Thyroid Hormones as Potential Early Biomarkers of Exposure to Nonylphenol in Adult Male Lizard (*Podarcis sicula*)

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Abstract: The thyroid has been shown to be a target organ of environmental chemicals, specifically endocrine disrupting contaminants. Reptiles are particularly suitable as contaminant biomonitors, due to their persistence in a variety of habitats, wide geographic distribution, longevity, and, in many cases, site fidelity. Nonylphenol, an estrogenic-like compound, can induce vitellogenin synthesis in males and immature reptilian species, but little is known about its effects on thyroid hormones balance. The present study evaluated the potential effects of an acute exposure to nonylphenol (i.p. injected) on the thyroid of the lizard *Podarcis sicula*.

Nonylphenol induced a significant decrease of T4 and T3 plasma levels, in agreement with the decrease of the epithelial cell height; the nuclei of the thyroid cells were small and elongated, with dense chromatin and a greatly reduced cytoplasm. The colloid was retracted with few reabsorption vacuoles. Moreover, nonylphenol administration significantly inhibited plasma thyroid-stimulating hormone levels, thereby altering the thyroid function.

This study highlights how the structural and functional disruption of the thyroid gland in non-target organisms as the lizard might also have an environmental aetiology. In conclusion, nonylphenol was suspected to inhibit the thyroid hormones balance, suggesting the thyroid should be included among the other endocrine glands, susceptible to endocrine disruption.

Keywords: Histology, nonylphenol, *Podarcis sicula*, thyroid hormones.

INTRODUCTION

Endocrine-disrupting chemicals are a broad group of substances that alter the functions of the endocrine systems in wildlife and humans (European Commission 1997). The endocrine disruptors are widespread in the environment and food chains and include some common environmental contaminants such as pesticides, plastic ingredients, dioxins, and biocides [1-3]. The possible impact of these endocrine-disrupting chemicals needs to be considered, because many of the compounds accumulate due to their persistence in the environment. Moreover, the endocrine-related adverse effects can occur at lower dose levels than those causing tumorigenicity or teratogenicity [4] with long-term consequences on health [5,6].

Over the past decade there has been an increasing focus on the effects of synthetic chemicals on human endocrine systems, especially on effects related to androgen and estrogen homeostasis. However, there is increasing evidence from animal and *in vitro* studies, that also the thyroid is vulnerable to endocrine-disrupting effects. Environmental chemicals may interfere with thyroid homeostasis through many mechanisms of action, i.e. at the receptor level, in binding to transport proteins, in cellular uptake mechanisms or in modifying the metabolism of thyroid hormones (THs). Several environmental chemicals have a high degree of structural resemblance to the THs thyroxine (T_4) and triiodothyronine (T_3), and therefore interfere with binding of THs to receptors or transport proteins [7].

Nonylphenol (NP) and octylphenol are industrial additives used in a wide variety of detergents, plastics and pesticides [8-10]. NP may be one of the more critical compounds due to its toxicity, persistence and estrogenic effects [11-16]. Interests toward this endocrine disruptor on thyroid hormones (THs) balance [17-23] are just beginning to be taken into consideration as shown by the *in vitro* effects of NP inhibiting thyroid peroxidases, catalysing iodination of triiodothyronine (T_3) and thyroxine (T_4) in ovariectomized rat [19].

Exposure of rats to NP dose-dependently increased thyroid stimulating hormone (TSH) [24], but no consistent effects on peripheral hormones were found [24,25]. In addition, another study performed in the rat, showed that NP increased T_3 and T_4 levels, without modifying TSH in ovariectomized rats. This pattern was not consistent with *in vitro* studies of protein extracts in which NP supplied inhibitory effects on thyroperoxidase (TPO) activity [19]. Interestingly, NP might also exert a notable impact during the development of fish and tadpoles as displayed by clearly decreased TH [21] along with the reduced rate of metamorphic progression and tail reabsorption in bullfrog tadpoles [26]. Conversely, fish treatment with dietary 4-NP

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during smoltification did not significantly influence plasma T_3 and T_4 concentrations in fresh water coho salmon [27].

