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Toxicity assessment of a binary nanometric mixture (ZnO/Fe2O3) *in Cornu aspersum* (Müller, 1774) (Gastropoda Helicidae)

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ABSTRACT The effects of harmful mixtures on living organisms are of greater importance due to exposure of these complex mixtures of contaminants in the environment. As nanoparticles (NPs) released can potentially interact with many pollutants, and even other NPs in this study, adult *Cornu aspersum* (Müller, 1774) (Gastropoda Helicidae) were used to estimate a mixture effect of two NPs "Fe_2O_3 and ZnO" on the exchange of metabolites (carbohydrates, lipids, and proteins), and some oxidative stress biomarkers: Glutathione (GSH), Glutathione-S-Transferase (GST), Catalase (Cat), and by performing hepatopancreas histological sections of this gastropod after four weeks of treatment. During this period, snails have ingested wheat flour containing the powder of this mixture at doses of 0, 1, 2, and 3 mg/g of wheat flour. The biochemical assays of metabolites reveal disturbances in metabolism by increasing the protein content and decreasing lipid and carbohydrate levels. The decrease of GSH level, GST activity, and Cat activity proves that failure of the detoxification system triggers oxidative stress. The histological study confirms the biochemical results by the tissue lesions, which are very serious, and in a surprisingly dependent dose manner with inflammations, necrosis, hypertrophies, degeneration of the connective tissue, and tubular membranes.

KEY WORDS *Cornu aspersum*; nanoparticles; Fe_2O_3, ZnO; oxidative stress; histology.

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INTRODUCTION

In the last decade, industrial use of metallic oxide nanoparticles has recently found applications in many fields such as consumer products, medicine, and the environment (Sengul & Asmatulu, 2020). Nanotechnology includes the research, development of new innovative materials, systems, and devices, the fabrication and processing of structures and materials on a nanoscale in various fields. Such have given solutions to many environmental problems like availability of clean water, nanocatalyst for hydrogen generation, dental materials, drug delivery systems, antimicrobial nanopowders,

nanosensors for the detection of pathogens or toxic materials, gas-separation nanodevices, and much more, especially in medicine and healthcare (Kaushik, 2020 ; Mazari et al., 2021). Each technology shows positive aspects but also creates problems and limitations (Khan et al., 2020). The use and disposal of NPs will lead to the intentional or accidental release of NPs into the environment, thereby increasing the environmental exposure of these nanoparticles to humans and other species (Feidantsis et al., 2020; Rather et al., 2021).

The released NPs can potentially interact with numerous pollutants, including other NPs, leading to biological effects (bioaccumulation and/or toxicity) that are poorly understood (Deng et al., 2017). Based on the individual behavior and toxicity of materials it is very important to understand if it is possible to predict the toxicity pattern made by their mixture or a whole new toxicity pattern will appear due to potential interactions with biotic creatures (Li et al., 2015).

However, it is very difficult to evaluate and detect the assessement of more than one chemical on a contaminated environment (Besha et al., 2020).

Studies investigating mixture effects, rather than individual effects, present a more realistic reflection due to the exposure of complex mixtures of contaminants in the environment (Ko et al., 2017). The evaluation of mixture effects is a difficult task in environmental assessment. The mixture toxic effect can be defined as an additive, synergistic (>additive), or antagonistic (<additive) action based on the results of these models (Ko et al., 2018).

Several studies have been done for the evaluation of nanoparticles toxicity, most of them agree that zinc oxide NPs is of a more remarkable toxic effect than several other NPs (TiO_2, CeO_2, NiO, CuO, Al_2O_3, Fe_3O_4, MgO, SiO_2, WO_3) as well as iron oxide (Yu et al., 2016; Ko et al., 2018). Because of its applications "Fe_2O_3 nanometric" in several techniques and products that go into diagnosing and treating diseases in humans (Luo et al., 2020; Hernández-Hernández et al., 2020), it is very important to make these assessments. The uses of nano zinc oxide have undergone an increase in use, especially in the field of cosmetology (Lee et al., 2020) because of its diffusion characteristics. Both ZnO and Fe_2O_3 NPs have effects on the embryonic stage through visible malformations and accumulations (Besnaci et al., 2016a; Amin et al., 2021). It is obligatory to adopt appropriate organisms, doses and methods to evaluate their environmental impact accurately, and understand the effects of these NPs mixtures.

