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Sana Besnaci

Cellular Toxicology Laboratory,
Department of Biology,
Faculty of Sciences, Badji-Mokhtar
University, Annaba, Algeria

Samira Bensoltane

a) Cellular Toxicology Laboratory,
Department of Biology,
Faculty of Sciences, Badji-Mokhtar
University, Annaba, Algeria
b) Faculty of Medicine, Badji-
Mokhtar University, Annaba,
Algeria

Fatma Moulka Hadjira Braia

Environmental biomonitoring
laboratory, Department of Biology,
Faculty of Sciences, Badji-Mokhtar
University, Annaba, Algeria

Labiba Zerari

Cellular Toxicology Laboratory,
Department of Biology,
Faculty of Sciences, Badji-Mokhtar
University, Annaba, Algeria

Sihem Khadri

a) Applied Biochemistry and
Microbiology Laboratory,
Department of Biochemistry,
Faculty of Sciences, Badji-Mokhtar
University, Annaba, Algeria
b) Sciences of Nature and Life
department, Faculty of Sciences,
20 Aout 1955 University, Skikda,
Algeria

Hawa Loucif

Cellular Toxicology Laboratory,
Department of Biology, Faculty of
Sciences, Badji-Mokhtar
University, Annaba, Algeria

Correspondence

Sana Besnaci

Cellular Toxicology Laboratory,
Department of Biology,
Faculty of Sciences, Badji-Mokhtar
University, Annaba, Algeria

Embryotoxicity evaluation of iron oxide Fe₂O₃ on land snails: *Helix aspersa*

Sana Besnaci, Samira Bensoltane, Fatma Moulka Hadjira Braia, Labiba Zerari, Sihem Khadri, Hawa Loucif

Abstract

In order to evaluate an ecotoxicological approach to assessing toxicological effects caused by iron oxide powder (Fe₂O₃) nanoparticles, we conducted a bioassay with helix as a biological model since the species is considered bioaccumulator and bioindicator of pollution. In this study, we evaluated the toxicity of Fe₂O₃ nanoparticles on the embryonic stage of *Helix aspersa* with different concentrations (1.25mg/ml, 1.5mg/ml, and 2mg/ml). Results reveal a deformation of the egg membrane and accumulation of this molecule at the back of the egg. We have also noted a low rate of hatching in 12th day, the mortality rate is found to be high at the highest concentration of Fe₂O₃. This bioassay highlights the toxicity of Fe₂O₃ nanoparticles on eggs of land snail: *Helix aspersa*.

Keywords: Iron oxide, nanoparticles, *Helix aspersa*, embryotoxicity, eggs

Introduction

With the tremendous growth in the field of science, nanotechnology has garnered a great interest in last decades and nanobiotechnology has come up as a major interdisciplinary subject. The development and application of nanotechnology have the potential to improve greatly the quality of life [1].

Iron oxide nanoparticles (NPs) are of considerable interest due to their wide range of applications in fields such as magnetic storage, chemical industries, water purification and medicine [2]. In addition to the nano-sized iron oxide, maghemite (γ-Fe₂O₃) and magnetite (Fe₃O₄) are the most commonly used magnetic NPs for biomedical applications because of their biocompatibility and suitable superparamagnetic properties [3]. The most prominent application of iron oxide NPs might be the use as a contrast agent in magnetic resonance imaging (MRI) [4, 5]. The dramatic growth and the therapeutic benefits that superparamagnetic iron oxide NPs have to offer, accompanies the risks and concerns associated with their exposure [6]. Therefore, there is a considerable need to address biocompatibility and biosafety concerns associated with their usage in a variety of applications [1].

The unique physicochemical properties of nanomaterials have a great positive impact on biomedical applications; the same properties can have a negative impact on the biosystem [7]. Therefore, as any new nanomaterial can have an impact on the biological environment with consequences in the medical field, a series of toxicology studies should be carried during its development process [3].

