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Review

TOXICITY FACTORS OF MAGNETITE NANOPARTICLES AND METHODS OF THEIR RESEARCH

E.M. Vazhnichaya[1*], O.V. Semaka[1], R.V. Lutsenko[1], N.O. Bobrova[1], Yu.A. Kurapov[2]

[1]Poltava State Medical University, Poltava, Ukraine
[2]Institute of Electric Welding named after E.O. Paton of the National Academy of Sciences of Ukraine, Kyiv, Ukraine

*Corresponding author: vazhnichaya@ukr.net

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Among nanoparticles (NPs) of metal oxides, magnetite NPs are the most well-known. The need for regulations related to the safety of magnetite NPs requires a deep understanding of their toxicological paradigm. The purpose of the presented review is to analyze the methods of studying the magnetite NPs toxicity and to summarize their toxicity factors based on the literature data. Literature sources were searched in the PubMed database, and 99 works were selected, supplemented with articles from other databases in some cases. It is shown that the study of the magnetite NPs toxicity became widespread during the last decade, reflecting the expansion of the list of synthesized magnetic NPs and the awareness that the prospects for their use depend on the safety of the created nanomaterial. The safety assessment of magnetite NPs on cell lines is the most popular. Primitive and more highly organized animals can be used to evaluate various aspects of the magnetite NPs toxicity. The toxicity factors of magnetite NPs depend on their characteristics (core composition, coating, size, and shape) and the mode of application (concentration, dose, exposure, type of cells, or animal model). One of the main mechanisms of nanomagnetite toxicity is the interference with iron metabolism and increased generation of reactive oxygen species leading to the disruption of cell proliferation, viability, and metabolism. Thus, the toxicity of magnetite NPs is studied by various methods and at different levels of living systems. Understanding the mechanisms of nanotoxicity should contribute to the targeted design of safe magnetic NPs.

Keywords: magnetite nanoparticle; nanotoxicity assessment; toxicity factor; toxicity mechanism.

Introduction

The European Commission defines nanomaterials as natural, random and industrial materials that contain particles of which 50% or more have one or more external dimensions in the range of 1−100 nm [1]. Due to their unique properties, such as solubility, specific surface area, aggregate state, conductivity, and high tensile strength, metal-based nanoparticles (NPs) attract the attention of scientists and practitioners [2]. Among NPs of metals and their oxides, NPs of magnetite, the iron oxide II, III (Fe_3O_4), are among the most well-known. They are characterized by superparamagnetic properties, biocompatibility and the presence of iron, which can be included in metabolic processes [3]. Biomedical application of magnetite NPs is based on their high biocompatibility, ability to cross biological membranes, appropriate surface architecture, and easy connection with ligands [4]. The surface of these NPs can be modified by coating with various materials to stabilize the NPs in certain media, allow the binding of other molecules or extend the time of recognition by the immune system [5]. On their basis, preparations for contrast in magnetic resonance imaging [6], remedies for the treatment of cancer [7] and anemia in patients with chronic renal failure [8] have been developed. Due to their unique chemical and physical properties, magnetic NPs are becoming an important class of biomedical functional nanomaterials in areas such as hyperthermia, drug release, tissue engineering, theranostics, laboratory diagnostics, and blood loss therapy [9−12]. Magnetite NPs can be used to protect the environment from contamination by arsenic, mercury [13, 14] or certain medicinal substances [15]. They have prospects of use for the intensification of biotechnological processes [16]. Nanomaterials based on magnetite NPs are used in catalysis, in particular in electrocatalysis, organic synthesis, catalytic synthesis of biodiesel [17], optics, nanophotonics [18], and electronics [19].

The factor limiting the use of magnetite NPs is their potential toxicity. As magnetic NPs have promising applications in life sciences, industry, ecology, and medicine, the risks to exposed professionals, the general public, and patients are increasing. The lack of regulations, guidelines and harmonized standards as well as limitations related

to their use in clinic in the context of safety and toxicity, requires a deep understanding of the toxicological paradigm of magnetite NPs. Therefore, their toxicological studies are widely conducted and mechanisms of the harmful effects of these NPs are analyzed [20]. The safety assessment of magnetite NPs concerns individual parts of ecosystems [21, 22], the anthropogenic environment [23], and the human body [24]. The ambiguity of the results and its dependence on many factors justify the need to determine the toxicity parameters in each specific case of the new iron oxide NPs fabrication, but to date there is no a single protocol for determining nanotoxicity [25]. This leads to the need to systematize the experience of studying the toxicity of magnetite NPs by various methods and to determine the main factors of toxicity that are established using these methods.

The purpose of the work is to analyze the existing methods of studying the toxicity of magnetite NPs and to generalize ideas about the factors of their toxicity based on the literature data.

Methods and objects of studying the magnetite nanoparticles toxicity

The literature search was conducted in the PubMed database using keywords related to the purpose of the review (magnetite NPs toxicity, magnetite NPs cytotoxicity, magnetite NPs animal toxicity, magnetite NPs toxicity factors, magnetite NPs safety, etc). After familiarization with titles and abstracts, 99 works were selected, full texts of which are used in this review. In few cases, they were supplemented with articles from other databases (4 references).

It is shown that the study of magnetite NPs toxicity became widespread during the last decade, which is confirmed by the total number of publications on this problem. If for the period 2000–2009, 56 publications were found in PubMed for the query "magnetite NPs toxicity", then for the period from 2010 to 2023, their number increased to 1511, reaching an annual maximum (191 sources) in 2019. At the same time, there was an increase in the number of published research results on the molecular and cellular toxicity of nanomagnetite, its toxicity in experiments using laboratory animals, and the improvement of methods of the magnetite NPs toxicological studies. Obviously, this process reflects both the expansion of the list of synthesized magnetic NPs with different functionalization and the recognition the idea that the prospects for their practical use largely depend on the safety of the created nanomaterial.

