Research Article

Impact of Nanometric Iron Oxide in the Hepatopancreas of Terrestrial Gastropod *Helix Aspersa*: Histological Changes and Biochemical Parameters

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ABSTRACT

Iron oxide nanoparticles are of considerable interest for application in nanotechnology-related fields. However, as iron being a highly redox-active transition metal, the safety of iron nanoparticles need to be further studied. In this study, adult snails, *Helix aspersa* are used to estimate the effect of these nanoparticles on biochemical parameters and histological changes in the hepatopancreas of this gastropod has after a treatment of six weeks. During this period, snails exposed by ingestion and contact to wheat flour, which contains NPs powder. The doses of ferric oxide nanoparticles were 0, 1, 2 and 3 g/kg of wheat flour. Although the percentage of consumption reduced in a dose-dependent manner, it is still (90.26% for the control group and 75.17% for the group of the highest dose). The results of the biochemical assays (total carbohydrates, total proteins and total lipids) showed significant increases of total carbohydrates and total proteins at three doses (1, 2 and 3 g/kg of Fe_2O_3) and significant decreases of total lipids at two doses (2 and 3 g/kg of Fe_2O_3 nanoparticles). However, the histological examination of the hepatopancreas of snails showed alterations as a response to all treatments, narrowing of the tubular lumen degeneration of some digestive cells, tubule degeneration and necrosis of the connective tissue intertubular, starting from the second dose appearance of some inflammatory infiltrates, leading to a tissue severely damaged at the third dose (3g/kg of iron oxide nanoparticles).

Keywords: Nanoparticles, *Helix aspersa,* nano-iron oxide, hepatopancreas, biochemical study, histological study.

INTRODUCTION

Nanotechnology is considered the principal technology of the twenty-first century involving the use of new devices to study matter at the molecular or supramolecular level: the characteristic scales of nanotechnology range from 1 to 100 nanometers (nm). At these scales, matter acquires unexpected properties. It should be regarded as new chemical compounds whose characteristics and toxicities are different[1].

It is able to offer solutions to contemporary problems through smaller materials, lighter, faster and more efficient. The metal nanoparticles occupy an increasingly important in industrial processes and in biomedical research. However, the cellular responses to these nanoparticles may lead to toxic phenomena are still unclear and must be studied in detail and case-by-case[2]. Current toxicological data on NPs are still insufficient. However, several studies show that NPs can enter the body through the pulmonary tract, skin or intestinal[3] and have systemic toxic effects[4,5] their uses requires as with any new technology, impact studies on the health of humans and animals. Currently, there are no regulations on their use, and their long-term impact on the environment and humans is still unclear. However, the scientific community has focused very early on the subject and begins to provide some answers[6].

Because of their potential introduction into the soil as well as the aquatic environment, the inclusion of a set of ecotoxicity tests in the risk characterization of nanoparticles (NPs) is necessary. Only recently, however, has research focused on their impact on terrestrial organisms[7-11] and the literature on this topic is very limited. Among the metal oxides, our study looked to iron oxide. Their small size gives their advantageous properties, particularly magnetic[12] making them useful in many applications. Thus, in the nanometer range, some iron oxide structures such as magnetite (Fe_2O_4) or maghemite (γ-Fe_2O_3) have superparamagnetic properties. It has exhibited great potential for their applications as catalytic materials, wastewater treatment absorbents, pigments, flocculants, coatings, gas sensors, ion exchangers, magnetic recording devices, magnetic data storage devices, toners and inks for xerography, magnetic resonance imaging, bioseparation and medicine[13]. Although iron is an essential element for life, an increase in cell medium concentration can generate ROS formation[14].

However, in most of their applications, the iron oxide nanoparticles are in contact with the cells or the intracellular medium. It is, therefore, likely that nanoparticles interactions iron/oxide cells are not trivial. This is the case of magnetite nanoparticles, the toxicity toward human cells has been demonstrated[15-17]. The iron oxide nanoparticles used for biomedical purposes[18,19] is generally surface-functionalized via grafting of organic compounds[20-24]. This organic layer limits the direct contact between the iron nanoparticles and cell components. As indicated by the nature of the coating

plays an important role in the biological effects of nanoparticles.