Toxicity is usually determined by animal experiments according to the guidelines of the OECD (Organization for Economic Cooperation and Development). *Helix aspersa* is one of bioindicator toxicity species, whereas, the preferred choice of this species is mainly due its bioaccumulation capability for many metal pollutants to its global distribution, reflecting its ability to adapt to habitats, soil and varied climates, and ease rearing [8]. However, in this bioassay, our choice fell on contamination during the embryonic stage, because whatsoever the studies relating to the aquatic or those concerning terrestrial environment, all emphasize the benefits of the embryo as a model for evaluating the toxicity of contaminants [9-11]. Indeed, in addition to being an alternative to testing of animals, these bioassays may be considered subchronic toxicity bioassays because they extend over a full stage of the life cycle, which is the embryonic development [1]. Several parameters can be measured: malformations, development time, heart rate, hatching success. No standardized terrestrial bioassay exists to Really assess the embryotoxicity of pollutants for soil invertebrates, and only a few Experiments with metals or organic compounds have been performed [12].

The objective of this report is to provide a toxicological approach to nanoscale iron oxide by tree concentrations on embryonic development of "*H. aspersa*". By monitoring of eggs just after laying during the incubation period by observation of the general appearance of the eggs: the shape, color, and same size. After 14 days of incubation, hatching percentage was calculated after counting the eggs hatched in the 12th and 14th days. We have followed the neo-hatched for 28 days after hatching and calculated the mortality rate.

Material and Methods

Our work was conducted at the cellular toxicology laboratory of Badji Mokhtar University Annaba-Algeria. The Studied species are adults of snail collected from non-contaminated site in the North -East of Algeria, during October month. The incubation was carried for almost two months; we started our testing in late January.

Chemical

The Fe_2O_3 NPs was developed in the LMS2 (Magnetism and Spectroscopy of Solids laboratory) physics department in Badji Mohkhtar university (Annaba-Algeria), the development of α - Fe_2O_3 nanoparticles was performed by high-energy mechanical milling, from the elemental powder hematite. By two steel jars, Milling was carried in a planetary by Fritsch mill. Under an argon atmosphere, the preparation of the load (beads + powder) was performed in a glove box. The weight ratio of beads / powders is about 1/20 and the grinding speed on the order of 500 revs / min. the grinding was done with sequences of half an hour followed by 15 minutes of break and that for 3h, in order to minimize effects relating to the increase of the temperature inside the jars.

According to results obtained by Nations *et al.*, [13] that were

not significant and from the preliminary tests, we fixed doses used in our study (Tab. 1).

Table 1: Distribution of batches according to nanometric- iron oxide concentrations.

Group	Nano Fe_2O_3 concentrations
C	Untreated
C1	Snails eggs treated with 1.25 mg/ml
C2	Snails eggs treated with 1.5 mg/ml
C3	Snails eggs treated with 2mg/ml

C: control, C1: concentration 1, C2: concentration 2, C3: concentration 3.

Experimental design

Gastropod terrestrial snails (*H. aspersa*) were collected from an uncontaminated site, for results in standards. A breeding snails established to the controlled conditions described by Gomot [14] (temperature $20 \pm 2^\circ\text{C}$, photoperiod 18 hL/6hO, humidity 80 to 90%) they were exclusively fed with lettuce. A wet sponge is placed in the box in order to ensure the necessary moisture, and in the same boxes, we have placed small plastic boxes filled with potting soil so that snails after coupling can lay eggs inside the soil. The couplings carried under the conditions already mentioned. Clutches obtained are identified [10, 12].

From the very first day of laying, we have divided into four batches each consisting of 16 eggs (Table 1). Petri dishes were prepared for incubation of eggs as follows: we put in each dish three layers of absorbent paper (Whatman, 10 cm diameter) and we impregnated them by the above-mentioned solutions, the tubes were shaken to obtain homogeneous solutions especially before use. 16 eggs were placed in each dish and incubated under favorable conditions to the hatching (fig 1).