The engineered magnetite NPs mentioned in the article were obtained using chemical, physical and biological methods with a prevalence of chemical methods [26]. Among them, there were both uncoated magnetite NPs and NPs coated with inorganic and organic substances [3, 5].

In the *in vitro* experiments, the cell viability assay, cell cycle analysis, apoptosis assay, gene expression analysis, investigation of the NPs internalization in cells, hemolysis, lactate dehydrogenase assay, detection of lipid peroxidation and antioxidant protection are used the most often. In the *in vivo* experiments, there are conducted the behavioral and biochemical assessment, morphological analysis, histopathology analysis, apoptosis assay, biodistribution study, embryotoxicity and teratogenicity assays. Examples of these methods applications we can find in the review published by Malhotra *et al.* [20].

Safety assessment of magnetite NPs on cell lines (*in vitro*) is technically accessible, well managed and inexpensive [27, 28]. Optical microscopy, electron microscopy, and atomic force microscopy based on observations of the image of internalization of NPs in cells [29], remain the most widely used methods for the analysis of cell viability, proliferation, and differentiation. In addition, gene expression analysis, proteomics and metabolomics are new methods that facilitate the study of mechanisms of the NPs toxicity [30, 31]. Studying the cell in all these parameters is valuable for initial biocompatibility, interactions between magnetic NPs and the cell membrane, and for testing aggregation and basic indication of any physiological effect. At the same time, when using the mentioned methods to determine the toxicity of iron oxide NPs, it should be taken into account that the presence of metal ions and (or) reactive oxygen species (ROS) formed under their influence can give false results of classic tests. for example, tests with tetrazolium derivatives [32] or neutral red dye [33].

The toxicity of iron oxides NPs is studied in models using cells isolated from the human or animal body, primarily blood cells [34–36] (Table 1). One of these studies showed that magnetite NPs are compatible with donor blood and do not cause damage to erythrocytes, leukocytes, and platelets when incubated at +20 °C for 2 h [35]. It was also described that ultra-small superparamagnetic magnetite NPs coated with dextran (ferumoxtran-10) did not show any toxic effect when interacting with

human monocytes and macrophages at a concentration of 1 mg/ml for more than 72 h [37]. At the same time, according to other data, magnetite NPs stabilized by citric acid (11.44 nm) promoted the release of significantly more hemoglobin from erythrocytes than in the control, which indicated a hemolytic effect that was dose-dependent [34].

During the *in vitro* study of eryptosis indices, magnetite NPs with an average size of 72.6 nm caused a significant deterioration of the condition of erythrocytes at a concentration of 25 μg/ml and were able to produce pathological changes in cell membranes, abnormal calcium levels in the cytosol, and oxidative stress, which caused programmed cell death *in vivo* [36].

There are many toxicological studies of magnetite NPs on various cell lines (see Table 1). The following examples can be cited from a large number of works. The study of the toxicity of uncoated magnetite NPs and NPs coated with n-octyltrieth-oxysilane (<20 nn) in PC12 (rat pheochromocytoma) and ReNcell VM (human neural stem cells) cell cultures at concentrations of 0.003–0.39 mg/ml for 24 h was described, when the results indicated an increase in the cytotoxicity of NPs by presence of the hydrophobic coating [38]. In another work, uncoated NPs with a size of 20–60 nm (1 ng/ml) were used to treat cells of the NRK-52E line (rat kidney epithelium), which made it possible to establish an increase in glutathione-related proteins and chaperones for the protection of NRK-52E cells from apoptosis by the method of comparative proteomics [39]. The effect of uncoated and amino acid-functionalized magnetite NPs obtained by one-step "green" synthesis was studied on cells of the HFF2 line, which demonstrated the absence of cytotoxicity at concentrations of 0.049–0.373 mg/ml [40]. The lack of cytotoxicity of magnetic NPs coated with dimercaptosuccinic acid was also established when they were studied on NCTC 1469 non-parenchymal hepatocytes, when such NPs had no significant effect on the cell viability, oxidative stress, cell cycle, or apoptosis at a concentration of 0.5 mg/ml [41].

Table 1: Cells and cell lines used for testing of magnetite nanoparticles cytotoxicity (mentioned in this article)

Cells and cell lines for nanotoxicity testing	References
Blood cells (erythrocytes, leukocytes, thrombocytes)	[35]
Human monocytes and macrophages	[37]
Human erythrocytes	[34, 36]
Rat erythrocytes	[60]
PC12 (rat pheochromocytoma) ReNcell VM (human neuronal stem cells)	[38, 56]
NRK-52E (rat renal epithelium)	[39]
HFF2 (human embryonic stem cells)	[40]
NCTC 1469 (murine non-parenchimal hepatocytes)	[41]
Mesenchimal stem cells of the cord and bone marrow	[42]
3D spheroids of the primary neuron-like cells	[43]
Caco-2 (human colorectal adenocarcinoma), HepG2 (human hepatocellular carcinoma), MDCK (Madin-Darby canine kidney cells), Calu-3 (human lung adenocarcinoma), Raw 264.7 (murine macrophage cell line)	[47]
BeWo b30 (fetal choriocarcinoma cells)	[55]
TK6 (human spleen lymphoblasts)	[62]
SK-Hep-1, Hep3B (human hepatoma cells)	[63]
Fibroblasts of the human periodontal ligament, murine dermal fibroblasts	[67]
D384 (human meduloblastoma cells), SH-SY5Y (human neuroblastoma cells)	[70]
HaCaT (human skin keratinocytes), HepG2 (human hepatocellular carciunoma)	[75]
SH-SY5Y (human neuroblastoma cells), A172 (human glioblastoma cells)	[76]
MCF-7 (human breast cancer cells)	[77]

Human stem cells represent an innovative cell-based model for primary screening and testing for the magnetite NP toxicity. For this purpose, it has been proposed to use mesenchymal stem cells obtained from the membrane lining of the umbilical cord [42]. The data of this study were compared with the results obtained using bone marrow mesenchymal stem cells, and it was observed that cytotoxicity occurred in cord cells at a tenfold lower concentration and only in them there was a decrease in cell density and loss of monolayer properties at ≥50 μg/ml.

