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Nanoparticles in foods? A multiscale physiopathological investigation of iron oxide nanoparticle effects on rats after an acute oral exposure: Trace element biodistribution and cognitive capacities

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ABSTRACT

Iron Oxide Nanoparticles (IONPs) are used in several fields of application, mainly in the biomedical field for their magnetic properties and in food additive known as "E172" for their colour. In the present investigation, we focused on IONP effects on Wistar rat following acute oral exposure. We performed a multiscale physiopathological investigation in order to elucidate potential toxic effects linked to IONP ingestion, especially on cognitive capacities, trace element distribution, blood constituents, organ functions, organ structure and iron deposit. We demonstrated that oral exposure to IONPs induces disturbances of certain parameters depending on the dose. Interestingly, the histopathological examination evidenced inflammatory effects of IONPs in the liver with iron deposits in hepatocytes and Kuppfer cells. Neurobehavioral examination showed that oral exposure to IONPs did not affect nor rat emotions, exploration and locomotion capacities, nor spatial reference memory status. Furthermore, oral administration of IONPs did not disrupt the trace element homeostasis nor in the liver neither in the stomach. Altogether, our study evidenced low signs of toxicity, but some effects lead us to a careful use of these NPs. Thereby, their use in foods should be further studied to better evaluate the potential toxic risks of the oral exposure to IONPs.

1. Introduction

Nanoscience and nanotechnology present a scientific revolution that began in the 1960s (Yah et al., 2012). The "Nanoworld" includes any object of nanometric size with interesting physicochemical properties that can be used in various fields, including industrial food, cosmetics or electronics, environment and biomedicine (Cortajarena et al., 2014; Yoshioka et al., 2014; Gupta et al., 2018). The design of nanoparticles focused on therapeutic and diagnostic applications has increased exponentially for the treatment of several chronic diseases, particularly cancer. The NPs are smart carriers, able to diagnose, deliver and monitor the therapeutic response in real-time and appear as promising tools in medicine. Metal nanoparticles (NPs) are the most widely used and are of most interest in various applications, as in scientific research essentially because of their properties, in particular on their composition. Thus, some types, such as titanium dioxide (TiO_2) and zinc oxide (ZnO) NPs are used in sunscreens for their UV absorption properties (Raj et al., 2012). Silver NPs have been extensively studied and used in food industry and medicine because of their broad-spectrum antimicrobial activity against microorganisms (Huang et al., 2018). Iron oxide NPs (Fe_2O_3 and Fe_3O_4) are used in several fields of application, but mainly in the biomedical field for their magnetic properties that give them interesting properties for fighting against cancer or in food additive known as "E172" for their colour. This additive is used worldwide for cake and dessert mixes, meat paste, salmon and shrimp paste preparations. Therefore, these NPs could be found everywhere, in

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the air, in drinking water, in food, in products for everyday use. They can be inhaled, ingested or absorbed by the body through the skin and mucous membranes (Ai et al., 2011). Not only humans can be impacted by nanotechnology, but the environment too. The nanoparticles can be released into the air, water, and soil systems as waste and therefore contaminate all the food chain (Gupta et al., 2018). Since their emergence, nanotechnologies, especially NPs uses, have raised many questions about their risks. Indeed, due to their high reactivity, NPs pose a potential risk to human health. Several products and medicines in the global market contain "nano" ingredients, mainly metallic NPs (Titanium, Silica, Iron …). Several investigations reported that these NPs have the ability to cross the barriers of the skin and biological membranes to reach the level of vital organs, including the brain (Lashof-Sullivan et al., 2014; Maher et al., 2016). Similarly, several deleterious effects have been reported, such as inflammatory syndrome, oxidative stress, DNA damage and cognitive disturbances (Babadi et al., 2012; Barhoumi and Dewez, 2013; Huang et al., 2014; Sadeghi et al., 2015; Sheida et al., 2017). However, to date, and despite numerous investigations by worldwide researchers, the effects of NPs or the mechanisms by which NPs can interact in the body are not fully understood. Interestingly, in 2015, the European Food Safety Authority (EFSA) published a report on "the scientific opinion on the re-evaluation of iron oxides and hydroxides (E172) as food additives". They concluded that an adequate assessment of the safety of E172 could not be carried out because a sufficient biological and toxicological database was not available (Opinion, 2015). In this context, new multiscale surveys and new advanced studies are necessary to evaluate the "nano" risk of the "nano products" ingested, whose presence in the food and medication markets increases from one year to another. Few studies reported that defined experimental doses could be toxic following acute oral exposure to iron oxide nanoparticles, however, a higher dose such as 2000 mg/kg was reported as safe (Kumari et al., 2012a; Yun et al., 2015). In this way, the Lowest Observed Effect Level (LOEL) dose must be urgently defined. Therefore, we conducted a new *in vivo* study, taking the Wistar Rat as a model, to evaluate the potential acute oral toxicity of IONPs at different scales: Rat health and body weight, cognitive capacities, metal biodistribution, blood count, and organ function and structure.

2. Material and methods

2.1. Iron oxide nanoparticle synthesis

For the present study, IONPs were kindly provided by Pr. Lassaad El Mir from the Laboratory of Physics of Materials and Nanomaterials applied to the Environment at the Faculty of Sciences of Gabès, Tunisia. The modified sol-gel method described previously (Lemine et al., 2014) was used to prepare the suspensions of IONPs, precisely maghemite (γ-Fe_2O_3), under supercritical conditions of ethyl alcohol, in which the hydrolysis proceeded slowly to release water mixture by an esterification reaction in order to control the size of the nanoparticles formed. An amount of 6 g of iron (III) acetylacetonate [$C_{15}H_{12}FeO_6$] was used as a precursor of the synthesis and was dissolved in 36 mL of methanol. The solution was incubated and dried in an autoclave after magnetic agitation under supercritical conditions of ethyl alcohol: Tc = 243 °C and Pc = 63.3 bar. Autoclave heating was provided by a furnace controlled by a temperature controller. The particle size could be adjusted by variation of magnetic stirring time.