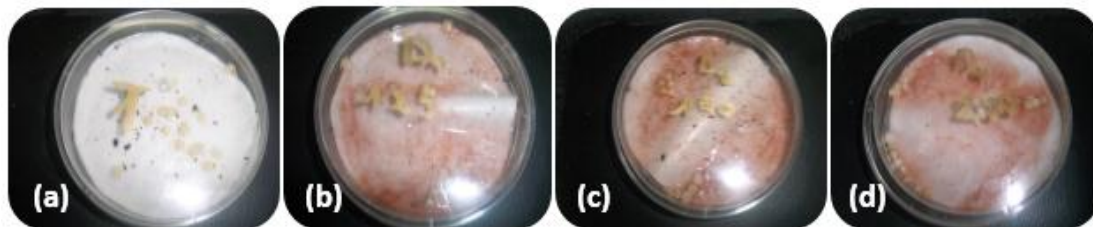


Fig 1: distribution of groups: (a) Petri dishes of control group, (b) Petri dishes of treated with 1.25 mg/ml of Fe_2O_3 NPs, (c) Petri dishes of group treated with 1.5 mg/ml of Fe_2O_3 NPs, (d) Petri dishes of group treated with 2 mg/ml of Fe_2O_3 NPs.

Results

As the eggs are opaque, observations are much more about the shape, egg size and color of the shell as NPs tested have a bright color, and their accumulation is easily observed. Embryos were observed with a binocular microscope equipped with a camera to take photographs at the same time.

We observed some modifications from the 5th day, and therefore, we took photographs the 5th (fig 2), the 8th (fig 3),

the 10th (fig 4), the 12th (fig 5) and 14th day (fig 6).

The counting of hatched eggs is done for each group the 12th day after laying and also the 14th day (two days extension for hatching). Table 2 summarizes the results.

The 28 days after hatching are included in the embryonic stage, the neo hatched are not yet juveniles. The results of the dead counts in this interphase are shown in Table 3.

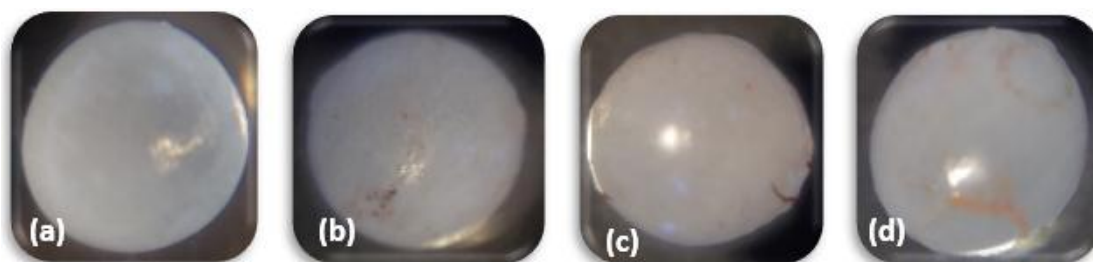


Fig 2: The development of eggs at the 5th day: (a) egg from control group: white and shiny egg regular shape, (b) egg from group treated with 1.25 mg/ml of Fe_2O_3 NPs, (c) egg from group treated with 1.5 mg/ml of Fe_2O_3 NPs, (d) egg from group treated with 2 mg/ml of Fe_2O_3 NPs (b, c and d: the same observations, deformation of the membrane and traces of molecule (Fe_2O_3 NPs)).

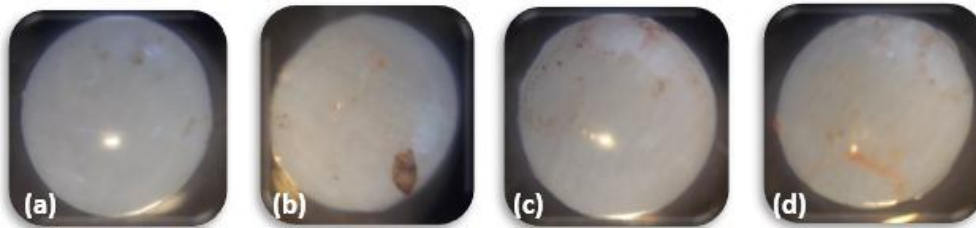


Fig 3: The development of eggs at the 7th day: (a) egg from control group nothing to report, (b) egg from group treated with 1.25 mg/ml of Fe₂O₃ NPs, (c) egg from group treated with 1.5 mg/ml of Fe₂O₃ NPs, (d) egg from group treated with 2 mg/ml of Fe₂O₃ NPs, (b, c and d the same observations with dependent concentration: deformation of the egg shape "irregular membrane", with accumulation of Fe₂O₃ NPs (rust color).