New *in vitro* models are proposed for evaluating NP-induced neurotoxicity based on the differentiation of human umbilical cord mesenchymal stem cells to three-dimensional (3D) spheroids of primary neuron-like cells [43]. When applied at the beginning of neurogenic induction and simultaneous formation of 3D structure, a nociceable concentration- and time-dependent cell death was observed: the effect started early (day 2) and at a low concentration (1 μg/ml). It increased (80% mortality) after a long exposure period (day 6) and the high concentration use (50 μg/ml), which was accompanied by a decrease in the level of ATP and neuronal markers. In this model, NPs applied at full 3D structure formation still caused toxic effects, although less severe.

Both primitive and more highly organized organisms can be used to assess various aspects of the iron oxides NPs toxicity (Table 2), while animals of different gender and at different stages of ontogenesis, including embryo- and fetogenesis, are used.

Invertebrate organisms are used as models for studying the toxic effects of magnetite NPs. For example, it is known a study conducted for toxicological evaluation of iron oxide NPs and NPs integrated with zeolite in mussels for 1, 3, and 7 days. Both types of NPs caused changes in the physiology and oxidative stress of mussels at concentration ranges of 10 and 50 mg/l iron oxide NPs and 50 and 100 mg/l iron oxide NPs-zeolite [44].

Among vertebrates, fish are quite often used to study nanotoxicity. There is a work that compared the toxicity of iron oxide NPs with iron salts in *Capoeta fusca* fish. The study demonstrated that magnetite NPs were the least toxic, but their exposure, like iron salts, caused histopathological abnormalities in the gills and intestines of fish [45]. It was found that uncoated magnetite NPs (15 nm) can cause behavioral and biochemical changes in zebrafish. Incubating adult zebrafish with a low dose (1 ppm) or a high dose (10 ppm) of these NPs for 14 days revealed significant changes in aggressiveness, speed and locomotor behavior in combination with changes in the content of neurotransmitters and stress hormones in the brain of fish at a high dose of NPs [45].

Amphibians can replace rodents in toxicological experiments with magnetite NPs [46]. For example, the toxicity and biodegradation of magnetite-zinc NPs were studied *in vivo* on embryos of the frog *Xenopus laevis*. Abnormal phenotypes with swelling and deformation of the gastrointestinal tract were detected. A short-term exposure for 72 h showed the preferential uptake of NPs with overexpressing of metal transporter proteins, whereas after a long-term exposure for 120 h, activated genes involved in metal accumulation returned to baseline levels for both iron and zinc, indicating that at the stage of the long-term exposure, the absorption process of NPs is significantly lower due to the processes of metabolism, distribution and excretion [47].

Table 2: Species of animals used for testing of magnetite nanoparticles toxicity (mentioned in this article)

Species of animals for studying of nanotoxicity	References
Fruit flyes (*Drosophila melanogaster*)	[82]
Mussels (*Mytilus galloprovincialis*)	[44]
Snails (*Cornu aspersum)*	[73]
Ray-finned fish (*Capoeta fusca*)	[45]
Zebrafish (*Danio rerio)*	[58, 68, 73]
Prussian carp (*Carassius gibelio)*	[73]
Spur frogs (*Xenopus laevis*)	[47]
Wistar rats (*Ratus norvegicus)*	[48–50, 52]
Sprague-Dawley rats (*Ratus norvegicus)*	[75]
Laboratory mice (*Mus musculus*)	[51, 59, 81]

To this day, numerous toxicological studies of magnetite NPs are conducted on mice and rats. In particular, the dependence of the toxicity of magnetite micro- (1 μm) and nanoparticles (10 and 50 nm) on their size was studied when administered to rats three times a week for 5 weeks at a dose of 500 mg/kg [48]. In another study, the toxicity of iron oxide NPs obtained by the method of "green" synthesis with carob leaf extract (10 mg/kg) was evaluated for inclusion in certain areas of the brain of Wistar rats. It was observed the degeneration of neurons in the hippocampus and striatum against the background of a violation of iron homeostasis in the brain [49]. The research of the effect of sublethal doses (equivalent to 0.1, 1, and 10% LD_{50}) of magnetite NPs coated with sodium oleate on the liver structure of female Wistar rats is also reported. It demonstrated a violation of the activity of antioxidant liver enzymes 1 week after the NPs administration with the most pronounced changes in the glutathione peroxidase and glutathione-S-transferase activity, increased mitochondrial respiration 2 weeks after the injection of 10% LD_{50} and histopathological changes (slight necrosis and lipidic changes of the sinusoidal space) [50]. It is also described that pregnant female mice were used to study the toxic effect of NPs on the liver of newborns. In this case, magnetite NPs produced more violations in the morphology of the organ compared to the previous study (lymphatic infiltration, vacuolization of the cytoplasm, and apoptosis of hepatocytes), which allowed to conclude about the moderate toxicity of the NPs for the liver of newborns, assuming that they can penetrate into the blood or lymphatic circulation of the embryo and accumulate in the embryonic liver [51].

The gonadotoxicity of magnetite NPs was studied in male albino rats [52]. The experimental groups were intraperitoneally injected with NPs at doses of 5 and 10 mg/kg of body weight three times a week for 60 days, and a significant decrease in the body weight was noted in the group receiving a high dose of NPs. Rat testicular tissue displyed degenerative changes. Parameters of the count, motility, and viability of spermatozoa were reduced. Along with the morphological abnormalities of the sperm, a decrease in the testosterone level, an increase in the content of malondialdehyde, a decrease in the activity of antioxidant enzymes and damage of testicular DNA were found [52].