2.2. Iron oxide nanoparticle characterization

Before performing any nanotoxicological study, nanoparticle characterization represents a primordial step to understand their effects on the studied organism. To characterize the IONPs, we used three different techniques: Transmission Electron Microscopy (TEM) and X-ray diffraction (XRD) for the powder and Dynamic Light Scattering (DLS) for the suspension. The XRD provides information on size and crystallized structure of the NPs. This technique was performed using the PANalytical Xpert Pro diffractometer with Cu k radiation (k = 1.54178 Å) in the range of 2θ = 20–70% at the 0.02°. The size and shape of IONPs were determined by a JEOL transmission electron microscope (JEM-2100F) operating at 200 keV. The same batch of IONPs was used in our previous paper (Askri et al., 2018a).

For the suspension characterization, a stock solution of 5 mg/mL of IONPs in sterile water was prepared to make a range of cell treatment concentrations. In suspension, IONPs were sonicated by the sonicator Sonics® for 60 min with 1 min ON and 1 min OFF and then dispersed in an ultrasonic bath for 60 min immediately before use. This suspension was diluted 1:100 in water to characterize the IONPs in suspension by the Dynamic Light Scattering (DLS) technique using a Zetasizer Nano (Malvern). This technique allows us to measure the hydrodynamic diameter and the *ζ-potential* of the NPs in solution and to study their dispersion state.

2.3. Animal housing and exposure route

Young adult male Wistar rats (9 weeks old) used in this study were purchased from SIPHAT (Ben Arous, Tunisia) and weighed 120–130 g at the beginning of the experiment. They were randomly divided into 3 groups, one control and two treated with n = 7 each. The two treated groups received 100 and 200 mg/kg of Fe_2O_3-NPs respectively once by oral gavage. IONPs were suspended in pure water and then sonicated for 60 min in a hand-held sonicator (Sonics®). Before each administration, the solution was also vortexed for 1 min. Control rats received water. The animals were housed under suitable conditions of temperature (25 °C) and light (12:12 h light/dark cycle) and they received water and food *ad libitum*. The experimental protocols were approved by the Medical Ethical Committee for the Care and Use of Laboratory Animals of Pasteur Institute of Tunis and the Faculty of Sciences of Bizerte (approval number: LNFP/Pro 152012). Before being sacrificed, the rats were anesthetised. The sacrifice was performed 10 days after the oral gavage and different organs (liver, kidneys, spleen, lungs and brain) were extracted for analyses and calculation of relative organ/body weight ratio (mg/g).

2.4. Cognitive capacities investigation

Twenty-four hours after the acute oral administration of IONPs, three behavioural tests were performed to evaluate the effects of these NPs on emotional state, locomotion and exploration, learning and memory performances in rats:

2.4.1. Elevated plus maze

The first test was the elevated plus maze and was used as described before (Pellow et al., 1985; Maaroufi et al., 2009). Briefly, the maze consists of four arms of 50 cm long and 10 cm wide, opposite one another in pairs, and connected by a central platform. The device was raised to a height of 60 cm. The closed arms were surrounded by a 50 cm wall, while the edges of the open arms were 0.5 cm in order to favour entry into the open arms (Treit et al., 1993). The test was of 5 min and we started by placing a rat in the centre of the labyrinth, facing an open arm. The rat explored the device freely. Thus, the number of entries and the time spent in the different parts of the labyrinth (the open arms, the closed arms and the central part) were recorded using a video camera placed above the device. The percentage reflecting anxiety status of the rats, also known as anxiety index, was calculated by the following formula (time spent in closed arms x 100/time spent in open and closed arms) (Pellow et al., 1985). Between each rat, the device was cleaned with 10% alcohol.

2.4.2. Open field

Exploratory behaviour, motor activity and anxiety were evaluated

by the open field test. Control rats and rats treated with IONPs were tested for a daily observation session of 5 min for three consecutive days with a 24-h interval. The device was a metal-gray colored enclosure with a circular shape. It was divided into seven sectors: a central sector and six peripheral sectors that were all of equal size. Three identical objects were placed in the same position in the peripheral part during the 3 days. At the beginning of the test, the rat was placed in a peripheral part. Different behavioural parameters were measured such as the number of sectors crossed and the time spent in the peripheral part and the central part, the total immobility time, the number of contacts with an object. Between each rat, the device and the objects were cleaned with 10% alcohol.

2.4.3. Morris Water Maze

The Morris water maze test (Morris, 1984) was performed to evaluate the spatial reference memory in rats. The experimental device of the test was a circular pool filled to a depth of 30 cm with water (23 °C ± 1 °C) which was rendered opaque by the addition of 2 kg of black plastic pellets (Maaroufi et al., 2014). A polystyrene platform (diameter 10 cm) was placed in the centre of the Northeast quadrant inside the device. The pool was placed in a room with various additional visual cues such as geometric figures in different colours fixed on the outside part of the pool, which helped the animal to find its way into the space. The trajectory taken by the animal in the pool was recorded using a video camera placed above it. The experimenter stayed behind a curtain near the maze in the experiment room to control the recording of the video. The experiment of the Morris Water Maze was carried out for 4 consecutive days, each rat underwent a daily session of four tests (maximum 60s/test). A 10 min delay was applied between the tests. During it the rat was dried and placed back in its cage. During the first day assays, the platform remained slightly visible above the surface of the water and then the next 3 days was immersed 1 cm below the surface. Each day, four starting points were possible (Northeast, South-East, North-West or South-West). Their sequence varied from one test to another and from one day to the next. At the beginning of each test, a rat was gently released into the pool facing the wall in one of the four starting positions (North, South, East, and West). Once in water, the rat swam to reach the platform and climbed above it for 10s before returning to its cage (Deguil et al., 2010). If the animal did not find the platform for 60s, it was guided to it by the experimenter and left there for 20 s. Then, the rat was lifted from the water and was moved to a new starting point. Twenty-four hours after the 4th day, a 60s retention test called “probe test” was performed. During this test, the platform was removed from the pool and the time spent by the rat in Northeast quadrant containing the platform for the previous 4 days was calculated.

