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Short-term *in vivo* exposure to graphene oxide can cause damage to the gut and testis

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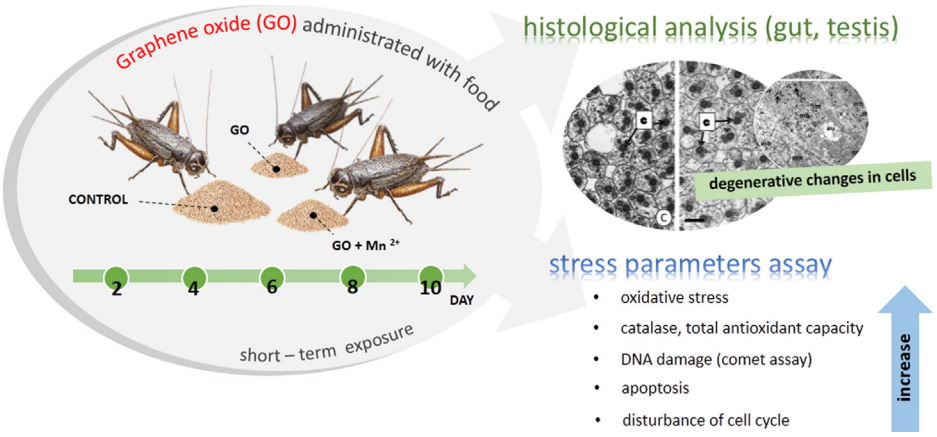
HIGHLIGHTS

- The *in vivo* toxicity graphene oxide (GO) administrated with food was measured.
- Stress parameters were measured in *A. domesticus* after exposure to graphene oxide.
- Administration of GO and GO + Mn^{2+} with food had an effect on the organism.
- Many histological changes were found in gut and testis of *A. domesticus*.

GRAPHICAL ABSTRACT



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ABSTRACT

Graphene oxide (GO) has unique physicochemical properties and also has a potentially widespread use in every field of daily life (industry, science, medicine). Demand for nanotechnology is growing every year, and therefore many aspects of its toxicity and biocompatibility still require further clarification.

This research assesses the *in vivo* toxicity of pure and manganese ion-contaminated GO that were administrated to *Acheta domesticus* with food (at 200 mg kg^{-1} of food) throughout their ten-day adult life.

Our results showed that short-term exposure to graphene oxide in food causes an increase in the parameters of oxidative stress of the tested insects (catalase – CAT, total antioxidant capacity – TAC), induces damage to the DNA at a level of approximately 35% and contributes to a disturbance in the stages of the cell cycle and causes an increase of apoptosis. Moreover, upon analyzing histological specimens, we found numerous degenerative changes in the cells of the gut and testis of *Acheta domesticus* as early as ten days after applying GO.

A more complete picture of the GO risk can help to define its future applications and methods for working with the material, which may help us to avoid any adverse effects and damage to the animal.

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1. Introduction

Even though nanotechnology is a relatively new field, nanoparticles (NP) are not. The first use of nanoparticles dates back to the 10th century BC [1]. Nanoparticles are a connecting link between bulk materials and atomic or molecular structures. The properties of many popular materials change upon the formation or addition of nanoparticles. This is possible because nanomolecules have a surface area to volume ratio that is larger than bigger particles. As a result, the chemical reactivity of the materials increases [2].

From a scientific point of view, the most attractive materials are from the graphene family [3]. One of these is graphene oxide (GO), which is a derivative of graphene sheets that have a large number of oxygen-containing hydrophilic groups in their structure [4]. The presence of hydroxyl, carbonyl or carboxylic groups makes GO a suitable material for functionalization and chemical modifications. GO is hydrophilic and thus can create a stable suspension in water. Due to its ability to bind different molecules, the practical application of GO in many different fields of science has become more popular [5]. Graphene oxide can be used in industry to produce graphene-based composites, but also has other applications such as advanced electronics, hydrogen storage, transparent film production and catalysis [6–10]. GO is also potentially the best candidate from the graphene family for biological and medical utilization. The use of GO as drug carriers, biosensors and vectors for gene or cancer therapy is now being considered [11–14]. However, before graphene oxide is used on a large scale in the medical field, a thorough understanding of its toxicology at various levels of physiological reactivity is needed [15].