Toxicity factors of magnetite nanoparticles

The toxicity of iron oxide NPs is associated with such factors as the nature of NPs, size, shape, surface modification, concentration (dose), duration of exposure as well as the type of cells on which these particles act (Fig. 1) [53, 54].

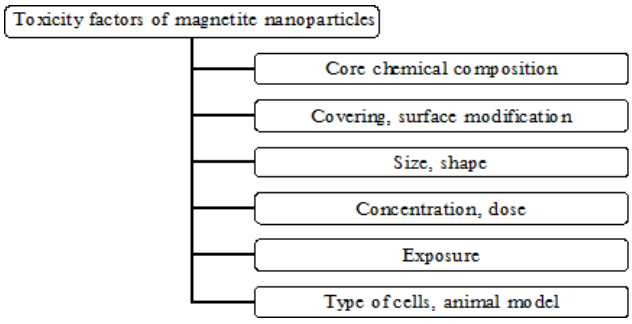

Figure 1: Toxicity factors of magnetite nanoparticles, depending on their characteristics (core composition, coating, size, and shape) and the mode of application (concentration, dose, duration of exposure, and type of cells or animal model)

The dependence of the NPs toxicity on their nature can be illustrated by the work in which uncoated magnetite NPs, magnetite NPs coated with sodium oleate, and rhodamine-labeled silicon dioxide NPs (25 and 50 nm) were used to evaluate toxicity parameters in the BeWo b30 placental barrier cell model by lactate dehydrogenase leakage. The results demonstrated that the cytotoxicity of iron oxide NPs was more evident at low doses after the short exposure compared to silica particles [55]. When comparing the cellular toxicity of uncoated magnetite and maghemite NPs on rat pheochromocytoma PC12 cells at concentrations from 0.1 to 0.5 mg/ml for 1 to 72 h, it turned out that different NPs of iron oxides interact differently with cells [56]. Uncoated magnetite NPs did not enter the cells, were on their outer surface, did not show a cytotoxic effect up to a concentration of 0.1 mg/ml, and 51% of the cells remained viable at 0.25 mg/ml after 72 h. Under the same conditions, maghemite NPs showed maximum interaction and penetration into cells without a cytotoxic effect at any of the studied concentrations.

In many toxicological investigations, the dependence of the magnetite NPs toxicity on the coating has been demonstrated. In the study mentioned above [56], it was also found that unlike

uncoated magnetite NPs, which did not penetrate cells and left more than half of the cells viable at 0.25 mg/ml after 72 h, starch-coated magnetite particles formed heterogeneous aggregates, and after 72 h reduced cell viability to 70% already at a concentration of 0.1 mg/ml. In another experiment, magnetite NPs were functionalized with polyethylene glycol and polyurethane with two different chain sizes (5 and 15 kDa) and showed that the cytotoxicity of coated NPs with a short chain is much higher than with long chains [57]. To understand the effect of coating on the toxicity of magnetite NPs, the authors used transcriptome sequencing and real-time quantitative polymerase chain reaction in the gills and liver of zebrafish exposed to uncoated NPs and starch-stabilized particles [58]. It was shown that the result of a 7-day exposure to magnetite NPs depended on both the coating and the tissue in which it was studied. Uncoated NPs showed greater toxicity than starch-coated ones in gills; conversely, magnetite-starch NPs caused more severe liver damage. It is believed that the administration of uncoated magnetic NPs *in vivo* leads to structural and functional toxicological modifications of vital organs, while the coating of these NPs with biocompatible and biodegradable polymers can significantly reduce the toxicity of these NPs, which was confirmed by comparing the effects of uncoated NPs and particles with a triple polymer coating on the albino mice [59]. When studying the hematotoxicity of magnetite NPs in a sodium chloride matrix synthesized by the electron-beam physical vapor deposition and their analogues with post-synthesis functionalization with ethylmethylhydroxypyridine succinate, polyvinylpyrrolidone or both of these agents, it was found that the presence of a synthetic antioxidant in the NP coating reduces their damaging effect on erythrocytes, which was confirmed by a lower content of cells with a pathological shape, weaker hemolysis and a smaller violation of the asymmetry of the erythrocyte membranes [60]. It was also tested the idea of whether it is possible to reduce the toxicity of magnetite NPs if their surface is functionalized with inert substances. Through a comprehensive assessment of the behavior, biochemistry, and morphology, it was obtained the evidence which supports the idea that surface modification of magnetite NPs with a carbon coating can indeed increase their biosafety *in vivo* [61].

However, there are conflicting data regarding the role of coating in the toxicity of magnetic NPs. When tested *in vitro* on TK6 lymphoblastic cells and human primary blood cells, uncoated magnetite NPs were found to be non-cytotoxic, and conversely, oleate-stabilized NPs were cytotoxic in a dose-dependent manner and induced DNA damage, suggesting genotoxic potential [62]. In this case, the coating clearly changed the behavior and cellular uptake of NPs, causing pathological morphological changes in the cells.

It was established that magnetite NPs have higher toxicity than microparticles, but within the nanorange no significant differences depending on size are observed [48]. At the same time, there is a work where size-dependent differences in the cytotoxicity were found in two types of human hepatoma cell lines (SK-Hep-1 and Hep3B) and within the nanorange [63]. 6 nm magnetite NPs showed the little cytotoxicity, 9 nm NPs affected cells through the mitochondrial dysfunction and induction of ROS-mediated necrosis, while 14 nm particles caused the cytotoxicity by disrupting plasma membrane integrity and promoting massive leakage of lactate dehydrogenase.

In the whole organism, the toxic effects of iron oxides NPs are related to their pharmaco/toxicokinetics [64], in which size plays a major role. It is known, that magnetite NPs smaller than 10 nm are quickly eliminated by the kidneys, while particles larger than 200 nm are captured by the organs of the reticuloendothelial system, especially spleen [65].