Only recent study [23] evaluated the potential effects of a single acute exposure to NP on the thyroid and reproductive axis of adult male shubunkins (*Carassius auratus*). Reptiles are particularly suitable as contaminant biomonitors due to their persistence in a variety of habitats, wide geographic distribution, longevity, and, in many cases, site fidelity [28].

The aims of the present study were (1) to develop a biological model for monitoring the ecotoxic effects of NP in the environment of the Campania region, based on a sentinel species, *Podarcis sicula*, because it is the most abundant species living in the open country and in cultivated fields and (2) to evaluate the possible adverse effects of NP on an endocrine organ such as the thyroid gland. Thus the sensitivity of the sentinel species to NP and the effects of this industrial additive on the thyroid gland morphology and thyroid hormones plasma levels were handled in the present study.

MATERIAL AND METHODS

Animals and Housing Conditions

Adult male lizards of *P. sicula* (weighing 13–15 g) were live-captured in the neighbourhood of Naples in June (n=20), when the thyroid gland was in full functional activity [29]. After capture, the animals were housed in large soil-filled terraria containing heather, and exposed to natural temperature and photoperiod. Water dishes were present in the terraria, and the animals were fed on live fly larvae daily. Captivity lasted 20 days to reverse capture-related stress [30]. All animals have been captured with the authorization of 06/01/2000 no. SCN/2D/2000/9213 of Italian Ministry of Environment.

Experimental Procedure

The animals received i.p. injections of Nonylphenol (Nonylphenol, CAS 84852-15-3, Sigma Aldrich, St. Louis, MO). Nonylphenol was dissolved in 50µl of peanut oil, with an injection volume of 0.1 ml. Injections were between 8.00 a.m. and 8.30 a.m.. The specimens were divided into three groups, each consisting of 20 animals, in order to obtain an adequate plasma volume.

Group 1. The animals received three weekly i.p. injections of NP (1.72 µg/100g body wt) for 7 days and were sacrificed 24 hr after the last injection.

Group 2. The animals received five weekly i.p. injections of NP (1.72 µg/100g body wt) for 14 days and were sacrificed 24 hr after the last injection.

Group 3. The animals received five weekly i.p. injections of NP (1.72 µg/100g body wt) for 28 days and were sacrificed 24 hr after the last injection.

A control group for each NP-treated group was kept under the same conditions as the treated ones, but it was intraperitoneally injected with peanut oil. Animals were sacrificed 24 hr after the last injection. The animals were anaesthetized by hypothermia, chilling them in chipped ice. Blood samples were collected by intracardiac puncture and put into heparinized tubes. Blood collection lasted less than 3 min; plasma obtained by centrifuging (2,500 g for 10 min at 4°C) the blood samples, was stored at- 20°C until assay.

Light Microscopy

Immediately after collection of blood samples, the animals were decapitated, and the thyroid glands were removed and fixed in Bouin's fixative and processed for light microscopy (LM). Serially cut paraffin sections (7 µm) were stained by Galgano I stain [31]. Observations were performed using a Zeiss Axioskop microscope; images were captured with a camera attached to an IBM computer running the Kontron Elektronik KS 300 image analysis system and then they were processed by Adobe Photoshop. The height of the follicular cells was measured in 30 cells every 3 slides, always on the second section of normal and treated specimens using a digital system of imagine (KS 300).

Hormone Assay

Plasma levels of T_3 and T_4 were determined by radioimmunoassay (RIA) [32,33]. In the T_3 assay, a measured amount of sample serum and standards was added to a tube coated with anti- T_3 rabbit antibody, with a trace (4.4 (Ci) amount of radioactively labeled T_3 ($[^{125}I]$ T_3) (Byk-Sangtec Diagnostica, Dietzenbach, Germany) and an agent-blocking Tris buffered saline 4 mM, ANS (8-anilino-1-naphthalenesulfonic acid) 6 mM sodium salicylate with 0.2% sodium azide as a preservative (Sigma Chemical Co., St. Louis, MO) to release T_3 from serum-binding proteins. Sensitivity was 0.1 ng/mL with an accuracy of about 97%. The range of intra-assay variance in 20 assays was 1.0–2.6%, whereas the interassay variance ranged between 3.9% and 5.7% in 12 assays.