The objective of this study was to evaluate the effects of a binary metallic oxide nanoparticles (MONPs) mixture on hepatopancreas metabolites rates, antioxidant defenses system changements, and histology damages on the snail *Cornu aspersum* (Müller, 1774) (Gastropoda Helicidae).

MATERIAL AND METHODS

The land snail *Cornu aspersum* is a good bioindicator of terrestrial pollution. The snails were collected from an uncontaminated site in the Annaba region (situated in the northeast of Algeria), and they were raised in the following optimal environmental conditions: photoperiod 18h light/24h, temperature (20 ± 2 °C), humidity 80 to 95%.

Two types of MONPs were used: nano-Fe_2O_3 and nano-ZnO in an equal amount of mixture. They were synthesized in the department of the physics university of Annaba into two different laboratories. The first one in the laboratory of magnetism and spectroscopy of solids (LMS2) by mechanical grinding from the elemental powder hematite, for a final size of about 26nm. The second is in the laboratory of study and research of condensed states (LEREC) via the co-precipitation method with a crystallite size closed to 59 nm.

The forty snails were divided into four groups, in perforated plastic boxes, and the treatment was started (the cleaning of the boxes and the change of the food is carried out twice a week). Three increasing doses were chosen from our mixture (nano-Fe2O3in/nano-ZnO): 1, 2, and 3mg/g of food with a control group that receives only wheat flour.

After four weeks, the hepatopancreas was recovered for biochemical assays of metabolites (total carbohydrates, total lipids and total proteins) stress parameters (GSH, and GST, and Cat), and histological sections of the four groups.

A portion of each hepatopancreatic sample (100 mg) is used in the protocol for extracting and measuring biochemical metabolites (Shibko et al., 1966), carbohydrates according to the method of Duchateau & Florkin (1959), lipids according to Goldsworthy et al. (1972) and proteins according to Bradford (1976).

Another fragment of hepatopancreas (500 mg) of all the groups is ground in a phosphate buffer (pH 7.4) to serve for the other enzymatic and non-enzymatic assays, the enzymatic activity of GST (glutathione-S-transferase) is dosed according to Habig et al. (1974) method, the enzymatic activity of Cat (Catalase) according to Aebi and GSH (glutathione) after deproteinization with the sulfosalicylic acid according to Weckbecker & Cory (1988) technique.

Three hepatopancreas were kept in 10% formalin for each batch. To make the histological study on this organ, passing through the following protocol: fixation, inclusion, cutting, staining, and assembly. In the end, microscopic observation is made using a camera microscope.

The means ± standard deviation (SD) are calculated for each experiment group. The statistical analysis used are the AV1 "one-way analysis of variance" with two comparison tests that of Fisher and Tuckey. All calculations were performed using Minitab analysis and data processing software version 16.2.

Knowing that:

P≤0.05: Significant difference compared to the control (*).