Biodistribution of magnetic NPs in the organism is an important parameter for study on their toxicity. This parameter is essential for medical applications. It allows to determine, whether NPs are captured by the desired organs or accumulated in other tissues which may lead to potential toxicity [66]. The sites of their first uptake are usually the liver, spleen, and lymph nodes, but with the appropriate design of magnetite NPs, they can also be accumulated in other organs, such as the lungs, heart, or brain, and produce biochemical changes and histopathological lesions. The factors affecting the biodistribution of iron oxide NPs include their size, shape, surface charge, coating molecules, and a route of administration [64, 66].

The greater amount of magnetite NPs introduced into the cell culture or the body relates to the higher risk of their toxic effects [32]. This dependence was common both for magnetite NPs and for NPs of another origin (fullerenes (C60), single-walled carbon nanotubes) studied in the cultures of human periodontal ligament fibroblasts and murine dermal fibroblasts [67]. Malhotra *et al.* incubated adult zebrafish with a low dose (1 ppm) or a high dose (10 ppm) of uncoated magnetite NPs (15 nm) for 14 days and found no significant abnormalities in behavioral and biochemical parame-

ters in the low NPs dose group, while significant changes in the aggressiveness, speed, and motor behavior combined with changes in the neurotransmitters and stress hormones concentration were induced in the brain when zebrafish were treated with a high dose of nanomagnetite [68].

In addition to the size, coating, and dose of NPs, their toxicity, in particular genotoxicity *in vivo*, is determined by the duration of exposure [69]. This fact was reflected in the work where magnetite NPs were evaluated on human astrocyte (D384) and neuronal (SH-SY5Y) cell lines after the short-term and long-term exposure [70]. Short-term exposures caused significant concentration- and time-dependent changes in D384 cells: 25–75% reduction in the cell viability starts at 25 μg/ml for 4 h and at 1 μg/ml for 48 h. When the exposure period was extended (up to 10 days), toxicity was again observed in D384 cell culture (reduction in the number of colonies, morphological changes, and reduction in the colony size), but at lower concentrations (0.05–10 μg/ml). The same study demonstrated the dependence of the magnetite NPs toxic effect on the model system. In this case, SH-SY5Y neuronal cells were less susceptible to the action of NPs: at the short exposure, cytotoxicity occurred only after 48 h at 10–100 μg/ml, and at the long-term exposure, it occurred only at the highest concentration.

The apparent reduction in the toxicity of magnetite NPs upon the long-term exposure observed in some cases can, at least in part, be explained by biodegradation and neosynthesis of magnetic NPs in cells, which occurs inside endosomes and involves the H-subunit of ferritin. This appeared to be a key process to avoid the long-term cytotoxicity as effect on differentiation in stem cells associated with high doses of magnetic NPs [71]

The authors discuss the dependence of findings on the iron oxide NP toxicity on cell culture, tissue, or animal model [72]. The variability of the results of the cytotoxicity assesment of magnetite NPs *in vitro* may be due to the fact that conventional methods neglect such important factors as NPs sedimentation and the absorption of proteins and other important biomolecules on the surface of NPs as well as the fact that the effect of cellular "vision" is ignored (i.e. cell type). By studying superparamagnetic iron oxide NPs with different chemical surface properties in different cell lines, it was shown that modification of conventional toxicity assays and consideration of the concept of "cellular vision" are crucial for obtaining reliable and reproducible nanotoxicological data [54]. *In vivo* experiments on the hemocytes of the snail *Cornu aspersum* for the state of oxidative, proteolytic, and genotoxic action and in the tissues of the *Danio* fish and *Carassius gibelio* for the state of apoptosis proved that interspecies and intertissue differences in the responses are obvious, but a common mechanism is triggerig the magnetite NPs toxicity in the tissues of different animal species [73].

Various studies have reported conflicting results regarding the toxic effects of iron oxide NPs, some claiming no significant toxicity, while others reporting severe effects [74]. In general, most researchers indicate relatively low toxicity of magnetite NPs. In particular, when new citrate-coated magnetite NPs were synthesized and their potential acute toxic effects *in vitro* and *in vivo* were studied, these NPs did not affect the viability of two cell lines (HaCaT and HepG2). In addition, *in vivo* they did not cause changes in biochemical or morphological parameters of vital organs (such as the brain, liver, lungs and kidneys). The iron concentration was slightly increased in the liver, but this result was not considered unfavorable given the absence of concomitant functional consequences [75].

Wanting to facilitate the analytical perception of information on the toxicity factors of magnetite NPs, we combined in Table 3 the characteristics of NPs, research methods, experimental models, used doses, routes and exposure of NPs administration with the results obtained by the authors.

Targets and mechanisms of toxic action of magnetite nanoparticles

If magnetite NPs enter the cells, their toxicity can affect a nuclear activity, cause a leakage of certain substances, or block cell membranes, which can lead to the impaired cell proliferation, viability, and metabolism [76]. Malhotra *et al.* classified the toxic effects of iron oxides NPs according to the mechanism of action on the inhibition of cell proliferation, temporary or complete cessation of the cell cycle, genotoxicity, damage to cellular components, and blocking of cell membranes [20].