The central model of this study is the little snail gray *Helix aspersa*, known for its ability to accumulate chemicals at significant concentrations in their tissues, particularly, the hepatopancreas[25,26]. By virtue of many works[27,28] it has been made possible to use the cellular alterations on the gastropods hepatopancreas as biomarkers for the exposure to xenobiotics. However, some information is available in the literature concerning the study of the biochemical and the histological markers gastropods, which are exposed to nanoparticles and especially Fe_2O_3, for example, Neenu[29], Hussain[30] reported the cellular toxicity induced by superparamagnetic Fe_2O_3 NPs. Further, Noori[31] observed reproductive effects of magnetic Fe_2O_3 NPs on mice.

The objective of this work is to study the effects of nanoparticles, Fe_2O_3 on the biochemical and histological parameters of the land snails *H. aspersa*, it is one of the most abundant gastropods in the North-East of Algeria.

MATERIALS AND METHODS

Chemical

The nanoscale iron oxide was developed in the laboratory of Magnetism and Spectroscopy of Solids (LMS2) of physics, the development of α- Fe_2O_3 nanoparticles was performed by high-energy mechanical milling, from the elemental powder hematite. Milling was carried out in a planetary by Fritsch mill type (P7), using two steel jars.

The preparation of the load (beads + powder) was performed in a glove box under an argon atmosphere. The weight ratio of beads/powders is about 1/20 and the grinding speed T at the order of 500 rev/min. To minimize the effects relating to the increase of the temperature inside the jars, the grinding was done with sequences of half an hour followed by 15 minutes of break and that for 3h.

Toxicological tests on the nanometer iron oxide are still insufficient and especially gastropods, according to results obtained by Nations[32] that were not significant and from the preliminary tests we fixed doses used in our treatment (Tab. 1).

Table 1: Group distribution in the basis of nanometric-iron oxide doses.

Group	Fe_2O_3 nanometric doses
C	Untreated
D1	Snails treated with 1g/kg of food
D2	Snails treated with 2g/kg of food
D3	Snails treated with 3g/kg of food

C: control, **D1**: dose 1, **D2**: dose 2, **D3**: dose 3.

Experimental design

Gastropod terrestrial snails (*Helix aspersa*) were collected from an uncontaminated site, situated in the North-East region of Algeria. Snails average weight of 8.86±1.15g were transferred to the laboratory where they will be adapted to the controlled conditions described by Gomot[33] (temperature 20±2°C, photoperiod 18 hL/6hO, humidity 80 to 90%) for almost a week. However, they were exclusively fed with wheat flour supplied in the Petri dish.

The 60 chosen snails were divided into four groups of 15 animals each and were reared in transparent plastic boxes (30×20×15cm) with a perforated lid and a wet sponge to retain moisture. The four groups of snails were fed with dry wheat flour (control snails), or fed with wheat flour contain the NPs powder according to as shown in table 1. The boxes were cleaned and the food was renewed three times a week. Under the controlled laboratory conditions previously mentioned, the experiment was done for six weeks and the physiological changes are notes (comportment and mortality).

Biochemical dosages

The determination of biochemical parameters (total proteins, total carbohydrates and total lipids) has been realized from the hepatopancreas of 13 specimens chosen from each group (control and treated). The methods used are the Method of Duchateau and Florkin[34] for quantifying total carbohydrates, the method of Bradford[35] for total proteins and method of Goldsworthy[36] for total lipids. The extraction of carbohydrates, lipids and proteins according to Shibko[37]: on a fragment of the hepatopancreas homogenized with trichloroacetic acid (TCA) in 20%. The amount of metabolites were measured in an aliquot of 100µl. Dosages were expressed as µg/mg of the analyzed tissue.

Histology

The histological sections were performed on fragments of three specimens for each group (the control and the treated groups). These glands were fixed in the liquid of Bouin[38] for 24 h.