For T_4, a measured amount of sample serum and standards was added to a tube coated with anti- T_4 rabbit antibody, along with a trace amount of radioactively labeled T_4 ($[^{125}I]$ T_4), 4.4 (Ci, (Byk-Sangtec Diagnostica, Dietzenbach, Germany) and a blocking agent, Tris-buffered saline 4 mM, ANS 6 mM sodium salicylate with 0.2 % sodium azide as a preservative (Sigma Chemical Co. St. Louis, MO) to release T_4 from serum-binding proteins. Sensitivity was 0.45 ng/mL, with an accuracy close to 100%; the mean intra-assay and inter-assay coefficients of variance were 4.6% and 4.3%, respectively. A logit–log curve fit using a % B/Bo calculation was used. T_4 and T_3 concentrations were determined by computing the % B/Bo for each sample and then finding the results on the standard curve. Crossreactivity for T_4 in the T_3 RIA (1.3%) was not considered for data calculations, neither was that for T_3 in the T_4 RIA (0.1%).

Plasma TSH was determined by immunoradiometric assay (IRMA) [32,33]. Sample serum and standards were added to antiligand-coated tubes. The Tracer/Capture Reagent, a blend of ligand-tagged TSH specific antibody and ^{125}I-labeled TSH (10 µCi), was added to each tube. A cubic spline function with the zero standard as one of the standard points was used for calculations. The minimum detectable dose (MDD) was 0.01 µIU/mL, with an accuracy close to 100%, and the mean intra-assay and interassay coefficients of variance were 5.0% and 7.5%, respectively.

Statistical Analysis

All data were expressed as means ± standard error of mean (SEM). The control and the experimental data of all

Table 1. Variations of Epithelium Height of the Follicular Cells Of the Thyroid Gland in *P. sicula* Subjected to NP Treatment (see Materials and Methods Section). Note: Values are Shown as Means ± SEM. * *P*<0.05 from Control Specimens, ** *P*<0.001 from Control Specimens

Group	Treatment (µg/100g Body Weight/Day)	Height of Follicular Epithelium (µm)
Control	Peanut oil	15.1 ± 0.02
1	1.72 µg/100g wt/7d	8.32 ± 0.05*
2	1.72 µg/100g wt/14d	5.10 ± 0.04**
3	1.72 µg/100g wt/28d	3.02 ± 0.02**

the groups were tested together for significance using one-way analysis of variance (ANOVA), followed by Duncan's test for multigroup comparison and Student's t-test for between group comparison. Differences were considered significant at P<0.05.

RESULTS

Morphological Observations of the Thyroid Gland

The thyroid gland of *P. sicula* control specimens is a single discrete ribbon-like structure, which transversely crosses the middle of the trachea. It is formed by follicles, surrounded by an epithelium formed by thyrocytes, and containing the colloid; the follicles are connected by an inter-follicular connective tissue, containing blood vessels. A superficial connective tissue capsule envelops the gland and sends branches which form a network surrounding the follicles. Control thyroids showed a medium-high follicular epithelium (15.1±0.02µm) (Table **1**; Fig. **1a**).

The thyroid gland of NP-treated lizards showed dose-dependent morphological changes. Indeed, in Groups 1 and 2, the follicular epithelium was low and the nuclei of the thyrocytes were small and elongated with dense chromatin and a greatly reduced cytoplasm. The colloid was retracted with few reabsorption vacuoles (Figs. **1b**-**1c**). In Group 3, the thyroid gland showed very evident signs of poor functional activity. The height follicular epithelium was very low and the thyrocyte nuclei were small and elongated with dense chromatin and greatly reduced cytoplasm. The colloid showed rare reabsorption vacuoles (Fig. **1d**). Data about the