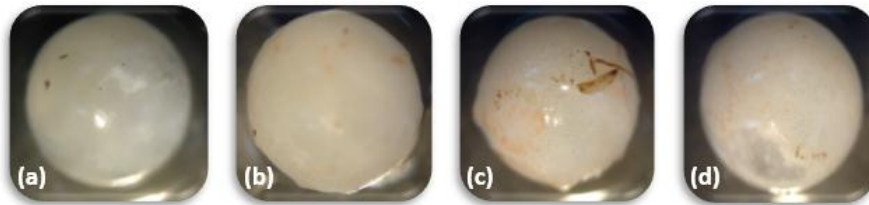


Fig 4: The development of eggs at the 10th day: (a) egg from control group nothing to report (normal color and shape), (b) egg from group treated with 1.25 mg/ml of Fe₂O₃ NPs, (c) egg from group treated with 1.5 mg/ml of Fe₂O₃ NPs, (d) egg from group treated with 2 mg/ml of Fe₂O₃ NPs (b, c and d the same observations with dependent concentration: deformation of the membrane, accumulation of Fe₂O₃ NPs).

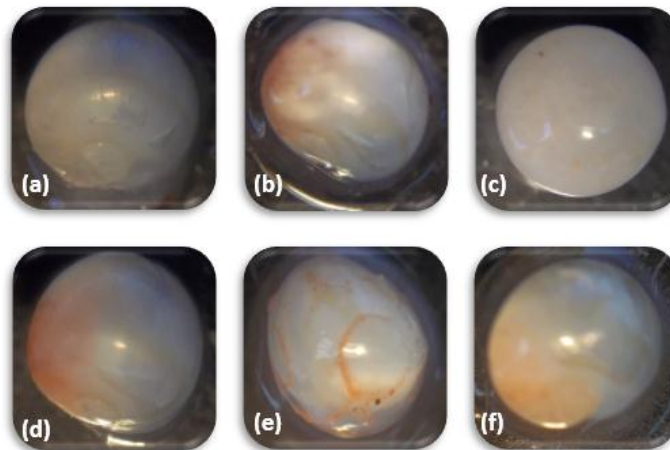


Fig 5: The development of eggs at the 10th day: (a) snail from control group: transparent shell, (b) snail from group treated with 1.25 mg/ml of Fe₂O₃ NPs, (c) egg from group treated with 1.5 mg/ml of Fe₂O₃ NPs: deformation of membrane, (d) snail from group treated with 1.5 mg/ml of Fe₂O₃ NPs, (e) egg from group treated with 2 mg/ml of Fe₂O₃ NPs: malformation of membrane, (f) snail from group treated with 2 mg/ml of Fe₂O₃ NPs (b, d and f: accumulation of Fe₂O₃ NPs on shell).

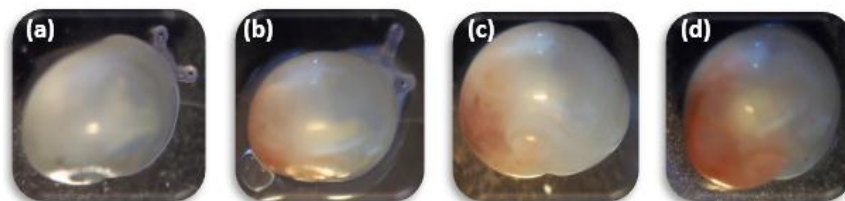


Fig 6: The development of eggs at the 14th day: (a) snail from control group with a transparent shell, appearance of the eyes, (b) snail from group treated with 1.25 mg/ml of Fe₂O₃ NPs the appearance of the eyes, accumulation of Fe₂O₃ on the back of the shell, (c) snail from group treated with 1.5 mg/ml of Fe₂O₃ NPs: accumulation of Fe₂O₃ on the back of the shell, (d) snail from group treated with 2 mg/ml of Fe₂O₃ NPs: the same observations as "c".

Table 2: Number of hatching eggs in the 12th and 14th day.