Samples were prepared for analyzes after several stages of rinses in dematerialized water, dehydration in baths of alcohol in increasing degree and impregnation in a bath of paraffin wax[39]. Then, the samples of hepatopancreas were included in the paraffin by means of molds (bars of leuckart).

Finally, the paraffin sections were cut into 5µm slices by a Leitz microtome and stained with hematoxylin and eosin for light microscopic examination[40]. The sections were viewed and photographed.

Statistical analysis

Data were calculated as Mean ± SD and analyzed using Student's test followed by the analysis of variance (ANOVA) in one way of classification.

The probability of 0.05 or less was considered significant. All statistical analysis was done according to the software MINITAB of analysis and data processing version 17.Ink.

RESULTS

Comportment

The continues followed of the movement and the feeding consumption of snails treated with the three doses of nanometric Iron oxide (1, 2 and 3 g/kg) and after comparison with those of the control we noticed; a decrease with dose-dependent manner of the nutritional attraction and activity, and a decrease of the mobility also with a dose-dependent manner.

At the dose 1 g/kg, most snails showed an activity almost identical to that of the control ones with little disruption of feeding consumption and comportment. However, snails treated with 2g/kg dose consume less flour and move more slowly than the control. The signs are more apparent in animals treated with high dose (3 g/kg). Snails spend most of their time at the top of the boxes, isolated from each other, with low nutritional activity.

Percentage of consumption

In order to calculate the percentage of consumption, it is necessary to dry and weigh the flour residue by using a precision balance, after each box cleaning and food change. At the end of the experiment, the amount of residuals for each group is eliminated from the total amount of six weeks of each group experience (Tab.2).

Table 2: Effects of Fe_2O_3 nanoparticles on the consumption of flour.

Group	Residues weight	% of consumption
C	26.6 g	90.26 %
D1	43.1 g	85.49%
D2	55.8 g	81.27 %
D3	72.9 g	75.17 %

C: control, **D1**: dose 1, **D2**: dose 2, **D3**: dose 3.

Biochemical parameters

The results of effects of the different doses of iron oxide nanoparticles (1, 2 and 3g/kg) on digestive gland constituents (total proteins, total lipids and total carbohydrates) in *H. aspersa* which are obtained after six weeks of treatment are summarized in Figures 1a, 1b and 1c. As compared to the control group, biochemical analysis revealed significant differences in the rate of different metabolites in snails exposed to iron oxide nanoparticles.

We observed a highly significant increase in total proteins concentration in snails exposed to 1 and 2 g/kg of iron oxide nanoparticles and a very highly significant increase in the dose 3 g/kg (fig. 1a) as compared to the control value. The figure 1b shows the carbohydrate rate with a very highly significant increase for the three treatment groups (by 1, 2 and 3g/kg of Fe_2O_3 nanoparticles) as compared to the control value. Concerning the content of total lipids which was not significantly reduced for the low dose; it was reduced in a very highly significant for the medium and the highly doses of iron oxide nanoparticles (fig. 1c) as compared to the control value.