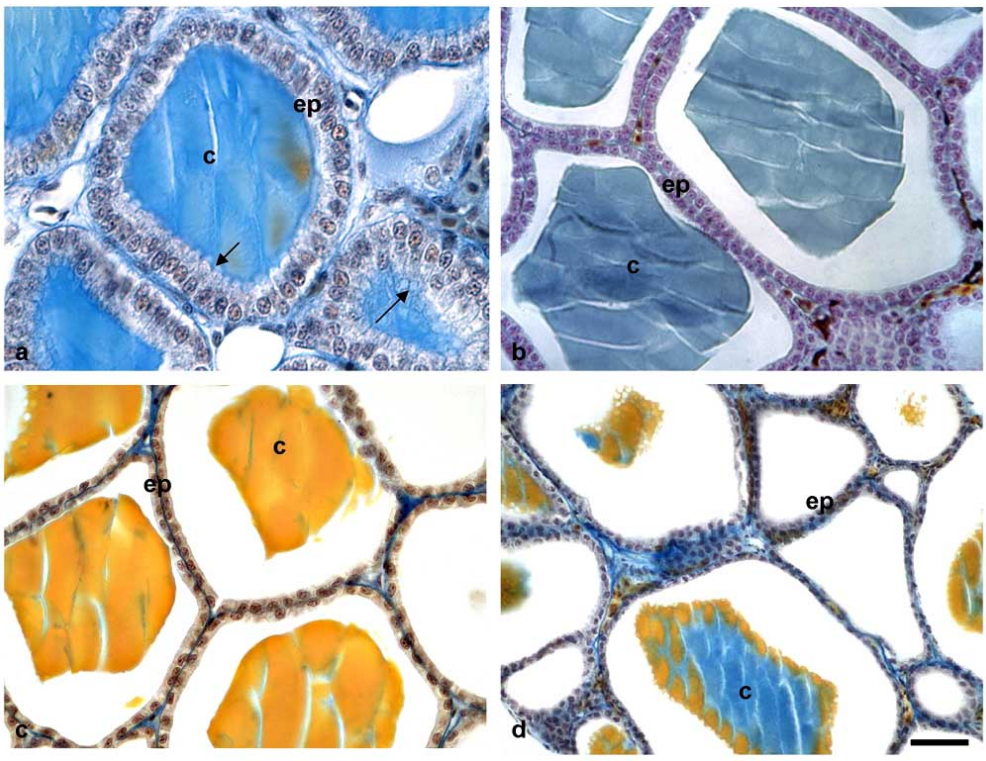

Fig. (1). Normal and NP-treated thyroids of exposed lizards *P. sicula* (stain Galgano I); **a**. Normal specimen; note the cuboidal follicular epithelial cells (ep), the colloid (c) and the reabsorption vacuoles (arrow); **b** specimen treated with NP (1.72 µg/100g body wt) for 7 days and sacrificed 24 hr after the last injection; the follicular epithelium (ep) is lower than in normal specimen; **c** specimen treated with NP (1.72 µg/100g body wt) for 14 days and sacrificed 24 hr after the last injection; the follicular epithelium (ep) is very low; **d** specimen treated with NP (1.72 µg/100g body wt) for 28 days and sacrificed 24 hr after the last injection; note the follicular epithelium (ep) very low and no reabsorbing vacuoles in the colloid. Scale bar: 20 µm.