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

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

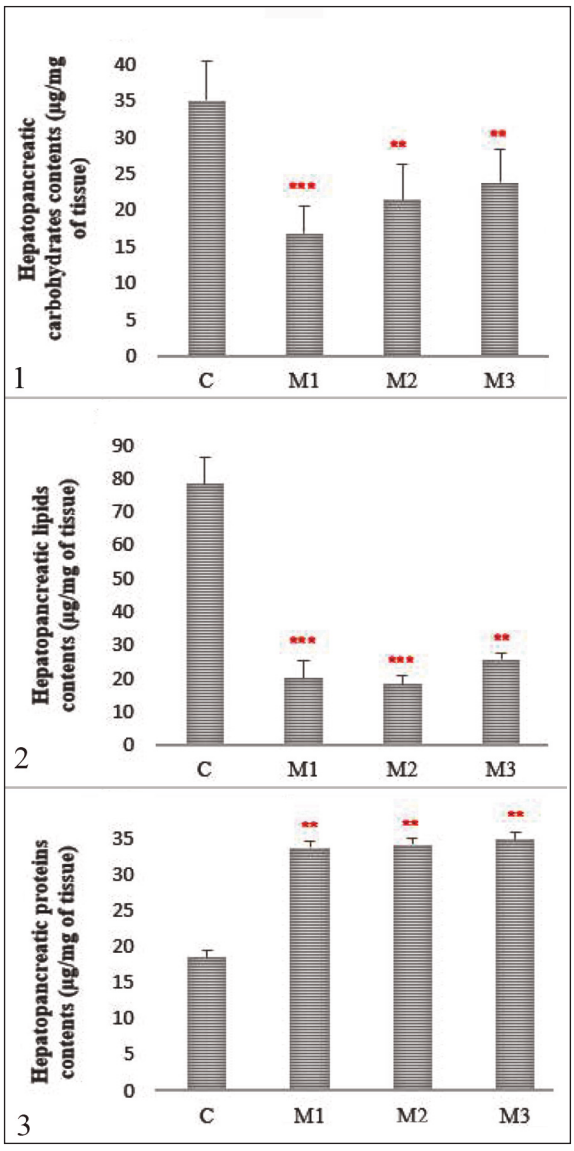
RESULTS

Results show variations between the treated groups and the control one using Fisher and Tuckey statistical tests for all the studied parameters and at all used doses (C: Control, M1: mixture at the dose mg/g, M2: mixture at a dose of 2 mg/g and M3: mixture at a dose of 3 mg/g).

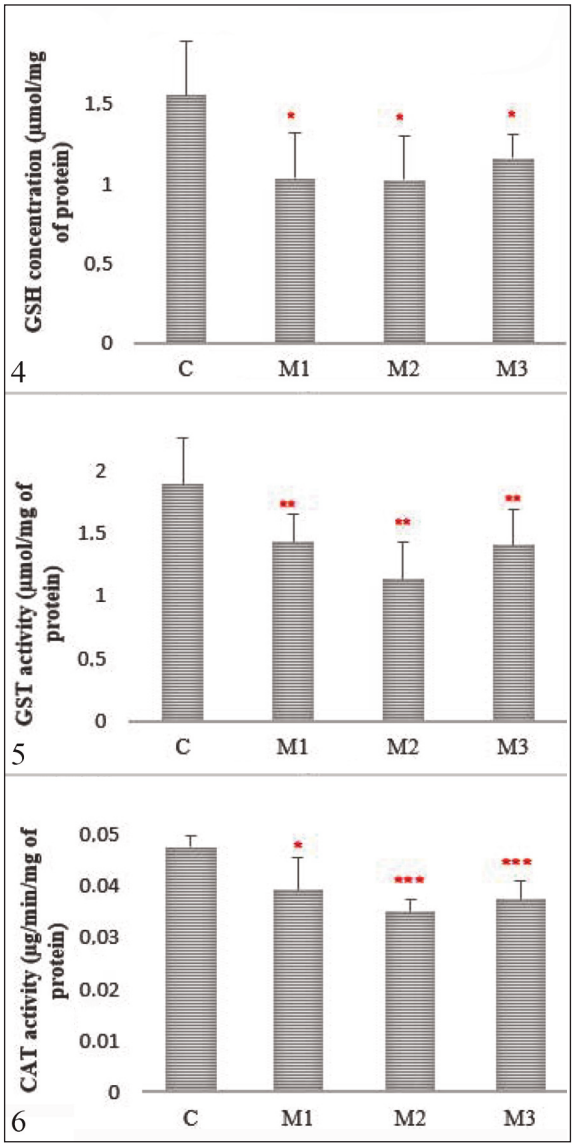
This difference was illustrated as a decrease in the carbohydrate level in a highly significant manner at the first dose and highly significant at the other two doses of the nanometric mixture (Fig. 1). The lipid level has also decreased in a very highly significant manner in the first two doses and highly significant in the third dose of the mixture (Fig. 2). Conversely, the protein level significantly increased at the three doses studied (Fig. 3).

