.5 mg/l TNT for 6 h and 24 h. StAR mRNA expression was measured by quantitative real-time PCR with specific primer pairs. Values are mean ($n = 4$) ± standard error. *Significant difference with respect to DMSO group ($p < 0.05$).

mRNA expression levels were restored to background level (Figures 3 and 4, respectively). No further modulation was evident after 24 h of exposure for both genes.

Plasma cortisol level increased in a concentration-dependent manner after 6 h at 0.5 and 1 mg/l, while a concentration-specific significant decrease was observed after 24 h at the same concentrations ($p < 0.01$). No modulation of cortisol level was observed at the highest TNT concentration of 2.5 mg/l (Figure 5).

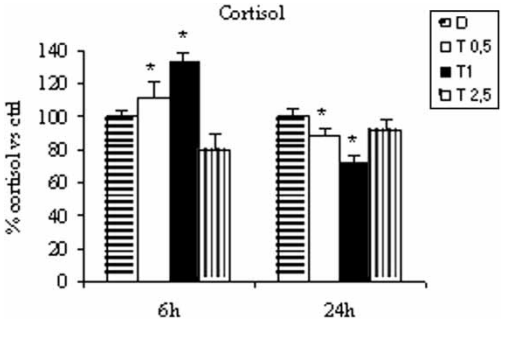

Figure 5. Ematic cortisol levels in European eels exposed to 0.5, 1 and 2.5 mg/l TNT for 6 h and 24 h. Cortisol level was measured by a time resolved fluoroimmunoassay. Values are mean ($n = 4$) ± standard error. *Significant difference with respect to DMSO group ($p < 0.05$).

4. Discussion

In the marine environment, TNT is present mostly in limited hot spot areas. Therefore, a chronic exposure of fish to this compound is unlikely [18]. Once absorbed by aquatic organisms, TNT is readily metabolised with a half life of 0.05 h and rapidly eliminated (0.77 h) (laboratory exposed fish) [19,20]. In this view, the acute exposure via water was selected in order to evaluate noxious effects of the compound in the most realistic exposure condition occurring in the marine environment at dumping areas. Overall, our results suggest that exposed eels are experiencing a TNT-mediated alteration of their neuroendocrine system.

The endocrine disrupting potential of TNT was assessed considering different potential pathways: one involving receptor-mediated responses and one interfering directly with crucial step of neurosteroidogenic pathway. The endpoints investigated displayed different responses to TNT, thus suggesting the existence of distinct regulation mechanisms for these proteins and thereby different target of interaction with the compound. The absence of a clear trend of modulation of the ER gene expression by TNT suggests that endocrine effects of the compound may not be mediated by the receptor. The decrease of P450*scc* and StAR mRNA at 6 h, indicates the ability of TNT to affect brain steroidogenic pathways by producing an inhibition of the initial phases of neurosteroidogenesis. Both StAR and P450*scc* are acutely regulated in neurosteroidogenic tissues, making these responses highly effective in acute and short-term exposure scenarios [9]. In our study, the acute decrease of both genes observed at 6 h was restored to background levels within 24 h of exposure to TNT. This fast recovery does not exclude the potential occurrence of adverse consequences for the entire organism as a consequence of StAR and P450scc impairment. The disruption of StAR and P450*scc* gene expression may infact represent the first step of co-related adverse events that determine severe disturbances for the whole organism.

Like all steroids, cortisol is generated from the precursor cholesterol and the first step in the cholesterol-steroid hormone pathway is the transfer of the precursor across the mitochondrial membrane –mediated by the StAR and its conversion to pregnenolone by P450*scc*. In this view, the decrease of plasma cortisol level observed after 24 h of exposure to TNT could reasonably represent a physiological effect of the decrease of StAR and P450*scc* mRNA expression. Cortisol production represents an outstanding response of fish species to different stress condition. Therefore, an alteration of the synthesis of this glucocorticoid leads to a reduction of organism stress response capability as well as regulation of intermediary metabolism, ion regulation and immune responses [10]. Given that CYP19B plays crucial role in estrogen production catalyzing the final step of the conversion of androgens to estrogens, CYP19 induction observed after TNT exposure can interfere with several physiological functions in multiple tissues such as sexual differentiation, development, reproduction and behaviour. Despite the slight changes in steroidogenic pathways observed in the present study, the data represents a novel aspect of neuroendocrine effects of TNT in fish not previously reported. As the investigated proteins play a crucial role in several physiological function of the organism, our results are predictive of potential physiological and reproductive adverse effects occurring in fish populations living in dumping areas. The environmental relevance of these findings is of course strictly dependent on TNT concentrations and sensitivity of different fish species. Finally CYP19B, P450*scc* and StAR showed great potential as sensitive biomarkers for the assessment of TNT effects on the endocrine system.

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