	C	C1	C2	C3
12th day				
Hatched	16	14	12	11
Unhatched	00	02	02	05
Decomposed	00	00	02	00
14th day				
Hatched	16	15	13	14
Unhatched	00	00	00	02
Decomposed	00	01	03	00

Table 3: Mortality rate at 28th day.

	C	C1	C2	C3
08 th day	0%	18.75%	18.75%	06.25%
18 th day	0%	18.75%	18.75%	12.5%
28 th day	0%	31.25%	18.75%	12.5%

Discussion

Very little work has been found on the toxicological effect of NPs Fe₂O₃ compared with the micrometer metal oxides and even compared to the TiO₂ and ZnO NPs and this is what we

left cautious about the explanation of our results.

Firstly, we were interested at the toxicity of nano Fe_2O_3 on the evolution of the development of *H. aspersa* (from laying until hatching), based on the study of Gimbert ^[15] who has shown that, often from metallic nature, xenobiotics which penetrate without any difficulty inside the cell which can accumulate and generate cytotoxic process. Embryotoxicity bioassays proved the advantage of this early stage of the life cycle, as shown by Druart *et al.* ^[10, 12].

Lacoue-Labarthe *et al.* ^[16-18] studies about bioaccumulation of ten metals in eggs of cuttlefish showed that, depending on the metal and the moment at which the eggs are exposed, the metal cross or not the shell of the egg. In contrast to our hypothesis, these authors show that during the early embryonic stages, metals are bonded to the shell and are not in contact with the embryo. Then after organogenesis, the shell becomes permeable and metals can exert their toxicity on embryo. Other authors also estimate that egg shell or albumen form a bulwark to the embryo against the toxicity of contaminants ^[19, 20], showing that the embryonic stages are less sensitive than the larval stages of the organism ^[21, 22, 19, 17]. However, Druart *et al.* ^[10] showed that seven days of embryonic development (the beginning of the larval stage), the Cd had already crossed the barrier of the egg may well reach the embryo and exert its toxicity. In general and especially in certain aquatic species (snails, fish), early stages (larvae, embryos) are more sensitive than the juvenile or adult stages ^[23-26].

Ahmad *et al.* ^[27] showed extensively high toxicity due to aggregation / agglomeration and mechanical damage by NPs (CoFe_2O_4). Larger agglomerates of Fe_2O_3 are more toxic to the lipid membranes because of higher affinity for membranes and higher cytotoxicity ^[28, 29]. Adhesion of aggregates, sedimentation, internalization of NPs and ions were also main contributing factors in inducing developmental toxicity in Zebrafish embryos ^[30]. Accompany the aggregation and sedimentation of Fe_2O_3 NPs and with the characteristics of nanoparticles, the direct adherence/adsorption of Fe_2O_3 NPs aggregates could be observed on the surface of embryos (Fig. 5) and this direct adherence/adsorption may cause depletion of oxygen exchange, resulting in hypoxia of embryos on exposure; this has been reported to cause delayed hatching and development of embryos, may also cause excessive production of reactive oxygen species (ROS) *in vivo*, resulting in oxidative stress for the embryos, which may be critical in inducing the observed developmental toxicity. Another study conducted by Aerle *et al.* ^[31] on silver NPs causes strongly marked malformations of the zebrafish embryo by using a Next Generation Sequencing approach in an Illumina platform (High-Throughput Super SAGE), a significant alterations in gene expression were found for all treatments and many of the gene pathways affected, most notably those associated with oxidative phosphorylation and protein synthesis. They provide that this toxicity is associated with bioavailable silver ions in exposed zebrafish embryos, that ions may cause like Singh *et al.* ^[32] in his hypothesis, a lead to an imbalance in homeostasis and aberrant cellular responses, including cytotoxicity, DNA damage, oxidative stress, epigenetic events, and inflammatory processes, which would eventually lead to the observed toxicity. An experiment on a pest slug, reporting the high sensitivity of eggs to metal salts and it is a confirmation of Aerle *et al.* results about ions metal effects ^[33]. Druart *et al.* ^[10, 12] showed for Cd; a significant transfer from exposure medium to eggs was emphasized, particularly affecting the albumen. Abnormalities of embryogenesis in non-hatched embryos depended on the substance and the concentration

considered.