The rate of GSH, GST, and Cat show a decrease that is significant for GSH and highly significant for GST at the three doses of the mixture (Figs. 4, 5).

The observed decrease in Cat is significant at the first dose and very highly significant at the last two-dose (Fig. 6).The control group presents a healthy hepatopancreas in which all its tissue constituents are in a normal state, it appears formed by the juxtaposition of numerous tubules, the spaces which separate them being occupied by a connective tissue within which the hemolymph circulates. The lumen is bordered by a simple epithelium combining several cell types of various morphologies, which fall



Figures 1–3. Rate of metabolites (µg/mg tissue) - Fig. 1: Carbohydrates. Fig. 2: Lipids. Fig. 3: Proteins - in the hepatopancreas of *C. aspersum* after four weeks of exposure at 0, 1, 2 and 3 mg/g of the nanometric mixture nano-Fe_2O_3/nano-ZnO.



Figures 4–6. Rates of hepatopancreatic stress parameters - Fig. 4: GSH. Fig. 5: GST. Fig. 6: CAT - of *C. aspersum* after four weeks of treatment at 0, 1, 2 and 3 mg/g of the nanometric mixture nano-Fe_2O_3/nano-ZnO.

into three main categories: secretory cells, digestive cells and calcium cells. The cells that compose them are of various morphologies that present three types: digestive cell, calcium cell and excretory cell. Each tubule is surrounded by a tubular membrane that forms a rigid wall between two tubules (Fig. 7).

The tissue examination of the hepatopancreas of the snails treated with the 1 mg/g dose of the nanometric mixture shows a narrowing of the tubular lumen which led to a grouping of the tubular mass. A very small inter-tubular connective tissue with some inflammatory infiltrates has also been observed (Fig. 8).

Regarding the tissue of the group treated with the 2mg/g dose of the nanometric mixture, we note that the observations are the same noted in that of the 1mg/g dose with a more retracted tubular lumen (Fig. 9).

The observation of the tissue treated with the third dose (3 mg/g) shows in addition to the same observations noted in the first two doses an absence of tubular light, necrosis, hypertrophies with increase in number of excretory cells (Fig. 10).

DISCUSSION

Several studies were conducted to determine the toxicity of nanoparticles on human health (Baranowska-Wójcik et al., 2020; Bengalli et al., 2021), and the environment (Kik et al., 2020; Martínez et al., 2021). As said in the introduction, nanoparticles as well as small molecules can interact in air, water or soil, whether or not their combination had effects and that is what we wanted to know about our mixture ZnO/Fe_2O_3. Ko et al. (2018) evaluated the toxic effects of individual and mixture NPs: ZnO, NiO, CuO, TiO_2 and Fe_2O_3 through the chlorophyll level of an alga, they found that the chlorophyll level is especially affected at different doses of ZnO/Fe_2O_3 mixture.