Several *in vivo* and *in vitro* studies have revealed that GO exposure increases oxidative stress in the cells, enhances apoptosis and decreases cell viability [16–19]. In our previous studies, we focused on the *in vivo* effects of GO after intentionally injecting it into the body cavity of *Acheta domesticus.* The results indicated that graphene oxide can already increase the level of reactive oxygen species (ROS) within 48 h after the injection [20]. In the subsequent study, we decided to estimate the *in vivo* toxicity of two types of graphene oxide (pure and ones contaminated with manganese ions), which were administrated to *Acheta domesticus* with food throughout the ten days of their adult life. We assumed that this natural way of applying nanoparticles could show the effects of both potential medical treatment as well as any accidental contact with GO in the environment. Moreover, it appears to be highly possible that changes at the cellular level during this period may also be crucial and noticeable.

The main aim of this study was to assess the effects of GO at the cellular and tissue level after a relatively short-term (ten days) exposure. The selected stress parameters – catalase (CAT), total antioxidant capacity (TAC) and the level of DNA damage – were measured every two days. The cell cycle, the level of apoptosis and the total oxidative stress were checked at the beginning (second day) and at the end of the experiment (tenth day) using flow cytometry. A histological assessment of the gut and male gonad (testis) was performed after ten days of treatment using Transmission Electron Microscopy (TEM).

2. Materials and methods

2.1. Experimental model

Acheta domesticus, a house cricket, is frequently used as a model organism in studies because its biology and physiology are well-known [21]. Adult insects from a laboratory stock population (kept at the University of Silesia in Katowice, Poland) were divided into three groups, transferred to separate plastic insectaries ($46 \times 31 \times 17.5$ cm; 110 individuals in each) and bred in standard conditions (temperature: $29.4 \pm 3.5\,^{\circ}$C; photoperiod: 12 h: 12 h; humidity: $46.52 \pm 9.43\%$) with unlimited access to water and food. The diet contained two different types of graphene oxide (pure and ones contaminated with manganese ions). The graphene oxide nanoparticles were manufactured according to modified Hummer's method at the Wielkopolska Centre of Advanced Technology (Poznań, Poland). The level of manganese impurity in both GO samples was estimated using electron paramagnetic resonance method (EPR). According to our calculations, the concentration of manganese ions in the GO + Mn^{2+} sample reached 0.23 wt%. Some trace amounts of manganese ions were detected in the pure GO sample, but only at a low temperature (4.2–40 K). The molar concentrations of manganese ions for GO with a C/O ratio equal to 1.67 were 1.94 0^{-2} mol% in the GO + Mn^{2+} sample and ~1.26 0^{-4} mol% in the pure sample. The detailed characteristics of the GO samples used in this experiment as well as the AFM (Atomic Force Microscopy) images of the flakes are presented in Dziewięcka et al. [20] and Majchrzycki et al. [22].

The food with graphene oxide was prepared by grinding the standard food and mixing it with GO suspended in distilled water [23]. The final concentration of nanoparticles in the diet was set at 200 $\mu g\,g^{-1}$ for the GO and GO + Mn^{2+} groups (pure graphene oxide or graphene oxide with manganese ions were used, respectively). Such a concentration of GO was determined basing on the initial pilot tests, in which survivability and growth rate of the insects were assessed. Concentration of 200 $\mu g\,g^{-1}$ did not cause a significant increase in mortality but at the same time, the growth of insects slowed. The insects from the control group consumed uncontaminated food. Twenty-two randomly selected individuals were chosen at each of the five time points: 2, 4, 6, 8 and 10 days after the beginning of the experiment. The individuals were dissected and the hemolymph, gut and gonads of the males were isolated and prepared for further analysis. Stress parameters and the level of DNA damage were measured every two days. Health status of cells was checked at the beginning (second day) and at the end of the experiment (tenth day). A histological assessment of the gut and testis was performed after ten days of treatment (Scheme 1).

2.2. Stress parameters

The insects were lightly anesthetized on ice and then the hemolymph and gastrointestinal tract were isolated. A forty μL of hemolymph was mixed with an anticoagulant buffer at a 1:1 ratio. The gastrointestinal tract was homogenized on ice in a phosphate buffer (pH 7.4; 4 °C) and centrifuged in order to obtain a submitochondrial fraction [20,26].

2.2.1. Total antioxidant capacity assay (TAC)

The antioxidant capacity parameter (TAC) is the total amount of antioxidants that is present in the plasma and body fluids [24]. The level of TAC was measured through the decolourization of 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) at 734 nm, as was previously described by Dziewięcka et al. [20].

2.2.2. Catalase assay (CAT)

Catalase (CAT) [EC 1.11.1.6] plays the main role in the decomposition of hydrogen peroxide into water and oxygen in living organisms. CAT activity was assessed by measuring the rate of H_2O_2 removal from the samples. All of the procedures were carried out as first described by Aebi [25] with the minor modifications that were introduced by Dziewięcka et al. [20].