Histopathology

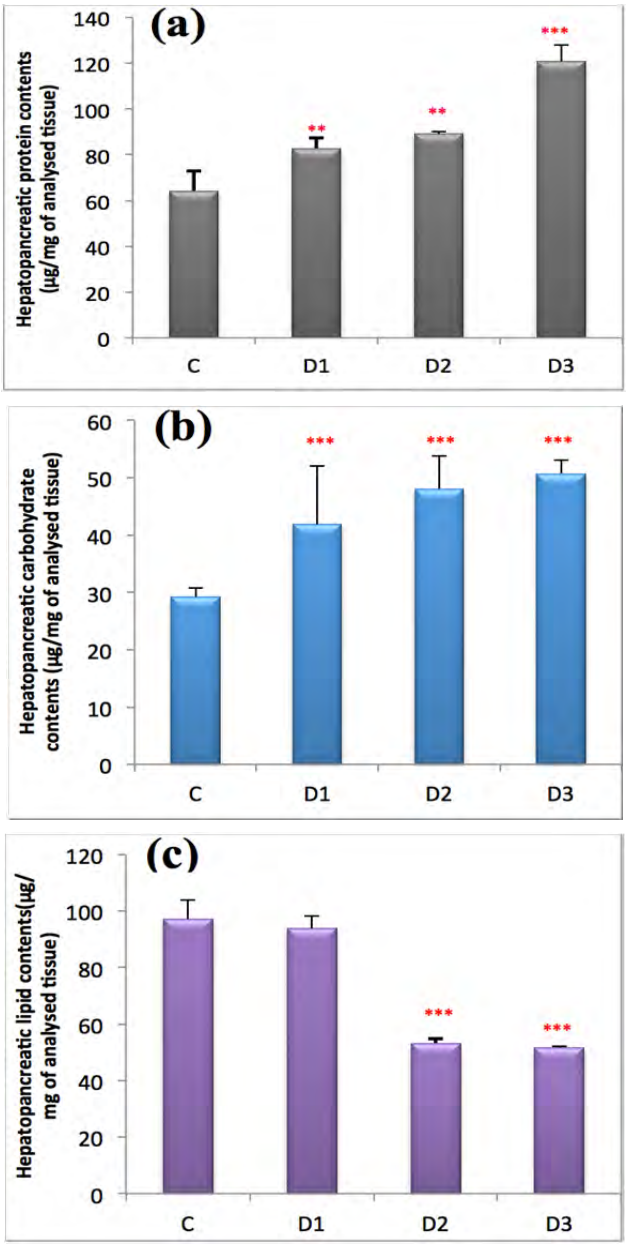

Figure 1: Concentrations of total contents (µg/mg of tissue analyzed) of proteins (a) carbohydrates (b) and lipids (c) in the hepatopancreas of *H. aspersa* after six weeks of exposure to 0, 1, 2 and 3 g/kg, of iron oxide administered by nanoparticles ingestion. Results are expressed as mean ± standard deviations (SD), n = 13.

**: Highly significant difference compared to the control (P ≤0.01).

***: Very highly significant difference compared to the control (P ≤0.001).

The digestive gland of normally feeding (untreated) *H. aspersa* consists of essentially the juxtaposition of numerous digestive tubules that have various shapes and sizes and are separated by intertubular space containing hemolymphatic sinuses and hemocytes. Circular muscle layer surrounds each tubule. A simple epithelium of several cellular types lines the lumen of the tubules. These cells have various morphologies, but they have three main cellular types and each cell has the essential components (membrane and nucleus). Digestive cells constitute the most abundant cellular component of the digestive gland tubular epithelium they are relatively polymorphic according to the stage of digestion[41], calcium and excretory cells are fewer than digestive cells[42] (fig. 2a).

At the end of the study, microscopic examination of histological sections snails treated with three doses (1, 2 and 3 g/kg) showed tissue changes by varying degrees with a dose-dependent effect. In the dose 1 g/kg of iron oxide nanoparticles (fig. 2b), partial degeneration of some digestive cells and intertubular connective tissue more important and more extends are observed, in addition, the regrouping tubules due to narrowing of the lumen which is accompanied by necrosis at the basement membrane. Also, at a dose 2g/kg (Fig. 2c), the same changes were observed, accompanied by breakage of the basal membrane in a dose-dependent manner and almost absent tubular lumen the connective tissue is too altered and the appearance of some inflammatory infiltrates. At a dose of 3g/kg (Fig. 2d), connective tissue, digestive tubules and their membranes are severely damaged with a highly advanced degeneration of digestive and calcium cells and the appearance of inflammatory infiltrates.