Many authors emphasize the oxidative stress caused by magnetite NPs [77–81]. It is believed that the toxicity of iron oxide NPs is associated with the production of ROS, which cause cell damage and death [52, 81]. Oxidative stress is a basis of the NP-induced cytotoxicity and genotoxicity [82]. There is an opinion that the toxicity of iron oxides nanoforms is caused by their accumulation in the endosomal compartments of the cell and the formation of metal ions as a result of the NPs biodegradation [83]. These iron ions are capable of inducing oxidative stress as the main factor in the toxicity of nanomagnetite. They contribute to

Table 3: Summary of magnetite nanoparticles toxicological studies published during the last 5 years (2019–2023) to indicate the variety of magnetite nanoparticles, experimental conditions and obtained research results (mentioned in the review)

Type of magnetite NPs	Size and shape of NPs	Cell culture or model organism	Method of toxicity analysis	Dose and exposure	Results	Ref.
Uncoated MNPs (1), Fe_3O_4@n-octyltriethoxysilane (2)	17.9–18.7 nm	PC12, ReNcell VM cell lines	Cell viability, differentiation, cytochemistry	0.39–0.003 mg/ml, 24 h	Changes in morphology, cell aggregation & death at high concentrations, (2) > (1)	[38]
Uncoated MNPs	20.3 nm, spherical	Cord lining membrane (1) & bone marrow (2) mesenchymal stem cells	Cell viability, differentiation, proliferation, NPs uptake	10-300 μg/ml, passages 3–9	Cytotoxicity at 10 μg/ml (1), at 100 μg/ml (2); cell density & proliferation ↓ at >50 μg/ml	[42]
Uncoated MNPs	20.3 nm, spherical	3D spheroids of human primary neuronlike cells	Formation, differentiation, morphology, cell death, neuronal markers, ATP, NPs uptake	1–50 μg/ml, 10 days	Cell death (day 2, 1 μg/ml), ↑ mortality (day 6, 50 μg/ml), neuronal markers & ATP ↓, morphology alteration (≥5 μg/ml)	[43]
Zinc-doped Fe_3O_4@dopamine, citric acid	12 nm	Caco-2, HepG2, MDCK, Calu-3, Raw 264.7 cell lines; *Xenopus laevis*	Quantitative PCR, elemental analysis, cell viability; morphology of embryos	0–1 mg/ml *in vitro*, 0.5–2 mg/ml *in vivo*, 72–120 h	Toxicity to all cell lines; after 96 h dose-dependent embryo mortality, abnormal fenotypes	[47]
Uncoated MNPs	15.62 nm, spherical	Wistar rats	Body weight, Fe, oxidative stress markers, AchE, histopathology in the brain	i/p, 10 mg/kg	Neuronal degeneration, ↓ Fe in some areas of the brain	[49]
Uncoated NPs	<100 nm	Albino rats	Body weight, immune status, histopathology, sperm parameters, testosterone, oxidative stress markers	i/p, 5 & 10 mg/kg 3 times/week for 60 days	Testicular degeneration, sperm damage, testosterone ↓, immunity ↓, oxidative stress	[52]
Uncoated NPs (1), triple polymer-layered MNPs (2)	<100 nm	Swiss albino mice	Biochemical, histopathological assessment	i/v, 5, 10 & 25 mg/kg,	Biochemical & histological changes in vital organs, ↑ mast cell infiltration & Fe deposition at hgh dose (1).	[59]

Table 3 -End

Type of magnetite NPs	Size and shape of NPs	Cell culture or model organism	Method of toxicity analysis	Dose and exposure	Results	Ref.
Fe_3O_4@NaCl (1), Fe_3O_4@NaCl-EMHPS (2), Fe_3O_4@NaCl-PVP(3), Fe_3O_4@NaCl-EMHPS-PVP (4)	22 nm (1), 39 nm (2), 72 nm (3), 65 nm (4)	Rat erythrocytes	Shape pathhology, hemolysis, cytofluorimetry	1-200 μg Fe/ml	↑ shape pathology (1)(3), ↑ hemolysis at 200 μg Fe/ml, ↑ phosphatidylserine expression at 100 μg Fe/ml (1)(3)	[60]
Uncoated MNPs (1), Fe_3O_4@C (2)	18 nm (1), 24 nm (2)	Adult zebrafish	Behavioral tests, biochemistry of the brain	1−10 ppm, 2 weeks, water-borne exposure	↓ locomotor activity (2) no stress response (2), (2) less toxic than (1)	[61]
Uncoated MNPs	15 nm	Adult zebrafish, zebrafish embryos	Behavioral tests, biochemistry of the brain	1−10 ppm, 2 weeks, water-borne exposure for adult, 1−1000 ppm, 96 h for embryos	Exploratory behavior & social interaction ↓, cortisol, Ach & catalase ↑, serotonin & dopamine ↓ at 10 ppm, LC_{50} > 1000 ppm	[68]
Uncoated MNPs	<100 nm	Wistar rats	Oxidative stress markers, apoptosis, histopathology in the kidney	i/p, 20 mg/kg, 6 days	Oxidative stress, DNA damage, apoptosis, tissue lesions	[74]
Citrate-coated MNPs	7.2 nm, spherical, hexagonal & square	HaCaT & HepG2 cell lines, Sprague-Dawley rats	Cell viability, acute toxic test, organs weight & morphology, biochemical analysis	0.0001−100 μg/ml, 72 h *in vitro*, 2000 mg/kg orally *in vivo*	IC_{50} > 100 μg/ml for both cell lines, no signs of toxicity in rats, Fe ↑ in the liver, biochemical changes	[75]
Silica-coated MNPs (1), oleic acid-coated MNPs (2)	<100 mn	SH-SY5Y neuronal & A172 glial cell lines	ROS generation, antioxidant protection, DNA fragmentation	Wide range concentrations, 3 & 24 h	Oxidative stress, DNA damage	[80]
Uncoated MNPs	2.3, 4.2, 9.3 nm	MCF-7 cell line, ICR mice	Cell viability, ROS generation, NPs uptake, in mice − acute toxicity, NPs distribution, ROS generation	Wide range of doses & time in cell line; i/v, 100 & 500 mg/kg *in vivo*	2.3−4.2 nm − lethal at 100 mg/kg (hepatic toxicity). 9.3 nm − no toxicity, ↑ ROS generation	[81]
Fe_3O_4@SiO_2@APTS ~ Schiff base-Cu(II)	20−25 nm	K562 cell line	Cell viability	1−1000 μg/ml, 48 h	IC_{50}: 1000 μg/ml	[94]

