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Graphene oxide and reduced graphene oxide promote the effects of exogenous T3 thyroid hormone in the amphibian *Xenopus laevis*

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ABSTRACT

The interest for graphene-based nanomaterials (GBMs) is growing worldwide as their properties allow the development of new innovative applications. In parallel, concerns are increasing about their potential adverse effects on the environment are increasing. The available data concerning the potential risk associated to exposure of aquatic organisms to these GBMs are still limited and little is known regarding their endocrine disruption potential. In the present study, the endocrine disruption potential of graphene oxide (GO) and reduced graphene oxide (rGO) was assessed using a T3-induced amphibian metamorphosis assay. The results indicated that GBMs potentiate the effects of exogenous T3 with a more marked effect of GO compared to rGO. T3 quantifications in the exposure media indicated adsorption of the hormone on GBMs, increasing its bioavailability for organisms because GBMs are accumulated in the gut and the gills of these amphibians. This study highlights that the tested GBMs do not disrupt the thyroid pathway in amphibians but indicates that adsorption properties of these nanomaterials may increase the bioavailability and the toxicity of other pollutants.

1. Introduction

Due to their unique properties, graphene-based nanomaterials (GBMs) such as graphene oxide (GO) and reduced graphene oxide (rGO) are triggering a great interest for the development of new applications (Smith et al., 2019). As these materials are forecasted to be produced at industrial level (Lin et al., 2019), this would undoubtedly lead to their release into the environment along the material life cycle (He et al., 2017; Mottier et al., 2017). Thus, it is necessary to determine their potential impact on the environment. GBMs were shown to possibly induce a wide range of biological responses in various organisms (Fadde et al., 2018; Freixa et al., 2018; Montagner et al., 2016). Among these ecotoxicological effects, potential endocrine disruption activities of GBMs remain poorly evaluated while dysfunction of the hypothalamus-pituitary-thyroid (HPT) axis could have strong deleterious consequences for organisms (Couderq et al., 2020). However, few studies aiming to evaluate the endocrine disrupting effects of GBMs are available and the results reported are contradictory. A recent studies indicated no endocrine disrupting effects of GO using Japanese medaka

(*Oryzias latipes*) as biological model (Dasmahapatra et al., 2020) while the metamorphic development of the amphibian *Xenopus laevis* was delayed following a 21-day exposure to GO at a concentration of 1 mg/L due to thyroid endocrine disruption (Li et al., 2019). However, according to the OECD guideline 231 for the testing of endocrine disruption potential of chemicals using amphibian metamorphosis assay (AMA), delayed development can occur through anti-thyroidal mechanisms (antagonism) but also through indirect non-specific toxicity that may lead to false-positive outcomes (OECD, 2009). Thus, the determination of endocrine disruption effects is remaining challenging in that particular case. In the work from Li et al., the alteration of the larval metamorphosis was associated to modifications of the fatty acids metabolism that is consistent with previous studies indicating that exposure to GBMs led to *X. laevis* larval growth inhibition (Lagier et al., 2017; Mottier et al., 2016), constituting a marker of general acute toxicity (Mouchet et al., 2008). As earlier metamorphic stages are known to be associated with lower T3/T4 hormone levels and lower expression of genes involved in the hypothalamic-pituitary-thyroid (HPT), as measured in this study, a possible false positive due to indirect toxicity cannot be fully excluded

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(Wen and Shi, 2016; Yaoita, 2019). In order to provide specific information about the mode of action of potential endocrine disruptors, T3-induced metamorphosis assay was used in several studies (Crump et al., 2002; Lim et al., 2002; Wang et al., 2017). Contrary to the AMA that is based on spontaneous metamorphosis of *X. laevis*, this assay relies on the induction of metamorphosis in larvae through the early exposure to the active T3 hormone and allows to detect chemical disruption of the TH signaling based on quantitative morphological endpoints (Yao et al., 2017). If a pollutant inhibits or stimulates T3-induced morphological changes, it will be proposed as a TH signaling antagonist or agonist respectively (Wang et al., 2017). This assay was shown to provide results quickly with an improved sensitivity (Jagnytsch et al., 2006; Wang et al., 2017).