2.5. Trace element biodistribution

Different trace elements, Iron (Fe), Copper (Cu), Manganese (Mn) and Zinc (Zn) were determined using a quadrupole ICP-MS Thermo X serie II (Thermo Electron, Bremen, Germany) equipped with quartz impact bead spray chamber and concentric nebulizer. The Xt interface and collision cell technology options were used. Aliquots of liver and stomach (0.13–0.23 g) were completely mineralized at atmospheric pressure in 67% HNO_3 (VWR, ref = 83879.270), at the ratio of 100 mg tissue/1 mL HNO3, for 24 h at room temperature then in the oven at 60 °C at least 48 h and we verified the limpidity before analysis. If the mineralization is not complete, the tubes were placed again at 60 °C for additional 24 h. For each set of measurements we used sample reference material SRM (NIST bovine liver 1577). The SRM is mineralized in the same conditions as the animal samples and allow to validate the results. Before the analysis, the limpid acidic solutions were thoroughly mixed and aliquots were diluted at 1:100 in water. Standard solutions were prepared in nitric acid 1% (v/v). ^{56}Fe, ^{63}Cu, ^{65}Cu, ^{55}Mn, ^{64}Zn, and ^{66}Zn isotopes were measured and ^{71} Ga at 650 nmol/L was used as internal standard. The limits of detection and quantification of the apparatus were 10.2 nmol/L for Fe, 32.7 nmol/L for Cu, 0.96 nmol/L for Mn, and 15.5 nmol/L for Zn. The analytical method was performed the same way as reported in our previous papers (Askri et al., 2018a,b).

2.6. Blood count and biochemistry assays

Blood count is used by several scientists to evaluate the toxic effects of a product or a medicine at the level of blood cells and contents. Blood samples were collected in tubes for haematological analysis containing EDTA as anticlot using HORIBA ABX Pentra XL80 cell counter. Biomarkers of organ functions inform also about dysfunction or activity following exposure to any chemicals or products that could affect the organism. For biochemical analysis and iron plasma measurement, blood samples were collected in heparinised tubes. The parameters related to hepatic and renal functions, and to lipid and protein metabolisms as well as the iron levels were measured using COBAS Integra 400 plus ROCHE®.

2.7. Histopathological examination

For the histopathological examination, the tissue fragments of liver were washed with a 0.9% solution of sodium chloride. They were fixed with 10% formalin and then paraffin-included for histopathological analysis. The sections were cut about 5 µm thick, deparaffinised, hydrated, and stained with haematoxylin-eosin (H & E).

2.8. Perl's stain for iron deposit detection

Iron deposit in the liver was assigned by the specific iron staining known as Perl's blue stain.

We used the same preparation protocol as for the histopathological examination. The sections were stained with Perl's blue.

2.9. Statistical analyses

All results were entered in a Microsoft Excel Spreadsheet. In the present paper, the results are expressed as mean ± Standard Error to Mean (SEM) except for the relative organ weight box plot. Statistical analysis was performed with one-way ANOVA and Tukey's post hoc tests at a significance level of 5%.

3. Results

3.1. Characteristics of IONPs used in the study

The techniques we used for the characterization gave us different required information about the IONPs as mentioned in Table 1. As powder, they had a spherical shape with an average size of 30 nm. However, the DLS showed than these NPs had a hydrodynamic size much higher than 30 nm when they were in suspension (~175 nm). The z-potential was negative and the PdI (Polydispersity Index) was

Table 1
Iron Oxide Nanoparticle characterization as powder and in suspension.

Used technique	Transmission Electron Microscopy	Dynamic Light Scattering		
Parameter	Particle size (nm)	Hydrodynamic size (nm)	Pdl	Zeta Potential
IONPs (γ-Fe_2O_3)	30	174.2	0.302	−10.6

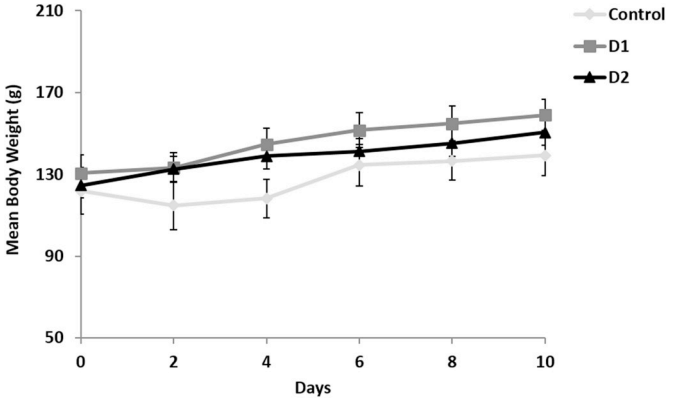

Fig. 1. Mean body weight evolution following acute oral exposure to IONPs at 100 (D1) and 200 mg/kg (D2). Data are expressed as means ± SEM, n = 7 animals *per group*.

inferior to 0.5.

3.2. Effects on the average weight of rats and the relative weight of organs

Our results showed that IONPs had no effect on rat weight (Fig. 1). Indeed, the weight of the rats treated with D1 (100 mg/kg) and D2 (200 mg/kg) evolved in the same way as the weight of the control rats. However, the variation of the relative weight of the organs was significantly different compared to the controls except for liver and spleen (Fig. 2). In fact, oral administration of IONPs at 200 mg/kg (D2) decreased significantly the relative organ weight of brain, kidneys, lung, and stomach compared to control group. The lowest dose decreased only the relative weights of kidneys, lung, and stomach.

3.3. Effects on haematological and biochemical parameters

The study of haematological and biochemical parameters showed that single doses of 100 and 200 mg/kg of IONPs significantly impacted these parameters. The lower dose (100 mg/kg) induced, on the one hand, an increase in red blood cell, haemoglobin, haematocrit and eosinophil levels and, on the other hand, a decrease in the number of neutrophils (Table 2). Following the dose of 200 mg/kg, we noted an elevation in haemoglobin and haematocrit levels. Similarly, IONPs slightly modified the biochemical parameters of rats (Table 3). Following exposure to D1, we evidenced a decrease in iron levels with an increase in urea level. However, D2 induced an increase in alanine aminotransferase (ALT) and a decrease in iron concentrations compared to control.

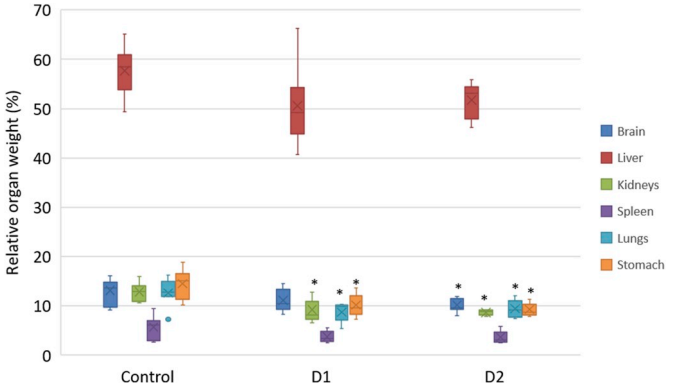

Fig. 2. Effects of acute oral administration of IONPs at 100 (D1) and 200 mg/kg (D2) on relative organ weight in rats. Data obtained at day 10 with n = 7 animals *per group*. * *p-value* < 0.05 using one-way ANOVA and Tukey's post hoc tests.