Footnotes. Ach − acetylcholine, AchE − acetylcholine esterase, APTS − (3-aminopropyl) triethoxysilane, ATP − adenosine triphosphate, DNA − desoxyribonucleic acid, EMHPS − ethylmethylhydroxypyridine succinate, IC − inhibitory concentration, i/p − intraperitoneally, i/v − intravenously, MNP − magnetite nanoparticle, PVP − polyvinylpyrrolidone, Ref − References, ↓ − a decrease of the parameter or process, ↑ − an increase of the parameter or process, () − individual type of NPs or cell lines if there are few ones.

the Fenton reaction, which leads to the formation of a large amount of hydroxyl anion radical and ROS. High levels of ROS produced in the cell usually put it in a state of oxidative stress, when proteins, DNA, and lipid structures are damaged, leading to cell dysfunction and toxicity. Also, magnetic NPs activate ROS-related signaling pathways [84], including JNK- and p53-mediated pathways to regulate the cell cycle and apoptosis, reducing neuronal viability [85]. In addition, oxidative stress is accompanied by the increased expression of pro-inflammatory genes and activation of neutrophils and macrophages [86].

The main properties of iron oxide NPs that lead to an increase in ROS production are the presence of pro-oxidant functional groups on the NP surface, particle-cell interaction, and the existence of an active redox cycle on the NP surface [87]. However, claims that oxidative stress is a constant and most prominent mechanism in the toxicity have not been proven in all cases. NPs that have an inactive surface or low solubility can induce toxicity without causing oxidative stress [88]. In addition, in some works, it was shown that during the experimental therapy of animal models of pathology (stress, blood loss), magnetite NPs exerted an antioxidant effect [89, 90].

In most cases, the excess production of ROS and resulting oxidative stress are a basis of the damaging effect of magnetite NPs, but there are other mechanisms as well. During the physicochemical interaction with the surface of the cell membrane, NPs can disrupt the membrane, affecting transport mechanisms, and inside the cells can affect the function of cell organelles, primarily mitochondria and peroxisomes, and intracellular transport (Fig. 2) [91]. At the same time, these violations, in turn, can increase the oxidative stress.

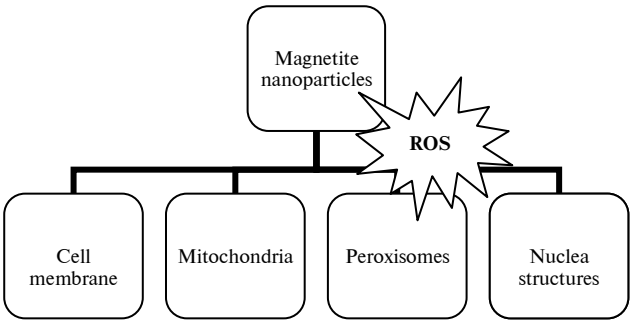

Figure 2: Intracellular targets for the toxic action of magnetite nanoparticles (cell membranes, mitochondria, peroxisomes, nuclear structures) and the reactive oxygen species generation as the main mechanism of the toxicity

Also, some physical mechanisms of magnetic NPs toxicity relating to their magnetism were disclosed in recent time, for example, the chain formation, tropism to membranes of endothelial cells, and participation in gating of mechanosensitive ion channels [92]. A magnetic-field activation of the magnetic particle chains on cell membranes may manifest itself in the following physical effects as arising shear stress in the cell membrane, a change in the activity of mechanosensitive ion channels; the formation of membrane pores followed by the membrane rupture and apoptosis.

Modern ideas about the mechanisms of cell toxicity of magnetite NPs can be summarized as follows [20, 93]. They can cause primary oxidative stress as a result of a direct reaction of the NP surface with cells, which induces the formation of ROS. Also, NPs based on iron oxide are able to release metal ions into cells, which increase the formation of ROS. Secondary oxidative stress can occur through indirect pathways, mainly through the NP-induced mitochondrial dysfunction or failure of antioxidant defense to restore redox balance. In this case, NPs are not directly responsible for increasing free radical oxidation, but affect mitochondria and phagocytes, indirectly increasing the level of ROS in the cell. The NP-induced intracellular ROS generation also modulates the structure and function of lipids, DNA, proteins, and carbohydrates as the main components of the cell, resulting in the damage to cell organelles and membranes. NPs can affect the levels of inflammatory factors, such as tumor necrosis factor-α and interleukins, causing mitochondrial destruction and DNA damage. All of these can ultimately induce activation of the apoptotic response and cell death. When the level of oxidative stress exceeds the body's ability to neutralize it, inflammation, fibrosis, genotoxicity and carcinogenesis can develop.

As we can see, there is a more and more complete awareness of the intracellular mechanisms of magnetite NP toxicity, which allows combining individual facts into a single consistent scheme. Knowledge of these mechanisms expands the prospects of computer modeling and forecasting in the design of new nanomaterials [94]. It also indicates ways to improve the safety of magnetic NPs, for example, with the help of antioxidants [87, 95, 96], by delivery through polymers or by metal-organic frameworks [97].

Although it was possible to prove the principle possibility of practical implementation of magnetic hyperthermia, magnetically controlled targeted drug

delivery and the ability to contrast in magnetic resonance imaging [6, 10, 11], the use of magnetite NPs in medicine is a complex problem, solution of which is still far from being completed. The same applies to the use of nanostructures based on magnetite NPs in ecology, where they are suitable for air purification and carbon absorption, water disinfection and purification, for adsorption/separative removal of organics, dyes, oil, arsenic, phosphate, molybdate, fluoride, selenium, cations of heavy metals, radionuclides, and rare earth elements as well as for environmental monitoring [98].