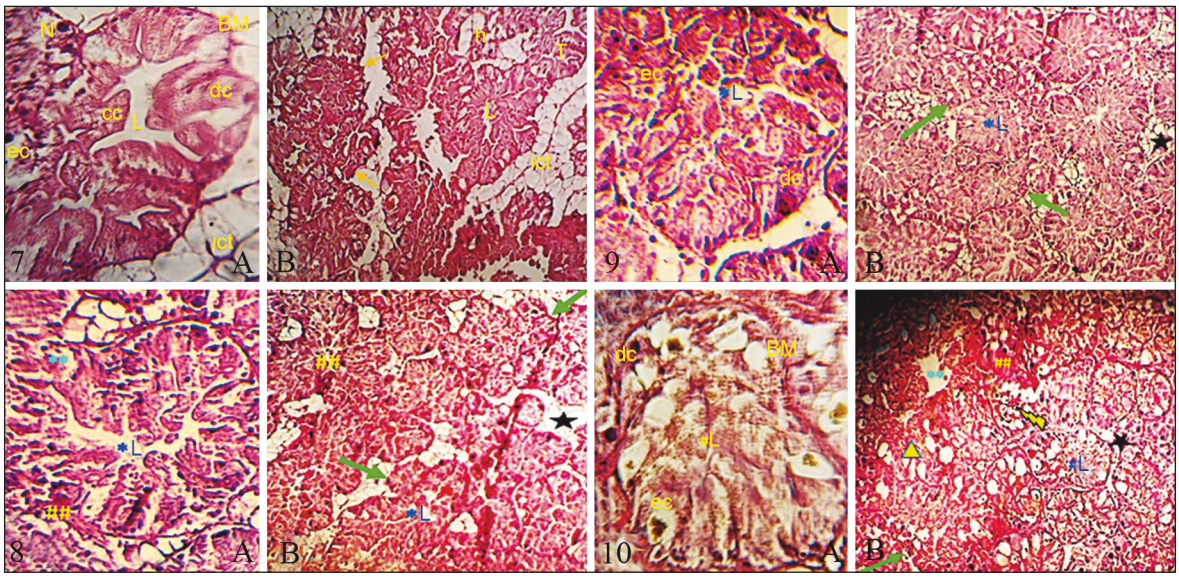
In our work, the effect of this mixture on metabolite rates was verified. From the results obtained we can say that a great disturbance in metabolic has been induced, firstly through the decrease of the carbohydrates level. With the same doses, the iron oxide NPs alone induced an increase in the carbohydrate content of *C. aspersum*'s hepatopancreas (Besnaci et al., 2016b). Chronic exposure of *Oreochromis niloticus* (Linnaeus, 1758) (Cichliformes Cichlidae) to metallic iron oxide NPs induced an increase in plasma glucose levels (Ates et al., 2016). On the other hand, Filippi et al. (2015) found an increase in carbohydrates through the increase of each gluconeogenesis and glycogenolysis following a treatment of human modified hepatocytes by ZnO NPs. On several other biological models, fish (Lee et al., 2014, Khosravi-Katuli et al., 2018), prokaryotes (He et al., 2011) and even unicellular ones (Khaldi & Grara, 2016) treatment

with ZnO NPs induces this increase in the carbohydrate rate. In our results, we can say that the mixture gave an opposite effect with respect to each of the separate NPs Fe_2O_3 and ZnO. This decrease in carbohydrates is explained by the increase in energy requirements following the detoxification reactions after exposure to xenobiotics (ZnO, Fe_2O_3) and the prolonged youth, which will therefore reduce the reserves and energy inputs.

The level of lipids also decreased in a very significant way at the three doses of the mixture, this can be explained by the chemical stress caused by the xenobiotic (ZnO and Fe_2O_3 NPs). Besnaci et al., (2016b) also found this result with *C. aspersum* snails after treatment with nano- Fe_2O_3. The application of nanoscale zinc oxide on another type of snail (freshwater) *Biomphalaria alexandrina* (Ehrenberg, 1831) (Gastropoda Planorbidae) revealed a decrease on total lipids and total cholesterol contents in hemolymph and tissues (Fahmy et al., 2014).