Table 2
Effects of acute oral administration of IONPs at 100 (D1) and 200 mg/kg (D2) on haematological parameters. Data are expressed as means ± SEM, n = 7 animals *per group*. WBC white blood cells, RBC red blood cells, MCV mean corpuscular volume, MCH mean corpuscular haemoglobin. * *p-value* < 0.05 using one-way ANOVA and Tukey's post hoc tests.

	Control	D1	D2
WBC 10^9/L	4.98 ± 0.58	5.73 ± 0.57	6.97 ± 0.51
RBC 10^{12}/L	5.31 ± 0.65	6.82 ± 0.23*	6.60 ± 0.15
Haemoglobin g/dL	9.38 ± 0.95	11.86 ± 0.32*	11.50 ± 0.20*
Haematocrit %	27.35 ± 3.08	35.00 ± 1.03*	34.17 ± 0.56*
MCV fL	52.00 ± 0.96	51.29 ± 0.64	51.71 ± 0.60
Platelets 10^9/L	615 ± 112	842 ± 44	818 ± 49
Neutrophil %	24.93 ± 3.67	13.21 ± 0.85*	18.79 ± 1.84
Lymphocyte %	70.00 ± 3.29	76.70 ± 1.43	71.01 ± 3.12
Monocyte %	2.97 ± 0.87	4.11 ± 0.55	5.70 ± 0.96
Eosinophil %	1.20 ± 0.55	5.14 ± 1.32*	3.31 ± 0.83
Basophil %	0.90 ± 0.09	0.83 ± 0.13	1.19 ± 0.17

Table 3
Effects of acute oral administration of IONPs at 100 (D1) and 200 mg/kg (D2) on biochemical parameters. Data are expressed as means ± SEM, n = 7 animals *per group*. ALP alkaline phosphatase, ALT alanine aminotransferase, AST aspartate aminotransferase.

	Control	D1	D2
ALP U/L	245.57 ± 24.07	239.83 ± 20.41	250.85 ± 10.09
ALT U/L	55 ± 3.71	49.57 ± 2.82	62.71 ± 3.33*
AST U/L	174.28 ± 25.66	142.42 ± 13.34	156.85 ± 11.64
Cholesterol mmol/L	1.32 ± 0.06	1.3 ± 0.06	1.24 ± 0.14
Creatinin µmol/L	13.57 ± 0.86	12.85 ± 0.55	13 ± 0.81
Glucose mmol/L	11.06 ± 1.08	9.8 ± 0.46	10.12 ± 0.66
HDL mmol/L	1.05 ± 0.06	0.85 ± 0.05	0.85 ± 0.05
Iron µmol/L	53.38 ± 6.31	32.13 ± 3.96*	37.04 ± 2.88*
Protein g/L	56.14 ± 0.82	60 ± 1.51	59.57 ± 0.81
Triglyceride mmol/L	0.59 ± 0.04	0.59 ± 0.05	0.68 ± 0.05
Uric acid µmol/L	101.28 ± 16.40	84.71 ± 8.05	85.85 ± 22.19
Urea mmol/L	5.87 ± 0.19	7.28 ± 0.46*	5.91 ± 0.18

* *p-value* < 0.05 using one-way ANOVA and Tukey's post hoc tests.

3.4. Effects on behaviour

3.4.1. No effects on rat emotions and anxiety

The behavioural performances measured by the elevated plus maze test evidenced that both rat groups exposed to IONPs showed no significant changes in comparison to the control group. Indeed, the time spent in the closed arms, the time spent in open arms and the number of entry into these arms were similar to those of the control group. Thus, the treated groups had an anxiety index similar to that of the control rats (Fig. 3).

3.4.2. No effects on rat exploration and locomotion capacities

The Open field was used to evaluate the exploratory behaviour of rats during 3 sessions. Following acute exposure to IONPs, no significant effects were found (Fig. 4). This proves that IONPs had no impact on exploratory behaviour, locomotion or anxiety in the rat.

3.4.3. Stable learning and memory capacities

The performances of rats related to learning and memory were studied by the Morris water maze test. This test showed that there was no difference in learning between control and exposed rats (to D1 and D2). Indeed, the swimming time to reach the platform was not significantly different between both treated groups and the untreated group. Swimming time in the area where the platform was deposited for the first 4 days remained also unchanged. This shows that iron NPs did not induce disturbances in memory or learning of rats (Fig. 5).

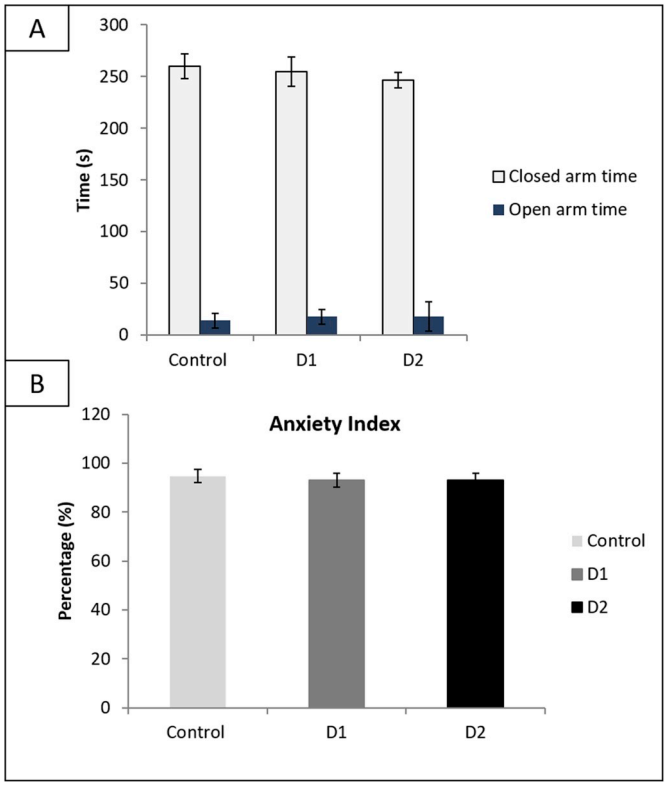

Fig. 3. Effects of acute oral administration of IONPs at 100 (D1) and 200 mg/kg (D2) on **A)** time spent in the closed and open arms and **B)** anxiety index from the elevated plus maze. (The test was performed 24 h after the oral gavage). Data are expressed as means ± SEM, n = 7 animals *per group*.