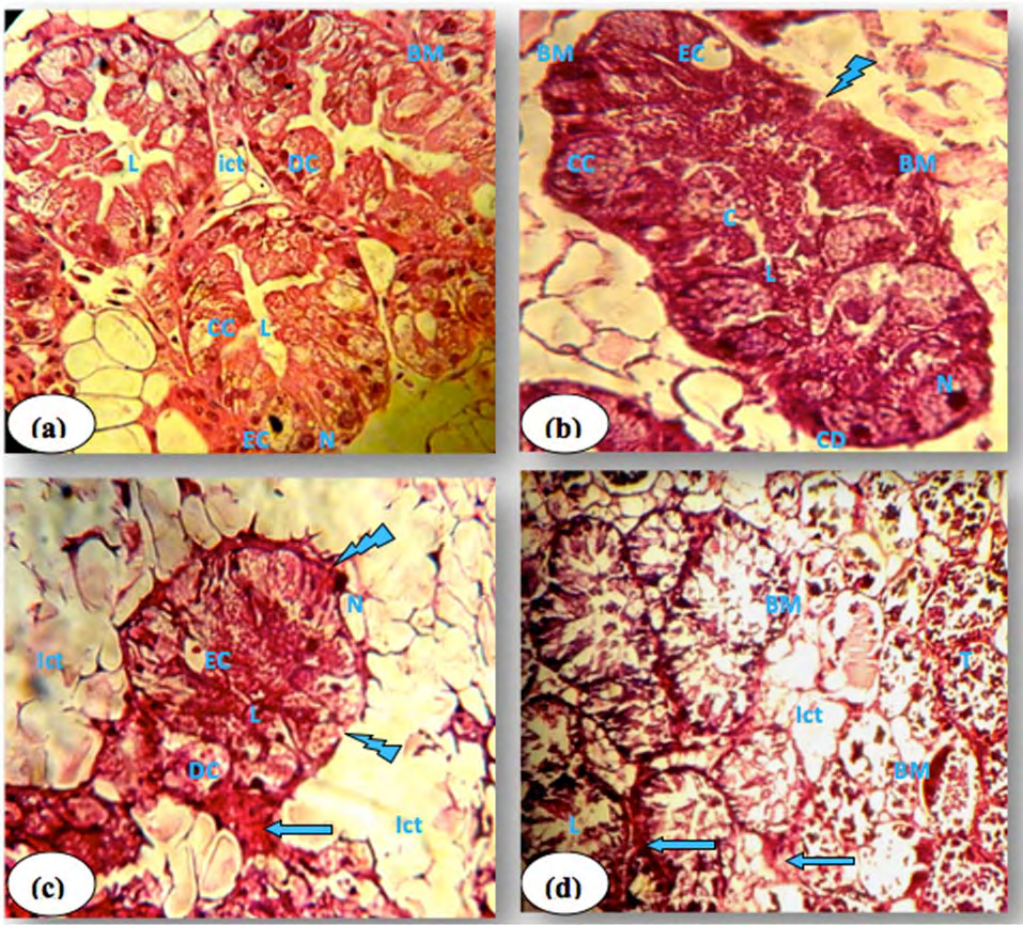

Figure 2: Histological pictures of the midgut gland of snails of *H. aspersa* in control and treated groups (with three doses 1, 2 and 3g/kg of iron oxide nanoparticles) stained with hematoxilyn-eosin coloration. (L) digestive tubule lumen; (DC) digestive cells; (CC) calcium cells; (EC) excretory cells; (Ict) intertubular connective tissue; (eg) excretory granules; (BM) basement membrane; (N) nucleos. **(a)** Control snails section, untreated showing the juxtaposition of numerous digestive tubules of various shapes and size. **(b)** hepatopancreas section of snails treated with 1g/kg of Fe_2O_3 nanoparticles showing a narrowing of tubular lumen partial degeneration of some digestive cells and tubule necrosis ( ). **(c)** hepatopancreas section of snails treated with 2g/kg of iron oxide nanoparticles, showing a remarkable degeneration of the intertubular connective tissue with a complete absence of the tubular lumen, a tubular necrosis with the appearance of some inflammatory infiltrates ( ). **(d)** hepatopancreas section of snails treated with 3g/kg of Fe_2O_3 nanoparticles, showing tissues severely damaged a very remarkable inflammatory infiltrates and especially in the basal membrane.