Thus, the objectives of this study were to go beyond the few existing data concerning the evaluation of GBMs endocrine disruption potential towards the amphibian *Xenopus laevis*. This work aims to characterize possible endocrine disruption mechanisms associated to GBMs exposure and to disentangle the toxicity-related effects from endocrine disruption. The main hypothesis being that the previously observed effects described in *Xenopus laevis* tadpoles were associated to toxicity rather than thyroid endocrine disruption.

2. Materials and methods

2.1. *Xenopus laevis* exposure conditions

Xenopus laevis tadpoles at stage 52 (Nieuwkoop and Faber, 1958) were exposed to GBMs at concentrations of 0, 0.01, 0.1 and 1 mg/L in the presence or absence of 2 nmol/L (~1300 pg/mL) of T3. *Xenopus laevis* originated from our certified breeding facilities (under the approval number A31113002) and the experimental procedure was approved by an ethic committee (CEE-073). Exposures were conducted during 96 h in reconstituted water. The exposure media, the contamination as well as feeding (1.5 mg of Serra Micron®) were renewed daily. Each condition was run in three replicates tanks (N = 18 larvae per treatment group) and the experiment was conducted three times (54 larvae per treatment group over the three experiments).

The tested GO was provided by Antolin Group (Grupo Antolin, Spain). In order to determine the influence of the oxygen content of the nanomaterial on its endocrine disruption potential, reduced graphene oxide (rGO) was produced by heat treatment of this GO in hydrogen atmosphere at 200 °C, leading to the loss of some oxygen-containing functions. Details on the synthesis and characterization of the tested GBMs are available in a previous work (Evariste et al., 2019), and characterization data are summarized in Table 1.

After 96 h of exposure, the larvae were anaesthetized by bathing in buffered MS222 solution to perform biometric measurements. Images were acquired under binocular magnifier (Olympus SZ×7) equipped with a photo camera (Olympus E 620). Morphological endpoints such as head area (HA), unilateral brain width/brain length ratio (ULBV/BL),

hindlimb length/snout-vent length ratio (HLL/SVL) were measured according to Yao et al. (2017) using ImageJ® software.

2.2. T3 quantification in media

T3 was quantified in the exposure media using Triiodothyronine (T3) competitive ELISA Kit (Invitrogen) according to manufacturer's recommendations. The hormone was quantified immediately after addition of T3 to the media and after 2.5 h in the presence or not of GBMs at 1 or 0.1 mg/L. The experiment was conducted in triplicate with technical replicate. Sample concentrations were determined from a standard curve ranging from 0 to 5000 pg/mL of T3.

2.3. Statistical analysis

Statistical analysis was performed using Minitab 16 Statistical software. To validate the efficiency of the T3 treatment on the biometric parameter measured, significant differences between the control group in the presence or absence of T3 were compared using Student-t test. After verifying the normal distribution and homogeneity of variance of the data, one-way ANOVA were performed separately to compare the effects induced by the different GBMs concentrations to their respective control group (with or without T3 hormone).

3. Results and discussion

During the experiments, no mortality was recorded in any of the treatment groups. The exposure to T3 led to morphological changes (Fig. 1), with significant modifications of all the biometric parameters measured in absence of GBMs (T-test, $p < 0.001$) (Fig. 2). For example, in presence of T3 hormone, the weight of the tadpoles decreased by $31 \pm 16\%$, the head area decreased by $31 \pm 11\%$ while the HLL/SVL and UBLW/BL ratio increased by $97 \pm 42\%$ and $24 \pm 13\%$ respectively. These changes were associated to gill resorption, limb outgrowth and brain remodeling (Heimeier and Shi, 2010). In addition, exposure to GBMs in absence of T3 hormone in the water did not influence either the morphological parameters measured (ANOVA, $p > 0.05$) or the weight of the larvae (ANOVA, $p = 0.100$), indicating that GBMs alone did not exert a T3-like action, in agreement with the work of Li et al. (2019).