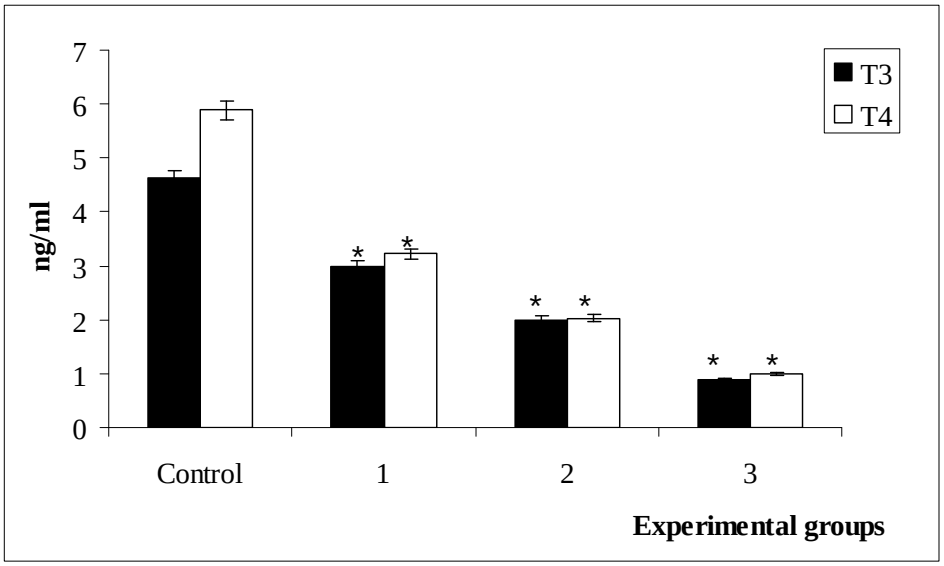

Fig. (2). Variations of T_3 and T_4 levels in the plasma of *P. sicula* subjected to different experimental treatments (see Materials and Methods section). Values are shown as means ± SEM. * *P*<0.05 from control specimens, ** *P*<0.001 from control specimens.

height of follicular epithelium after acute treatment are shown in Table **1**.

T4 and T3 Plasma Levels

Plasma levels of thyroid hormones in the lizard *P. sicula* were affected by the different NP doses after 28 days of treatment. In fact, the level of circulating T_4 and T_3 dose-dependently decreased in all treatment groups. Plasma T_4 decreased ($p<0.05$) from 5.89 ± 0.04 ng/ml in the control specimens to 3.22 ± 0.03 ng/ml in animals of Group 1, and to 2.03 ± 0.02 ng/ml in animals of Group 2 and reached its minimum value ($p<0.001$) (1.00 ± 0.02 ng/ml) in animals exposed to five weekly injections i.p. of NP (1.72 µg/100g wt) for 28 days (Group 3).

Plasma T_3 decreased ($p<0.05$) from 4.62 ± 0.02 ng/ml in the control specimens to 2.89 ± 0.04 ng/ml in animals of Group 1 and 1.59 ± 0.05 ng/ml in animals of Group 2 and reached its minimum value ($p<0.001$) (0.09 ± 0.02 ng/ml) in animals of Group 3 (Fig. **2**).

TSH Plasma Levels

Plasma concentrations of TSH decreased in all treatment groups. A NP dose of 1.72 µg /100g wt for 7 days (Group 1) produced a slight decrease in the level of TSH (3.43 ± 0.03 µIU/ml) with respect to the control group (5.15 ± 0.03µIU/ml). A mild to significant ($p<0.05$) inhibition in the plasma levels of TSH was observed in lizards exposed to a dose of 1.72 µg /100g wt for 14 days (Group 2) (2.09 ± 0.04 µIU/ml) and a dose of 1.72 µg /100g wt for 28 days (Group 3) (0.98 ± 0.04 µIU/ml) (Fig. **3**).

DISCUSSION AND CONCLUSIONS

The present study is the first report dealing with NP effects on the thyroid gland of the lizard *P. sicula*. NP is an