3.5. Effects on homeostasis of trace elements in the liver and stomach

The trace element Fe, Mn, Zn and Cu were measured in the liver and in the stomach (Fig. 6) by ICP-MS. The results showed that the acute oral exposure to IONPs had no impact on the distribution of Fe, Mn, Zn and Cu elements neither at the hepatic nor stomach level since there was no increase or decrease in their respective levels.

3.6. Modification of liver structure and iron deposit

The histopathological examination showed several aspects of liver inflammation and necrosis following the acute oral administration of IONPs at both doses of 100 and 200 mg/kg (Fig. 7). Perl's blue staining revealed minimal deposits of iron in the Kuppfer cells and in hepatocytes' nuclei (Fig. 8).

4. Discussion

The effects of nanoparticles (NPs) have been the subject of several *in vivo* toxicological studies. The effects on the nervous system have been investigated recently after administration of some types of NPs (Valdiglesias et al., 2016; Patel et al., 2017; Liu et al., 2018). In fact, data on nanoparticle effects on the nervous system, including brain trace elements levels, as well as cognition capacities, attracted the attention of scientists and civil society. Thus, we took into account these parameters to establish a new overall pathophysiological study following an acute exposure to IONPs at two doses 100 and 200 mg/kg. Those doses were fixed according to a large research in literature. We selected a dose largely lower than the limit oral dose 2000 mg/kg as mentioned by Yun et al. (Yun et al., 2015), as we aimed to study metabolic and functional effects and not the mortality of the rats. The daily doses of iron oxide nanoparticles or all the nanoparticles in general to which humans might be exposed are not yet defined. Furthermore, to improve the control of the declarations regarding the use of iron oxide nanoparticles in food or food packaging they should be urgently defined. Acute exposure is quite important in the case of nanoparticles as they are hazardous materials. Human exposure to iron oxide nanoparticles may be short or long, both are possible and important too. In this investigation we chose to use the acute exposure to have an evaluation of the immediate effects on the rats' physiology and mainly to study the effects on brain and cognition within the 24 h following the exposure. Indeed, the nanoparticles could be metabolized and/or cleared, in majority, by the organism in a short time as they will be directly in contact with the gastrointestinal system. In the present work, the techniques used to characterize the IONPs administered to Wistar rats showed that our nanoparticles were pure; no other elements were found as contamination with iron. The hydrodynamic size of the IONPs was higher than the powder size (30 *vs* 174.2 nm). This could be explained by agglomeration for IONPs as they exhibit magnetic forces and they tend to agglomerate. Nonetheless, the measurements of z-potential and PdI prove the good dispersity status of the particles in water. Moreover, it is quite difficult to estimate the agglomeration of the IONPs once they are in the body.

Our results indicated that acute oral exposure to iron NPs by gavage did not affect the overall health of the animals as no signs of toxicity or mortality were observed. Furthermore, no effects were observed on rats' body weight. The results obtained after a unique oral administration in terms of effect on weight are in agreement with our previous results obtained with intravenous administration (Askri et al., 2018b) but not with intranasal administration (Askri et al., 2018a). Moreover, in 2012, Kumari et al., reported that following chronic exposure to IONP at doses (30, 300 and 1000 m/kg/day during 28 days) the rats showed no significant loss in body weight and feed intake (Kumari et al., 2012b). The same team performed an acute exposure study applying three different doses (500, 1,000, and 2,000 mg/kg) and reported same observations (Kumari et al., 2012a). Nevertheless, according to the present study, IONPs administered by the oral route significantly decreased the relative weight of certain organs such as brain, kidneys, lungs, and stomach following the ingestion of iron NPs. These results are consistent with those obtained by Szalay et al. (Szalay et al., 2012), following intratracheal instillation of IONPs. This decrease in organ coefficients indicates a systemic action of iron NPs and may reflect a potential toxicity of these NPs. The reduction in the relative weight of the kidneys, lungs and stomach with the two doses of ingested IONPs could be explained by the loss of cells or the hypotrophy of these organs caused by the administered NPs. Organ hypotrophy could be the result of a systemic action of IONPs and may be caused by IONPs accumulation or excretion from the organs. In fact, in Abdelhalim and Jarrar, 2012), the authors worked on Gold NPs (GNPs) effects on rat liver and reported that cytoplasmic degeneration and nuclear destruction of the hepatocytes may be the result of GNPs interaction with proteins and enzymes of the hepatic tissue (Abdelhalim and Jarrar, 2012). This interaction is the origin of oxidative homeostasis destabilisation and reactive oxygen species (ROS) generation which in turn may induce stress in the hepatocytes to undergo atrophy and necrosis. These could be the same mechanisms by which the organ weight of the kidneys, the lungs and the stomach deacresed in our study.

The increase in blood constituents suggests a systemic effect of iron oxide NPs mainly in the bone marrow during the process of haematopoiesis following IONP exposure. According to the biochemical assays, administration of IONPs induced ALT disruption depending on the dose used without modification of other enzymes. This result was unexpected since it is known that following exposure to NPs, liver enzymes are usually over-expressed. The same result was, however, reported by Abdelhalim and Moussa few years ago (Abdelhalim and Abdelmottaleb Moussa, 2013) after intraperitoneal administration of GNPs (50 nm) for 3 successive days. In our experiments, ALT activity was unchanged using D1 whereas D2 increased ALT activity. This