In the case of co-exposure to GO and T3, the response to the T3 hormone improved significantly, leading to more marked T3-induced biometrical changes in presence of GO. Thus, compared to the control group with T3 only, the weight of the tadpoles significantly reduced following exposure to T3 and GO at 1 mg/L ($22.4 \pm 12.3\%$) (Fig. 2A). The HLL/SVL ratio was not significantly influenced (ANOVA, $p = 0.082$) either to GO or rGO exposure (Fig. 2B). In this case, HLL did not differ between the tested conditions, while SVL significantly decreased following exposure to T3 and GO at 1 mg/L. The more marked effects occurred for the head area parameters that significantly decreased in a dose dependent manner from 0.1 mg/L of GO in presence of T3 (Fig. 2C). Thus, the effects induced by the hormone on head area parameter was increased by $39.5 \pm 5.1\%$ in presence of T3 and GO at 1 mg/L. The effects of T3 on UBLW/BL ratio were also significantly increased following exposure to GO at 0.1 and 1 mg/L (Fig. 2D). When the larvae were co-exposed to T3 and rGO, the effects occurring following exposure to 1 mg/L of rGO were comparable to those following exposure to GO at 0.1 mg/L in the case of the weight, HA parameter and UBLW/BL ratio (Fig. 2).

The results obtained indicated that the exposure to GBMs did not lead to impairment of TH signaling as suggested by Li and collaborators (Li et al., 2019). Thus, the developmental delays observed by these authors may be attributed to the disturbances of the lipid metabolism more than to an endocrine disruption. The improved effects of T3 in presence of GO and in a lesser extend to rGO, suggest agonistic effects of these GBMs on the TH signaling. However, the exposure to the GBMs in absence of T3 hormone did not lead to the induction of T3-like effects.

Table 1

Physico-chemical characteristics of graphene oxide (GO) and reduced graphene oxide (rGO) used in the study. TEM: transmission electron microscope; HRTEM: high resolution TEM; BET: Brunauer-Emmett-Teller; at. %: atomic %.

	Graphene Oxide	Reduced Graphene Oxide
Carbon content	69.0 \pm 0.4 at. %	83.8 \pm 0.5 at. %
Oxygen content	31.0 \pm 0.4 at. %	16.2 \pm 0.3 at. %
Csp ² graphene	35.5 at. %	64.5 at. %
C-OH/C-O-C	24.7 at. %	7.8 at. %
C O	2.5 at. %	5.8 at. %
O C-O	5.3 at. %	1.3 at. %
Sat.	1.4 at. %	4.5 at. %
Number of layers (HRTEM)	1–5	1–5
Lateral size (TEM)	0.2–8 μ m	0.2–8 μ m
Specific surface area (BET)	228 \pm 7 m ² g ⁻¹	16 \pm 0.5 m ² g ⁻¹