For the protein level, the results show an increase at all three doses. Boucenna (2016), Besnaci et al. (2016b) and Sidiropoulou et al. (2018) had also noted this variation in *C. aspersum* after treatment with nanoscale iron oxide. Treatment with nanometric iron oxide on rats also induced an increase in the protein level (Zhu et al., 2008). In contrast, Fahmy et al. (2014) reported a decrease after treatment of *B. alexandrina* snails with nano-ZnO. While the results of Khaldi & Grara (2016) NPs ZnO show an increase in certain concentrations and a decrease in another higher, so it is a question of dose. Our variation is explained by the detoxification system reactions and its enzymes "stress proteins". The presence of metals may also induce the synthesis of proteins enabling to connection between them (Zidar et al., 2009), their transfer 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 (Pihan, 2001).

The oxidative stress caused by a particle is considered one of the most important mechanisms of toxicity of nanoparticles, especially for particles containing transition metals. Metals, such as Zn, Fe, Cu and Mn are considered as transitional metals due to their great participation in metabolic reactions, serving as cofactors for several enzymes (Saad, 2003; Sellami et al., 2017). In several cases, the effects of predator stress were affected by the accumulated metals, thus providing strong evidence for stressor interactions.



Figures 7–10. Histological sections of the *Cornu aspersum* hepatopancreas. Fig. 7: control. Figs. 8–10: treated with the nano-Fe_2O_3/nano-ZnO mixture, (a) tubule at 400-fold magnification (b) tissue organization at 100-fold magnification. L: lumen; dc: digestive cells; cc: calcium cells; ec: excretory cells; ict: intertubular connective tissue; BM: basement membrane; N: nucleos; (*L) retracted lumen; (#L) lumen absent; (arrow) tubular mass grouping; (##) inflammatory infiltrates; (**)necrosis; (triangle) hypertrophy; (zig zag) high number of excretory cells; (star) connective tissue almost absent.

In order to understand the mechanism of our nanoparticles mixture, we studied three markers of oxidative stress including total Glutathione concentration, Glutathione-s-transferase activity and Catalase activity.

The significant decrease noted at the three studied doses of our mixture of the rate of the three evaluation parameters of oxidative stress (GSH, GST and CAT) indicated that there was a failure in the detoxification system. Many metallic nanoparticles have been studied to exert their toxicity through oxidative stress. Metallic nanoparticles can liberate free ions in the cytoplasm upon surface oxidation (Kumar, 2006; Asharani et al., 2008; Dubey et al., 2015).

The results of our study confirmed that the decrease in GSH content in digestive gland appears to be a common response of mollusks to metal exposure (Xiong et al., 2011; Ali et al., 2012; Fahmy, 2014). Reduction in GSH after ZnO and Fe_2O_3 NPs exposure could be due to its increased use in free radical scavenging. Several studies on the NPs of zinc oxide and iron oxide have noted this variation. Following treatment with NPs of iron oxide, Boucenna (2016) on *C. aspersum* and Sarker & Sil (2014) on cell cultures noted this decrease. Studies by Fahmy et al., (2014) and Falfushynska et al. (2015) also reported a decrease in GSH level, the first in *B. alexandrina* following treatment with ZnO NPs and the second in *Unio tumidus* Philipsson, 1788 (Bivalvia Unionidae) treated with Zn NPs.

GST is an enzyme that participates in the detoxification process due to conjugation reaction between GSH and xenobiotics (Cummins et al., 2011). Thiol compounds, such as reduced and oxidized GSH, represent the initial protective substances against heavy-metal ions and other pollutants. In accordance with our result, Escobar et al. (1996) and Sanzgiri et al. (1997) reported that the enhanced free-radical concentrations resulting from oxidative stress conditions can cause loss of GST enzymatic activity (Fahmy, 2014). It has been considered that the low levels of oxidative stress induce the expression of protective mechanisms, whereas larger doses result in the activation of pro-inflammatory mechanisms, and cell death at the extreme levels (Nel et al., 2001). Freshwater snail Biomphalaria alexandrina were treated with different doses of nano-ZnO, and found a decrease in GST activity, which confirms the strong action of nano-zinc oxide with the release of ROS and especially in combination with iron oxide that is also able to release free metal ions (Fahmy et al., 2014).