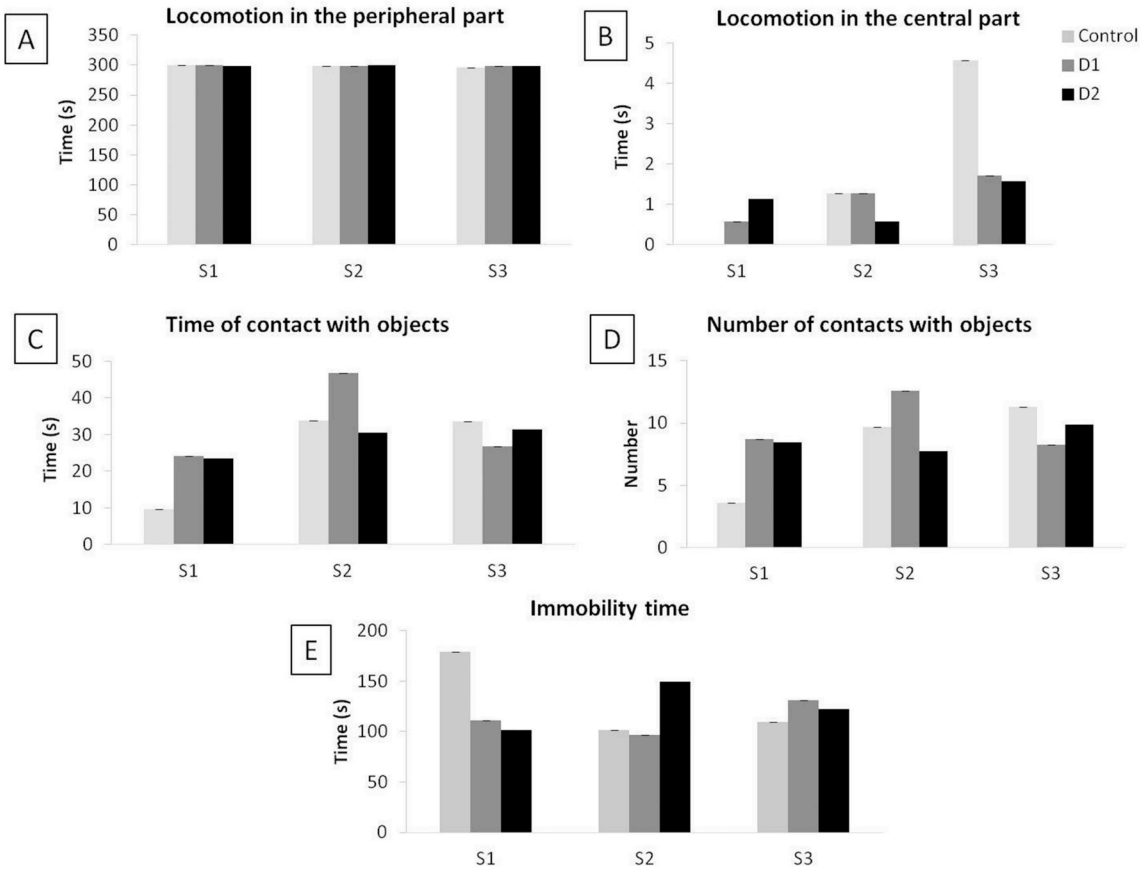

Fig. 4. Effects of acute oral administration of IONPs at 100 (D1) and 200 mg/kg (D2) on locomotion and exploration assessed with the Open field behaviour test (Sessions (S) 1, 2 &3). Data are expressed as means ± SEM, n = 7 animals *per group*.

suggests that the administration of oral NPs of iron disrupts liver function only at the highest dose or that the delay between iron NPs and ALT measurement was sufficient to restore the enzyme activities. Another study was conducted on IONP toxicity by Kumari et al., in 2012. The authors demonstrated that following chronic exposure, Fe_2O_3-30 increased significantly AST and ALT in serum. According to their second published study of the same year, they demonstrated that, similarly, IONPs induced liver damage detected by ALT and AST increase. Nevertheless, our present results disagree with those of the cited study as the authors reported that Fe_2O_3-30 low dose-treated (500 mg/kg) rats were asymptomatic, which is not the case in our study as 100 and 200 mg/kg showed various effects on rats. The effects of IONPs on liver had been demonstrated earlier with other routes by biochemical tests and histopathological examination. In the present study, the histopathological examination showed liver inflammation and necrosis following the acute oral administration of IONPs at both administered doses. These findings are in concordance with our previous observations after a subacute intranasal administration of IONPs (Askri et al., 2018a). Interestingly, we evidenced iron deposits in the Kuppfer cells and in hepatocytes' nuclei using Perl's blue staining with both doses. This demonstrated that even after a unique exposure at a dose largely lower than the lethal dose, IONPs could induce inflammation and necrosis and could be accumulated locally in the liver without significant overall increase in hepatic iron concentration as demonstrated by ICP-MS measurements. This finding is related to the decrease in plasma iron level detected in the IONP exposed groups. Indeed, in response to inflammatory processes there is an increase of macrophages that use the plasmatic iron to deal with inflammation (Recalcati et al., 2010; Gammella et al., 2014; Gustafson et al., 2016). Furthermore, Kumari et al., 2012a, showed that IONPs (Fe_2O_3-30 at the highest dose 1000 mg/kg/day) induced serious alterations in the liver tissue such as dilated central vein, focal areas of necrosis and perivascular round cell collection (Kumari et al., 2012b). The highest dose induced also focal tubular damage in the kidneys, red pulp congestion and prominent white pulp in the spleen. However, heart and brain sections were not affected by the IONPs with no alterations in their tissue architecture. The results obtained by Kumari et al., are in concordance with the results we obtained with the two doses 100 and 200 mg/kg.

Regarding the distribution of trace elements in the organs, we found a stability of Mn, Zn, Cu and Fe in the liver and stomach. Similarly, Chua and Morgan reported no change in manganese levels in the liver after oral iron overload (Chua and Morgan, 1996). Our results disagree with those of Vayenas et al., who reported an increase in Fe, Mn and Zn levels in the liver after iron overload (Vayenas et al., 1998). In addition, in the study performed by Sheida et al., intraperitoneal injection of iron NPs was accompanied by significant changes in trace element concentrations in the brain, 7 days after injection (Sheida et al., 2017). The increase in iron was associated with a decrease in Zn, Cu and Mn relative to controls. The effects on trace element biodistribution seems to be mostly dependent on the dose, the duration of overload, the study design as well as the administration route as underlined by our previous studies (Askri et al., 2018a,b; Sheida et al., 2017).