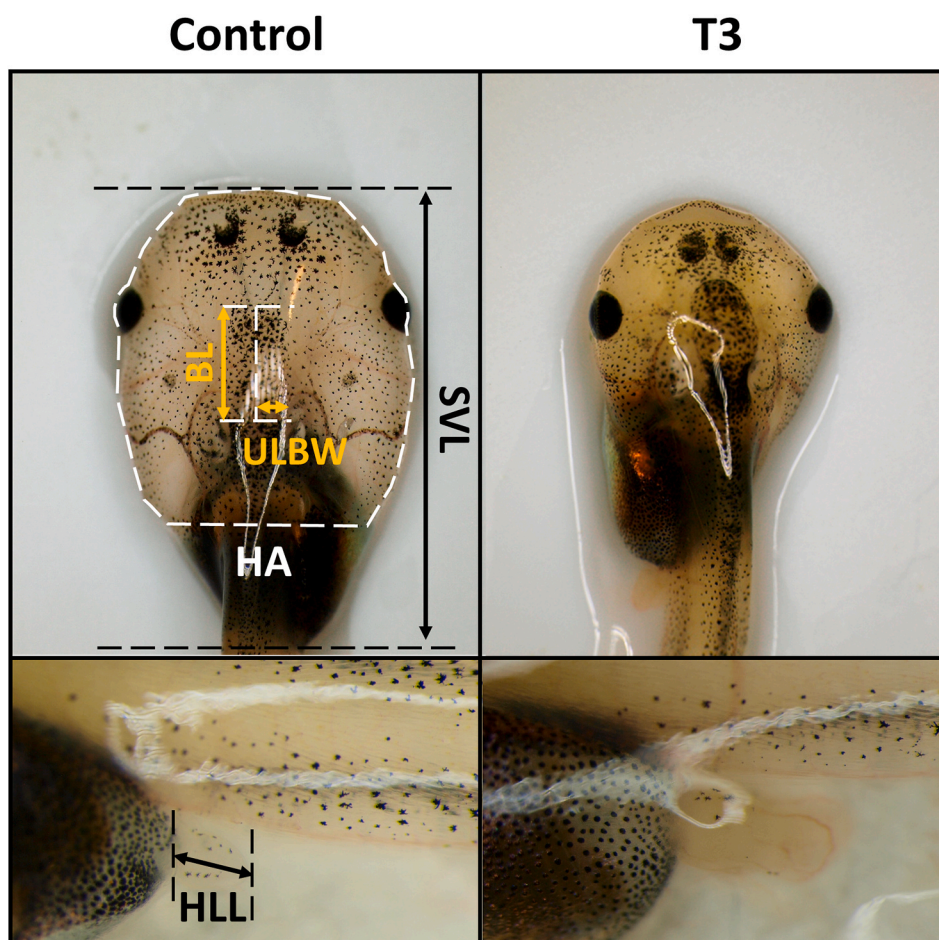


Fig. 1. Illustration of the morphological changes induced by a 96-h exposure to 2 nmol/L of T3 hormone in *Xenopus laevis* tadpoles at stage 52. HA: Head Area; SVL: Snout-Vent Length; BL: Brain Length, ULBW: Unilateral Brain Width; HLL: Hindlimb Length.

Thus, another mechanism is more likely to be involved in the observed effects. According to the literature, GO and rGO were shown to constitute promising adsorbent for bisphenol A (BPA), and could be used for the removal of this compound in the water (Kwon and Lee, 2015; Xu et al., 2012). It was also indicated that the composition and the nature of the oxygen-containing function of the nanomaterials influences its adsorption capacity on contrary to the specific surface area parameter (Bele et al., 2016; Cortés-Arriagada et al., 2013). BPA constitutes a model case for endocrine disruption studies (Ohore and Zhang, 2019). In amphibians, the antagonistic effects of BPA were associated to its ability to bind TH receptors similarly to T3, affecting metamorphosis process through impairment of T(3)-signaling pathways (Heimeier et al., 2009). Thus, due to the structural homologies between T3 and BPA, it is highly probable that GBMs are able to adsorb the T3 hormone on its surface in a similar way to BPA. This would result in a greater adsorption of T3 on oxygen-containing functions of GO compared to rGO.