Since their appearance, many researchers have studied the influence of the physicochemical characteristics of nanomaterials than on their toxicity. Therefore, the toxicity may vary according to the synthetic agents ^[34], the nominal diameter ^[23 35], and shape ^[36, 37] or coating ^[38]. According to nanomaterials, some or all of the toxic effects can be attributed to the dissolution of nanomaterials in the environment ^[38-42]. In addition, some studies clearly show that the nanoparticulate form is more toxic than the ionic form or the micrometric one ^[39, 42, 43].

Oxidative stress defines the potential of ROS to damage cellular components such as biomembranes, proteins, DNA and RNA ^[44]. The implication of oxidative stress has been extensively demonstrated as the mechanism responsible for nanomaterials toxicity ^[45, 46]. This effect is particularly well illustrated in a study of the impact of TiO_2 NPs on carp juveniles ^[47].

Ireland *et al.* ^[48] showed that TiO_2 and Al_2O_3 nanometric are redox-active transition NPs, which interfere with the metabolism of proteins by the formation of reactive oxygen species (ROS) that lead oxidative stress and cause cellular damage. The contact between nanomaterials and organisms can cause direct toxic effects or indirect effects (decreased of nutrition after adsorption of NPs on organism exchange surfaces) ^[49].

The impact of NPs on aquatic organisms like freshwater snails ^[50, 51], on chironomid larvae ^[52, 53], on cnidarians ^[54] and on polychaetes ^[55] had also been studied showing toxic effects through a reduction in nutrition and an increase in the number of malformations, oxidative stress, DNA damage correlated with an increase in mortality.

The deformation of the membrane and swelling of the eggs treated with different concentrations of nanoscale iron oxide is a consequence of their penetration through the membrane, which is accumulated in the cells. Nanoparticles are taken up by cells through different mechanisms, such as endocytosis, phagocytosis, and pinocytosis ^[56, 57]. According to Marigomez, *et al.* ^[58]: the transfer of the metallic elements, through the cell plasma membranes, is mostly effected by passive diffusion or by mechanisms necessitating energy such as transport by membrane proteins, specific or not, or by endocytosis of the molecules. They may agglomerate and reside in the cytoplasm or gets entry into the nucleus as a single particle through nuclear pore membrane ^[59]. Interestingly, they can also be deposited into various organelles, such as the lysosome, mitochondrial matrix, and endoplasmic reticulum ^[56, 57, 60-62]. Numerous reports have flourished its existence in the endolysosomal compartment ^[60].

The accumulation of the molecule at the back of the shell is not a coincidence, indeed, it is the location of the hepatopancreas, according to the work of Oberdörster *et al.* ^[7] showed that nanoparticles could cross the protective barriers, distribute themselves into the organism and accumulate in certain organs, mainly in the respiratory or digestive exposure. The digestive gland is the most important gastropod organs involved in pollutant detoxification ^[63]. The histological and histochemical changes are expected to be useful biomarkers of metal oxide nanoparticles exposure ^[64]. Thus, in *H. pomatia*, after exposure to Cd, 85% to 95% of this ETM were found in the hepatopancreas ^[65, 66]. Boucenna *et al.* ^[67] found hepatopancreas cell damage that is due to the accumulation of heavy metals at this level.

A recent study demonstrated that *in vitro* exposure of metal oxide causes inhibition of nucleotidases activities in the

hepatopancreas of *H. aspersa* [68]. Manzl *et al.* [69] also observed the acute toxicity of the metal oxides in *H. pomatia* hepatopancreas cells.

Conclusion

In light of results found in our work, we confirmed that our chosen NPs and with selected concentrations (1.25, 1.5 and 2 mg/ml of Fe₂O₃) has a toxic effect during the embryonic phase. Its toxicity is manifested in different ways. Firstly, on eggs hatch before the deformation of the membrane, subsequently it appeared in hatching success of eggs and the hatching period, and by the mortality rate of the new hatched. Finally, we could also show that *H. aspersa* is a good bioindicator bioaccumulator and the major site of accumulation is the hepatopancreas it appeared with rust color.

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