To explore the cognitive function and cerebral effects of IONPs, we performed various behaviour tests that cover anxiety, locomotion, exploration, learning, and memory. To our knowledge, we conducted the unique *in vivo* study reporting the effects of an acute oral exposure to iron oxide nanoparticles on brain neurotransmitters and cognition. Iron Oxide NPs had no impact on the cognitive performance of Wistar rats. Indeed, using the elevated plus maze no significant effect was found on behaviour related to emotions or more specifically to anxiety. This result has already been evidenced by two other routes of IONPs administration (Askri et al., 2018a,b) and has been demonstrated with other

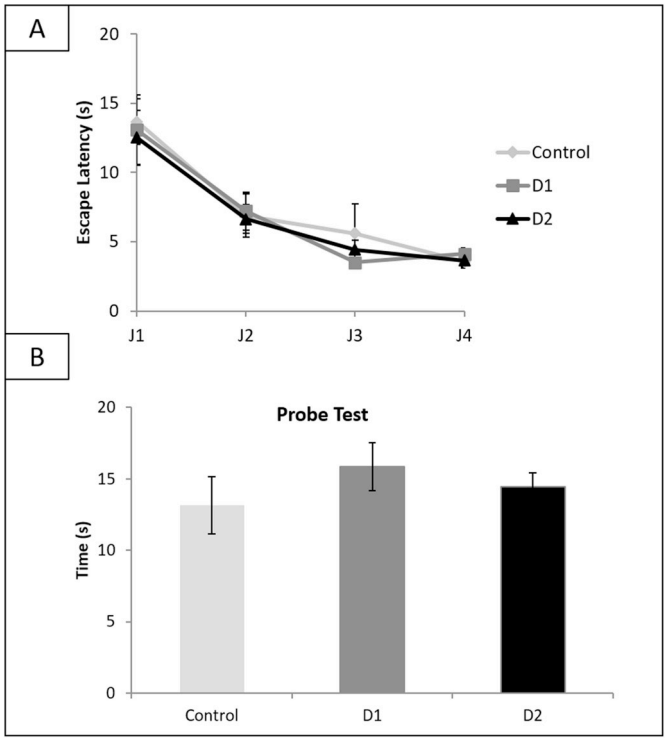

Fig. 5. Effects of acute oral administration of IONPs at 100 (D1) and 200 mg/kg (D2) on the cognitive capacities of Wistar rats evaluated by Morris water maze: **A)** Escape latency to reach the platform during acquisition after oral administration of IONPs and **B)** the spent time in the area where the platform was located on the previous day of the test. Data are expressed as means ± SEM, n = 7 animals *per group*.

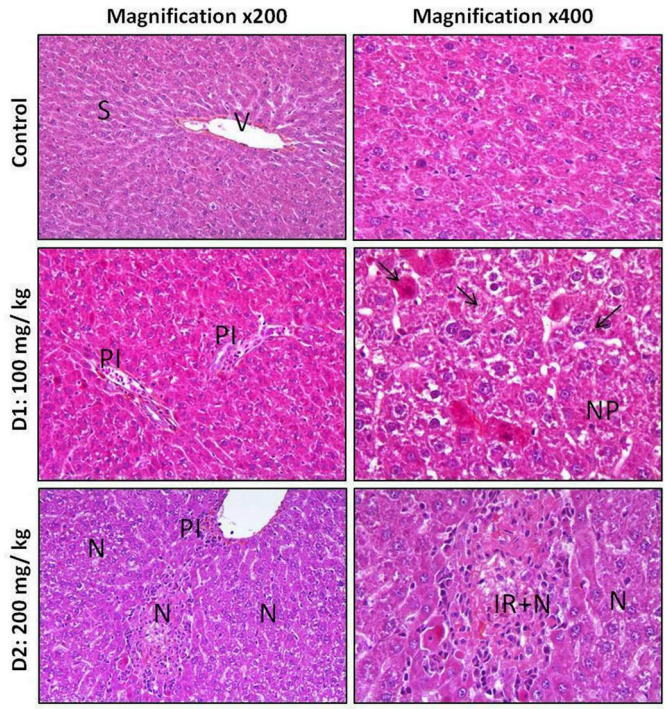

Fig. 7. Histopathological modifications of rat liver tissues following acute oral exposure to IONPs at 100 (D1) and 200 mg/kg (D2) compared to control rats using H&E stain. S: Sinusoid, V: Vein, PI: Portal Inflammatory Infiltrate, NP: Nuclear Pyknosis, arrows indicate hepatocytes with abundant eosinophilic granular cytoplasm and cytoplasm condensation. N: Necrosis and IR: Inflammatory Response.