To assess this hypothesis, T3 hormone was quantified in the exposure media. T3 adsorption on GBMs would lead to a decrease in the hormone concentration in the water column as these nanomaterials were shown to sediment over time in salt-containing media (Chowdhury et al., 2013, 2015; Evariste et al., 2019). T3 concentration determined immediately after media contamination was 823 ± 72 pg/mL which is lower than the expected nominal concentration. However, the initial concentration of T3 hormone in the water column decreased by $8.8 \pm 2.93\%$ over 2.5 h in control media while it significantly decreased by $45.8 \pm 2.3\%$ or $34.9 \pm 6.7\%$ in the presence of GO at 1 mg/L or 0.1 mg/L, respectively (Fig. 3), confirming our hypothesis. In the case of rGO, no significant effect was noticed despite an apparent trend in T3 concentration decrease. Thus,

the adsorption capacity seems to be linked to the degree of GBMs oxidation and could explain the lower increase of the T3-related effects following T3 and rGO co-exposure. Nevertheless, this result could also be associated to a slower sedimentation of the rGO compared to GO in the exposure media as previously measured (Evariste et al., 2019), since its dispersion stability is less affected by Ca^{2+} ions due to the lower amount of functional groups (Chowdhury et al., 2015).

GBMs are known to accumulate in the gut and gills of different organisms such as amphibians (Lagier et al., 2017), invertebrates (Cavion et al., 2020; Lv et al., 2018) as well as fish where it exert adverse effects (Zheng et al., 2019). Similar observations were made in zebrafish using radiolabeled graphene (Lu et al., 2017) while GO was able to translocate from the water to the brain of zebrafish (Hu et al., 2017). As the gills appear to constitute a possible route for GBMs to reach the inner organism, the effects observed in our study might possibly be associated to a translocation of GBMs-T3 complexes in the organisms through the gills in the anterior part of the animal, leading to increased bioavailability of T3 in the head region. This would also explain the improved effects of T3 specifically in this region and the absence of effect potentiation on the hindlimb length parameter, which is mediated mainly by dermal absorption of the T3 hormone in this region of the body. Such hypotheses are remaining to be validated using radiolabeled materials for precise detections in these biological matrices.

4. Conclusions

The results obtained in our study did not indicate a specific endocrine disruption of the thyroid pathway in the amphibian *Xenopus laevis*