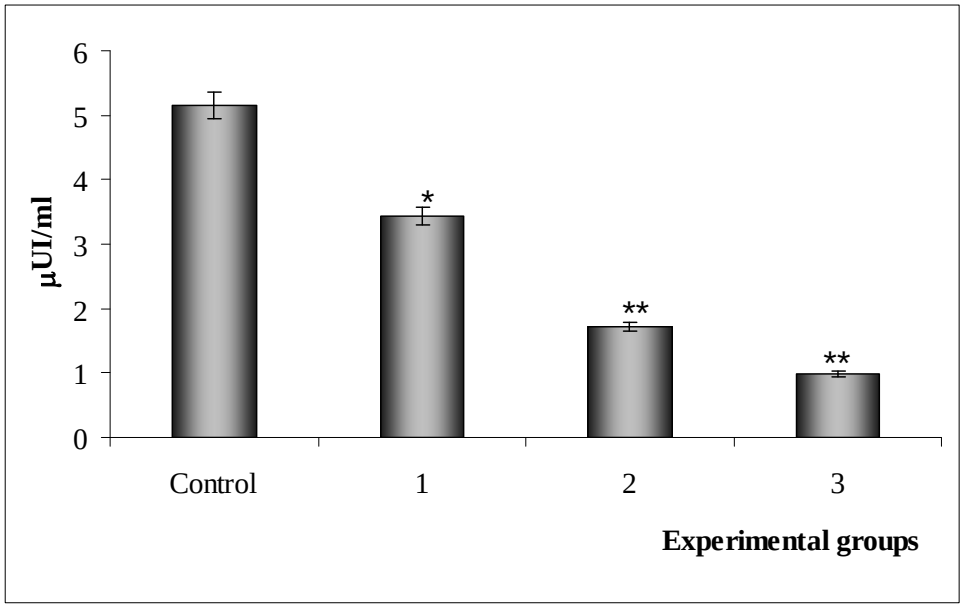

Fig. (3). Variations of TSH levels in the plasma of *P. sicula* subjected to different experimental treatment (see Materials and Methods section). Values are shown as means ± SEM. * *P*<0.05 from control specimens, ** *P*<0.001 from control specimens.

industrial additive used in a wide variety of detergents, plastics and pesticides [8- 10]. NP may be one of the more critical compounds due to its toxicity, persistence and estrogenic effects [11-16].

The results of this study indicate that both structural and functional differences in the thyroid gland of the lizard *P. sicula* exist, in the animals exposed to NP. Structurally, animals exposed to NP showed decreased epithelial cell height, and nuclei of the thyrocytes small and elongated, with dense chromatin and a greatly reduced cytoplasm. The colloid was retracted with few reabsorption vacuoles.

Functionally, the same animals exhibited decreased T_4 and T_3 plasma levels, compared to control animals. Both histological and hormonal data have been used to indicate thyroid endocrine disruption. Additionally, NP administration produced a significant inhibition on serum TSH levels. This result might have caused hypothyroidism induced by NP exposure; therefore, the authors suggest that the effects of NP could very well occur pituitary level *via* decreased TSH production accounting for a reduced thyroid activity.

Little is known about the effects of NP, and alkylphenols in general, on thyroid hormones (TH) balance [18-21, 27]. *In vitro* evaluation of several chlorinated phenols showed that NP had weak affinity for the transport protein TTR but not for thyroid receptor (TR) in chicken and bullfrog [18]. In addition, NP induced GH3 cell proliferation and has proved to inhibit thyroid peroxidases catalysing iodination of T_3 and T_4 [19, 20]. All *in vitro* studies have been performed on avian or mammal cells. To our knowledge, only three studies reported *in vivo* findings on fish species and in any case they consisted of contrasting results after two different treatments [21, 27]. McCormick and co-workers [21] found that intraperitoneal administration of 0.5 to 150 μg/g of 4-NP to juvenile Atlantic salmon led to a dose-dependent reduction of T_4 levels, while only higher doses reduced T_3 plasma levels. Both hormone levels were decreased by E_2 treatment at a 2 μg/g dose. On the contrary, Keen and co-workers [27] failed to observe such a reduction after dietary 4-NP treatment during smoltification in coho salmon, whereas a net decrease in T_3 levels was observed in E_2 treated animals. Recently, Zaccaroni and co-workers [23] evaluated the potential effects of a single acute exposure to nonylphenol (i.p. injected) on the thyroid and reproductive axis of 250 shubunkins (*Carassius auratus*). Nonylphenol induced a significant decrease of thyroxin levels, whereas no effect on triiodothyronine concentrations was detected. No histopathological changes were detected for thyroid or testes. Our data, in agreement with those of McCormick *et al.* [21] and Zaccaroni *et al*. [23], tend to point to a predominating effect of 4-NP on thyroid activity with T_4 turning out to be the most sensitive parameter of NP action. It has been suggested that NP could act on the thyroid through different mechanisms, like interference with the binding of T_3 to transthyretin, or antagonism to T_3 binding to TH receptors [18, 34]. Although these mechanisms have all been postulated for higher vertebrates, they may also apply to *Podarcis sicula*, confirming the direct effect of NP on the thyroid axis. These data confirm the existence of an interplay between NP and thyroid axis, suggesting the thyroid should be included among the other endocrine glands susceptible to endocrine disruption.

In conclusion, the toxicological results disclose the inhibitory effect of NP on thyroid hormones balance after a single acute exposure. This observation suggests that TH may serve as a potential early biomarkers of NP endocrine disruption, as TH changes, namely decreased T4 levels, occur before any others in all the tissues involved in thyroid hormone production and metabolism (i.e. thyroid, liver, kidney).

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