Despite the obvious successes of fundamental research in the field of nanoscience, the real practical biomedical application of magnetite NPs is not developing so quickly, and a number of nanopreparations based on them is limited [99]. The reasons preventing the widespread practical use ("translation") of magnetite NPs are divided into scientific, clinical, and marketing ones. Among them, there are insufficient understanding of the mechanisms of interaction of NPs with body structures, different behavior of NPs in the *in vitro* and *in vivo* experiments; insufficient knowledge of pharmacokinetic processes involving NPs, ambiguity of data on biosafety and toxicity, possible inadequacy of animal models to events in the human body, lack of strict standards for NPs, changes in the properties of NPs during the transition from laboratory synthesis to industrial manufacture, etc. [100]. In addition to these issues, some problems are caused by the magnetic properties of iron oxides NPs, in particular, the inability to be in a stable equilibrium under the action of magnetostatic forces alone [101], an increased tendency to aggregation due to magnetic dipole-dipole interactions [102], etc. Also, concentration of magnetic material (magnetite or maghemite) in the human heart, spleen, and liver varies from 13.7 to 343 ng/g [103]. It can be assumed that the presence of this biogenic material may have implications for the effect and toxicity of magnetite NPs administered to the human body with a purpose of diagnosis or treatment.

Therefore, to ensure the successful translation of magnetite NPs, it is necessary to find out many more of their features, including those related to the toxicity. However, special physicochemical properties of these NPs, which give them advantages in the creation of new drugs and methods of treatment and diagnostics, are quite real and determine the prospects for further research. Short and long-term toxicity assessment of nanomagnetite, as well as other metal/metal oxide NPs, is of paramount importance to ensure the human and global biome's safety.

Conclusions

The use of magnetite NPs in medicine is a complex multidisciplinary scientific and practical task, solution of which is far from complete. Despite the successes of fundamental research in the field of nanopharmacology and nanotoxicology, reflected in numerous scientific publications, in the real practical biomedical application of magnetite NPs, problems arise related to the complexity and multifactorial processes of the interaction of iron oxide NPs with various biological structures of the human body.

The toxicity of magnetite NPs is studied by various methods and at different levels of the living organisms. Different objects are used for assessment of the magnetite NPs toxicity *in vitro* and *in vivo*. The experimental data show that this toxicity can occur due to the disruption of cellular mechanisms and due to the normal functioning of the entire body. To better understand these mechanisms, multiomic studies using proteomics, genomics, and metabolomics are needed and should be applied in an integrated manner. The toxic effects of magnetite NPs depend on their size, presence and composition of the surface coating, dose or concentration, duration of exposure, and the model on which the toxicity is studied. In the living organism, one of the leading mechanisms of nanomagnetite toxicity is the interference with iron metabolism and increased production of ROS and oxidative stress.

Although magnetite NPs are generally considered biocompatible and have a low cytotoxic potential, these particles are still foreign to the human body and can have certain, including negative, effects on the organs and systems. These interactions and their consequences for the human body cannot always be predicted by conducting preclinical studies *in vitro* and on animal models.

There is an obvious need for the unification and further development of the toxicology of iron oxides NPs, especially magnetite NPs, which will give a more complete picture of the interaction of these particles with cells, tissues, and organs. Understanding the mechanisms of nanotoxicity should contribute to the directed design of edgeless magnetic NPs based on iron oxides as well as a prevention and treatment of possible manifestations of their toxicity. Unique physicochemical properties

of magnetite NPs, which can be considered as their advantages in the creation of new medicines or therapy and diagnostics methods, determine the need and perspective of further research in this field.

Interests disclosure

The authors declare that there are no conflicts of interest.

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О.М. Важнича[1], О.В. Семака[1], Р.В. Луценко[1], Н.О. Боброва[1], Ю.А. Курапов[2]

[1]Полтавський державний медичний університет, Полтава, Україна
[2]Інститут електрозварювання імені Євгена Патона НАН України, Київ, Україна

ФАКТОРИ ТОКСИЧНОСТІ НАНОЧАСТИНОК МАГНЕТИТУ ТА МЕТОДИ ЇХ ДОСЛІДЖЕННЯ

Серед наночастинок (НЧ) оксидів металів найбільш відомими є НЧ магнетиту. Потреба в нормативних актах, пов'язаних із безпечністю НЧ магнетиту, вимагає глибокого розуміння їхньої токсикологічної парадигми. Мета представленого огляду – проаналізувати методи дослідження токсичності НЧ магнетиту та узагальнити фактори їх токсичності на основі даних літератури. Пошук літературних джерел здійснювали в базі даних PubMed. Були відібрані 99 праць, які в деяких випадках доповнені статтями з інших баз даних. Показано, що дослідження токсичності НЧ магнетиту набуло значного поширення протягом останнього десятиліття, що відображає розширення переліку синтезованих магнітних НЧ та усвідомлення того, що перспективи їх використання залежать від безпечності створеного наноматеріалу. Оцінка безпечності НЧ магнетиту на клітинних лініях є найбільш популярною. Для вивчення різних аспектів токсичності НЧ магнетиту можуть бути використані примітивні та більш високоорганізовані тварини. Фактори токсичності НЧ магнетиту залежать від їхніх характеристик (складу ядра, покриття, розміру і форми) та способу застосування (концентрації, дози, експозиції, типу клітин або тваринної моделі). Одним із основних механізмів токсичності наномагнетиту є втручання в метаболізм заліза та посилене утворення активних форм кисню, що призводить до порушення клітинної проліферації, життєздатності та метаболізму. Отже, токсичність НЧ магнетиту вивчається різними методами і на різних рівнях живих систем. Розуміння механізмів нанотоксичності має сприяти цілеспрямованому дизайну безпечних магнітних НЧ.

Ключові слова: наночастинка магнетиту; визначення нанотоксичності; фактор токсичності; механізм токсичності.