DISCUSSION

Biochemical parameters in species exposed to toxicants are good biomarkers and can constitute an important diagnostic tool to assess the exposure and effects of xenobiotics[43,44]. The results of biochemical parameters dosage show an increase of proteins and carbohydrates level, a decrease of lipids level.

Proteins are the first biomarkers of metabolic disruption. The evolution of the rate of total protein in the hepatopancreas of treated snails was increased (with very highly significant differences) and was dose-related. This phenomenon could be considered an early biomarker of exposure to chemical contaminants. Proteins are mainly involved in the architecture of the cell; proteins may also bind toxins and function as transport proteins[45].

When among living organisms are subject to changes in their environment, they are submitted to intense stress, causing the death of organisms without the latter can react particularly when their detoxification enzymes are decreased, this stress may be less intense, allowing then the organization to deploy a battery of responses, through the activation of their detoxification mechanisms, to fight, to survive and in some cases, to acclimate to this new parameter[46].

The synthesis of total proteins involves several event in particular the induction of the enzymatic activities: detoxification enzymes/metabolization. This increase can be explained also by Kohler[47] hypothesis that said metal oxides trigger the synthesis of stress proteins (enzymes of detoxification).

From this concept and the result obtained by Zhu[48] that found a significant increase in protein levels in rats exposed to different concentrations of nanometric iron oxide, our result is proved to be true. In addition, Masaya's[49] results of the toxic effect of chemical stress among different biological models, Grara's[28] that showed a variation in snails treated with heavy metals, and in the same order of ideas Boucenna's[50] results that showed toxic manifestations of a metal dust on *Helix aspersa,* those results were accompanied by a dose-dependent induction of protein synthesis.

The presence of metals may also induce the synthesis of proteins enabling the connection between them[51] the transfer of a part of nanometric iron oxide in granules corresponds to intracellular detoxification pathways described in soil invertebrates. Metals tend to bind to cytoplasmic metalloproteins. Metals-metalloproteins can then be excreted in pellet form after lysosomal action[52].

Then the total carbohydrates for the treated groups showed a significant increase by contribution to the control group, this increase can be explained by several hypotheses as the increase in energy reserves which is proved by the feed consumption percentage which is decreased compared to the control with dose-dependent manner but not significantly (more than 75% of the feed is consumed for the three treated groups). Whereas the decrease of the total lipids confirms that. To ensure the necessary energy for the body to fight against a xenobiotic they should be used spare elements and degrade them to increase the rate of glucose, the energy producing a key molecule which is essential in the detoxification reactions. For the same species, Jumel and Lagadic[53] report an increase in energy demand of animals that results in rapid mobilization of glycogen of the mantle. Our work is in agreement with that of Saravanan[54] that have been proposed a hypothesis that it said the elevation of blood glucose level may be a response to respiratory disturbances due to the stress caused by Fe_2O_3 NPs.

Thus, for the lipid levels, our results show a dose-dependent decrease in snails treated with nanometric iron oxide compared to control snails. Our results are in agreement with those of Grara[28] that recorded a significant decrease in lipid concentration *Helix aspersa* to heavy metals.

Under stress conditions, the snails need more energy to detoxify the toxicant. Indeed, the dose-dependent decrease of lipid levels after exposure of *Helix aspersa* to nanometric iron oxide may be due to the chemical stress caused by xenobiotic tested. However Eissa[55] reported that the detrimental effect of chemical compounds could be attributed to the increased use of energy and/or the alteration of cell organelles (treated snails) and can interfere with protein synthesis.

Histological examination of the hepatopancreas of *Helix aspersa* treated with nanometric iron oxide highlighted important qualitative changes since the lowest concentration tested. Indeed, nanoparticles exposure can cause very significant cytological alterations in hepatopancreas, which plays a crucial role in the detoxification of pollutants[56]. In the first time, we have revealed some digestive cells and their nuclei became highly dilated resulting in a very narrow tubular lumen (fig. 2b, c) this result is in agreement with that of Dumee[57]. The histological study revealed also, degeneration of digestive tubules, fragmentation of digestive cells and ruptures at the basement membrane of tubules with dose-dependent manner, leading to a severe deterioration in the tissue of the digestive gland at the highest concentrations (fig, 2d). The deterioration of digestive cells entail, therefore, altering the global digestive process caused by ingestion of the nanoparticles, this could be a first biological response due to the presence of xenobiotics, these observations are in agreement with the work of Boucenna, Russell and Chabicovsky[50,58,59].