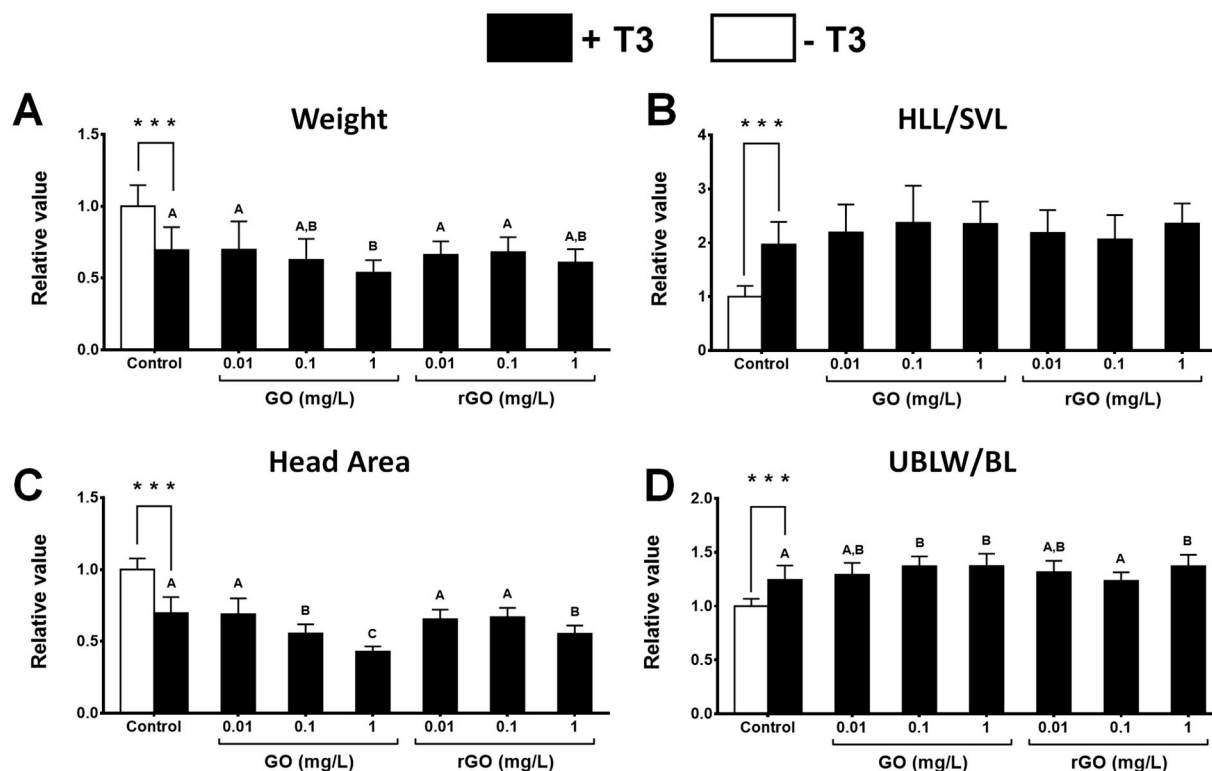


Fig. 2. Biometric measurement of *Xenopus laevis* tadpoles following a 96-h exposure to GO or rGO at concentrations ranging from 0.1 to 1 mg/L in the presence of 2 nmol/L of T3. The control group was tested in presence and in the absence of the hormone. Normalized data are presented as mean \pm SD. Asterisks indicates significant differences between the control group in the presence or absence of T3 (T-test, $p < 0.001$). Capital letters indicate statistically homogenous groups between T3-containing conditions according to the post-hoc Tukey when ANOVA $p < 0.05$. HLL: hindlimb length; SVL: Snout-Vent length; UBLW: Unilateral brain width; BL: Brain length.

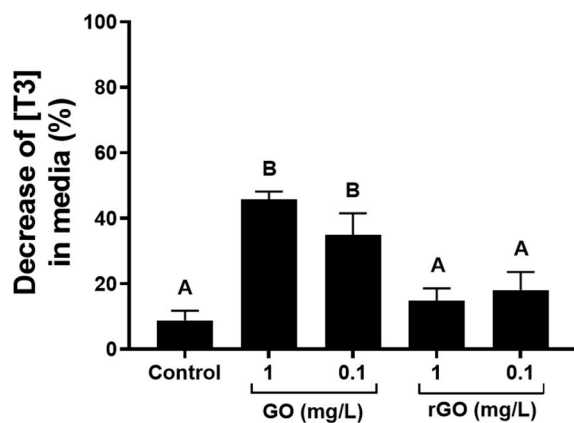


Fig. 3. Evolution of T3 concentration in exposure media over 2.5 h in control or GBMs-containing media. Data are presented as mean percentage decrease of T3 concentration \pm SEM. Letters indicate significant differences between conditions (ANOVA $p < 0.001$ followed by Tukey test).

following exposure to GBMs. However, these results highlight the potential of GBMs to transport hormones and improve their bioavailability, leading to agonistic responses. Due to their capacity to adsorb other compounds including pollutants (Peng et al., 2017; Wang et al., 2014) it is necessary to characterize the potential risk of these nanomaterials under co-exposure scenario with potential endocrine disruptors as synergistic effects may occur and threaten aquatic organisms.

Author statement

Lauris Evariste: Writing – original draft, Formal analysis, Investigation, Methodology, Antoine Mottier: Conceptualization, Methodology, Eric Pinelli: Funding acquisition, Conceptualization, Emmanuel Flahaut: Funding acquisition, Resources, Writing – review & editing, Laury Gauthier: Funding acquisition, Project administration, Florence Mouchet: Funding acquisition, Supervision, Writing – review & editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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