The inhibition of CAT activity was also noted, after exposure to ZnO/Fe_2O_3 NPs mixture in hepatopancreas tissue of treated snails may be due to the enhancement of the peroxidation end product, MDA, which is known to inhibit protein synthesis and the activities of certain enzymes (Fahmy et al., 2014). Several nano zinc oxide studies have noted this decrease, Panda et al. (2003) and Dimkpa et al. (2012) explained the absence of increased CAT activities with ZnO NPs by no-reactivity of these enzymes to Zn ions in wheat. Fahmy et al., (2014) report a decrease in Catalase activity at the level of the haemolymph in the snail *B. alexandrina* treated with different doses of NPs of zinc oxide, they suggested at the end of the study that ZnO inhibits catalase. Du et al. (2017) noted a decrease in Catalase in *Danio rerio* (F. Hamilton, 1822) (Cyprinidae) zebrafish embryos after treatment with ZnO NPs. According the results noted by sarkar and Sil, (2014) and Besnaci et al. (2019) after treatment with NPs of Fe_2O_3, not only zinc inhibits Catalase activity. So the effect of the mixture is a synergistic effect of each of the two NPs.

Histological damages are one of the consequences of oxidative stress, associated with behavioral, physiological and also biochemical disturbances. The histological study showed that the mixture causes unambiguous tissue damage. Indeed, histological examination of the hepatopancreas highlights the appearance of lymphoplasmacytic inflammatory infiltrates at the lowest dose; this could be a first biological response due to the presence of xenobiotics. The digestive gland is a very important organ in gastropods, responsible for the production of enzymes, the absorption and storage of nutrients, endocytosis and excretion of certain particles and mainly involved in the detoxification of pollutants. Histological and histochemical changes are used as biomarkers of xenobiotic exposure.

The observed tissue damage shows the toxicity of the metal nanoparticles studied and confirm the biochemical results obtained. In the same direction (evaluation of the toxicity of nano- Fe_2O_3 on *C. aspersum*) (Besnaci et al., 2016b, Boucenna, 2016) other studies noted the same observations: inflammatory infiltrates, hypertrophies, and necrosis. ..etc. NPs induce the release of ROS, disrupting the functioning of organelles such as mitochondria and lyso-

somes and causing cell death by necrosis or apoptosis (Marano et al., 2011). Hsieh et al. (2015) report that nanoscale iron oxide causes mitochondria-dependent apoptotic cell death in cell cultures. However, exposure of *U. tumidus* mussels to ZnO NPs results in inflammation and cell destruction leading to necrosis (Falfushynska et al., 2015). The bioaccumulator site in *C. aspersum* is hepatopancreas (Besnaci et al., 2016b); the penetration of NPs and their accumulation damages the membranes and thus disrupts intercellular exchange and membrane fluidity. Increased ETM diffusion causes cellular necrosis (Yager & Harry, 1963). Several studies show the internalization and penetration capabilities of NPs through observed tissue modifications (Zhu et al., 2008, Kumari et al., 2012; Esmaeillou et al., 2013; Al rasheed et al., 2014; Dubey et al., 2015).

CONCLUSIONS

Finally, the present toxicological study has made it possible to highlight, on one hand biochemical disturbances and tissue damage, and on the other hand an activation of biomarkers thus protecting hepatopancreas cells by triggering the detoxification system. We have shown very strong toxic effects of Fe_2O_3/ZnO mixture in an acute exposure situation on *C. aspersum* snails. Mixture treatments caused pronounced effects like each type of nanoparticles when tested individually (Fe_2O_3/ZnO) after comparison with other studies.

The choice of our model is not random, indeed, *C. aspersum* is known by their accumulating power. It is a species used as a sentinel for soils contaminated by metallic pollutants.

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