Note that the digestive cells are most abundant in the epithelium of the hepatopancreas according to Chabicovsky[59] the loss of digestive cells seems to be a general response after exposure to heavy metals in terrestrial gastropods and is connected mainly to the

deterioration of the digestive process caused by the presence of metal particles[42].

Whereas Zhu[48] in his comparison between the submicron-sized and the nano-sized of iron oxide particles, it has been found that iron oxide nanoparticles cause cell lysis at the rat lungs and also increases microvascular permeability, it is hypothesized that these cellular alterations caused by transition metal or its oxide particle exposure may be due to the induction of reactive oxygen species (ROS) and reactive nitrogen species (RNS). We demonstrated that the nano- and submicron-sized iron oxide particles could generate inflammation and mediate oxidative stress.

These results are in accordance with those of Triebskorn and Köhler[60,61] which have revealed structural alterations of basophils and digestive cells of the digestive gland of *D.reticulatum* slug exposed to Cd, Pb and Zn and also that of Manzl[62] they equally observed the acute toxicity of the metal oxides on the cells of the hepatopancreas on the species of *H.pomatia*. Thus, in *Helix pomatia*, an isoform is rapidly induced following exposure to Cd, binder 85% to 95% of this ETM in the hepatopancreas[63]. An *in vitro* study has shown that the metallic oxide exposure causes inhibition of nucleotidases activities in the hepatopancreas *H.aspersa*[64]. Yager and Harry[65] say the high diffusion of ETM in the cells causing cellular necrosis; it is obvious to say that the necrosis observed the result of NPs that have a smaller size than the ETM easier to broadcast.

Therefore, it seems obvious that the nanometric iron oxide has a very important cytotoxic potency, which is not very different from that of heavy metals macrometric. On the other hand, the structural changes observed at the higher concentrations could be due to starvation caused by the repulsion of contaminated food and thus, by induced aestivation[66].

The nanoscale iron oxide may present everywhere, and snails can be in contact with him all lifetime. Our study is a sub-chronic experimental approach in the laboratory conditions can be inadequate to generalize the results obtained. It may be important to test whether these responses are alike[67], because Wu[68] concluded that after laboratory experiments while some biomarkers like enzyme induction and lysosomal integrity are clearly reversible after pollution reduction, other responses like cell damage and pathology may be permanent and not reversible. In the future, it is important to elucidate this issue by playing on biomarkers; the xenobiotic concentrations and sensitivity of the biological model, the parameters that affect the recovery time exchange[68]. It would be interesting to quantify iron oxide nanoparticles and its metabolites in snail's tissues to elucidate the effects of this nanoparticle on land snails.

CONCLUSION

We showed at the end of this work that the sub-chronic exposure of *Helix aspersa* to iron oxide nanoparticles is not sufficiently different to that of macrometric metallic oxides. With selected doses we revealed significant increases of detoxification enzymes and proteins transport (rate of total proteins) decrease in energy needs (rate of total carbohydrates and total lipids), and remarkable alterations on the hepatopancreatic tissue due to the induction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which induces by the histo-accumulation of the iron nanoparticles in the individuals of land snail *H. aspersa* exposed to these nanoparticles. We have not confirmed the degree of toxicity of iron oxide nanoparticles only but we also confirmed *Helix* is an excellent bioindicator and bioaccumulation of environmental degradation.

The results of this study highlight the need for safe disposal and release of metallic NPs in ecosystems. In addition, *Helix aspersa* species are edible for humans; it should pay attention on the origin of snails harvested in the wild, so it could contaminate humans.

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