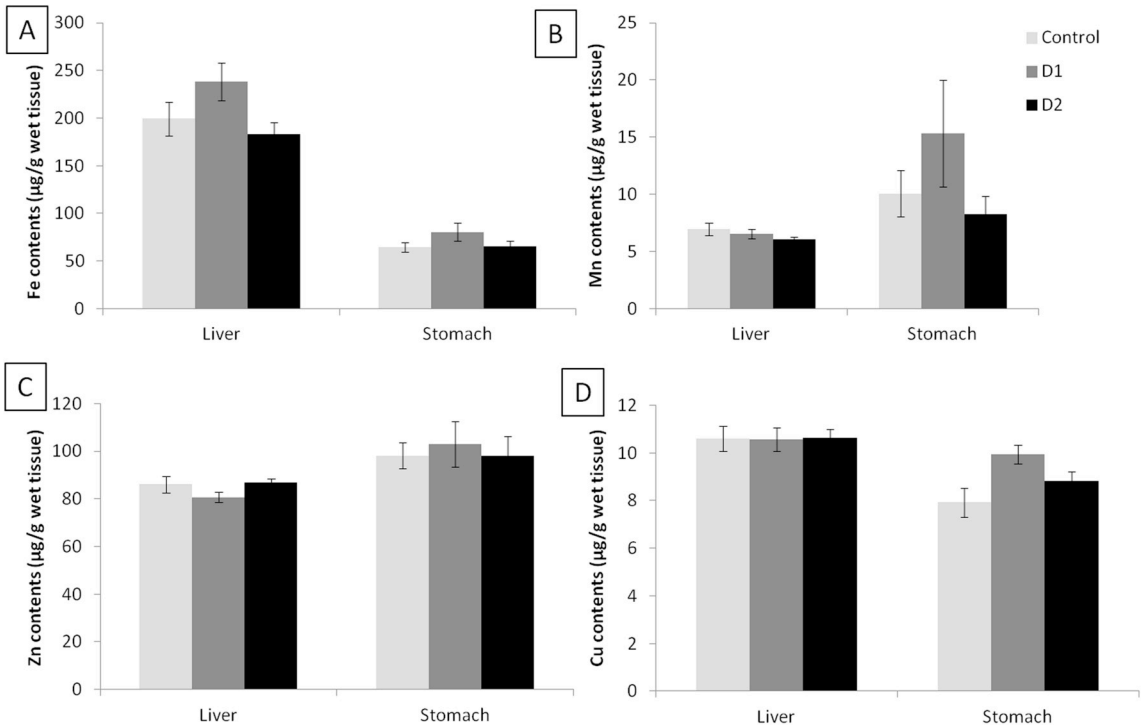

Fig. 6. Effects of acute oral administration of IONPs at 100 (D1) and 200 mg/kg (D2) on trace element contents in liver and stomach. Data are expressed as means ± SEM, n = 7 animals *per group*. * *p-value* < 0.05 using one-way ANOVA and Tukey's post hoc tests.

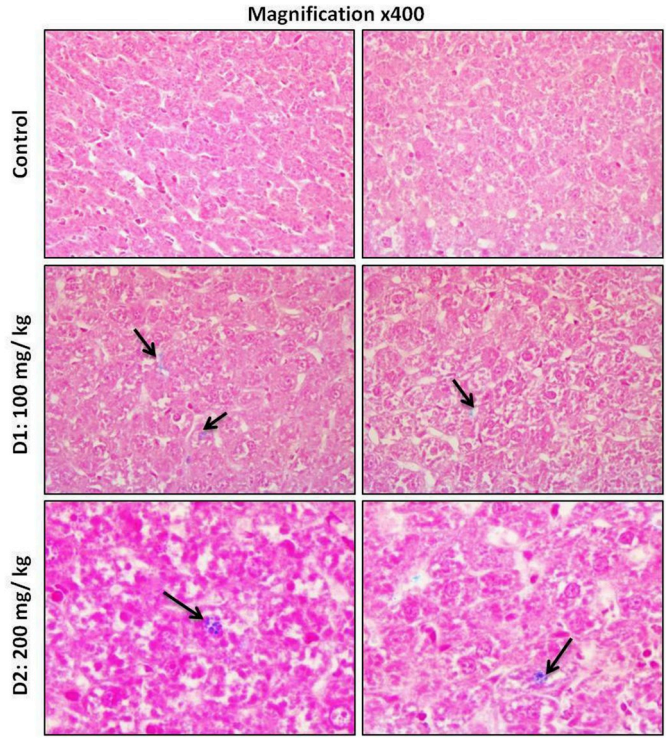

Fig. 8. Iron deposits of rat liver tissues following acute oral exposure to IONPs at 100 (D1) and 200 mg/kg (D2) compared to control rats using Perl's blue stain. Arrows indicate iron deposit in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

NPs (Amara et al., 2014; 2015; Slama et al., 2015; Younes et al., 2015). In contrast, Sheida et al., using the "Open field" test, showed that intraperitoneal administration of IONPs increased anxiety and fear in rats and decreased locomotor activity in rats treated at a dose of 14 mg/kg (Sheida et al., 2017). In addition, experiments with Morris water maze test demonstrated that rats' exposure to IONPs did not alter spatial learning and memory. Control and treated rats were able to improve their performance during tests in the same way, suggesting similar cognitive flexibility. Interestingly, in 2018, Du et al., evidenced that increased levels of brain iron correlated with decreased cognitive function in Alzheimer's disease (Du et al., 2018). Thus, the unchanged cognitive capacity observed in the present study could be related to the stability of iron in the brain following the acute oral administration of IONPs. Moreover, it has been reported that a decrease in Zn levels in brain may be considered as one of the mechanisms of disorders related to higher nerve activity, namely cognitive decline, impaired memory and changes in motor activity (Sheida et al., 2017). To sum up, we hypothesize that the stability of behaviour and cognitive abilities of rats after exposure to iron NPs observed in our study might be related to the stable concentration of this trace element in the brain.

As a mineral, iron is known to be more toxic when administered intravenously. Intramuscular injections are less toxic, and oral iron is the least toxic, probably because the amount of iron absorbed orally is not equal to 100% of the ingested dose. However, we showed that when iron is in nanoparticulate form, the intravenous route seems to be safer than oral and intranasal administrations (Askri et al., 2018a,b). Additionally, in the case of oral administration, the effects of NPs were more pronounced at the hepatic and stomach levels compared to intranasal administration. Interestingly, this kind of studies i.e. integrative studies using the same IONPs was recently performed on zebrafish by Villacis et al. (2017). In their study, the authors combined classical (genotoxicity, oxidative stress) and molecular (transcriptomic) methodologies (Villacis et al., 2017). In the experimental protocol they exposed adult zebrafish for 96 h to five sub-lethal IONP concentrations, ranging from 4.7 to 74.4 mg/L. They concluded that IONPs induced considerable genotoxic effects combined with cell growth decrease and disability of the cell produce new proteins. Nevertheless, oxidative stress induction by the IONPs was limited.

5. Conclusions

Our findings contribute to a better understanding of the physiological effects of the ingested IONPs on rats. The unique oral administration of IONPs has shown that they have no major effects on Wistar rats' health. The neurobehavioral examination conducted the day after gavage showed that oral exposure to IONPs did not affect nor rat emotions, exploration and locomotion, nor the spatial reference memory status. However, at the haematological and biochemical levels, oral administration of IONPs induces disturbances of certain parameters depending on the dose, among others polycythaemia and the decrease in plasma iron concentrations. The histopathological examination evidenced inflammatory effects of IONPs on the liver with iron deposits in hepatocytes and Kuppfer cells 10 days after the administration. Finally, oral administration of IONPs did not disrupt the trace element homeostasis. Altogether, our data are worthwhile for other researchers to establish further studies and provide deeper knowledge on animal and human exposure to IONPs. Therefore, the oral ingestion of IONPs should be further investigated and well documented to better evaluate both the acute and chronic toxic risks of oral exposure to NPs by nutrition, medication and intoxication in order to define the LOEL and NOEL (No Observed Effect Level) doses for Human.

Declaration of interests

The authors report no conflicts of interest in this work.

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Transparency document

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