# EXPLORING THE IN VIVO TOXICITY OF NANOPARTICLES

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EXPLORING THE \textit{IN VIVO} TOXICITY OF NANOPARTICLES

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Abstract

Toxicological tests of a xenobiotic play a key role to determine the safety of the new compound before it reaches the market. In this review article, we describe the main types of toxicological studies that can be performed in vivo to detect a possible undesired effect of a xenobiotic with especial emphasis on the data available for the different types of nanoparticles. The different procedures described in this review allow to obtain valuable information about the possible toxic effects of a xenobiotic in order to minimize the possible risks for patients once the compound has been approved for therapeutic use.

Keywords: nanoparticles, in vivo toxicity, toxicology, experimental procedures
1. Introduction

Drug or chemical compounds toxicity in animals is one of the key steps in the approval of a new drug and has the objective of identifying possible risks for human health before the drug reaches the market. After thalidomide disaster, toxicity testing has been routinely performed prior to a drug being released into the market. These studies with regulatory purposes are performed following standardized protocols and meeting the good laboratory practice and experimental animal’s protection regulations. Toxicological tests focus on different aspects of the new drug candidate such as molecular mechanisms and the biological targets of the possible toxicity of the compound; species-specific toxic susceptibility, gender and population groups; kinetics and metabolism in different organisms. Finally, all available information for risk assessment of the use of xenobiotics in different therapeutic applications is integrated to predict the undesired consequences in the interaction between xenobiotics and living beings.

In this review article, we describe the main types of toxicological studies in vivo to detect a possible undesired effect of a xenobiotic with especial emphasis on the data available for the different types of nanoparticles (NPs) (Figure 1).

2. Types of toxicological studies.

A good toxicological evaluation of a xenobiotic means that the toxicity of the compound must be study using different tests and during different periods of time. According to this last parameter, we can classify the toxicological studies in:

a) Acute toxicity studies.

The objective of this type of studies consists in completely characterize the acute biological effects of the xenobiotic. They include not only the study on lethality rate (the dose that causes the death in the animal species chosen) but also xenobiotic effect on target organs and the clinical signs associated to those effects. This type of studies also provides us information about the range of doses that can be used during repeated administration toxicity studies in animals.
Acute toxicity studies require the use of animal groups that must be homogenous in age, gender and weight. Most used specie is rat, although other rodents might be also used. Regarding gender, females are preferred since literature surveys of conventional LD$_{50}$ tests show that, although there is little difference in sensitivity between sexes, in those cases where differences are observed, females are generally slightly more sensitive $^3$. However, if previous knowledge of toxicological properties of structurally related chemicals indicates that males are likely to be more sensitive, then this sex should be used for testing. When the test is conducted in males, adequate justification should be provided. Nevertheless, when knowing the maximum tolerated dose is required it is very common to use other species such as rabbits, dogs and apes.

In acute studies it is necessary to keep the animals in an identical environment for at least 5 days before performing the testing procedure. In the unusual circumstance that administering a full single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. After the xenobiotic administration, animals must be observed individually at least once during the initial 30 minutes, periodically during the first 24 hours, with more frequent observations during the first 4 hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely euthanized for animal welfare reasons or are found dead. Observations should include: body weight and food/water consumption; hematological tests; clinical biochemistry and histopathological studies. Additional observations might be necessary if the animals continue to display signs of toxicity $^3$.

In general, acute toxicity tests are used for initial in vivo testing looking for information on whether a “go” or “non-go” decision can be made about either the drug or the nanoparticle. A significant number of articles studying acute toxicity for different types of nanoparticles have been published. Some recent examples for different types of nanoparticles including a polydopamine (PDA) nanoparticle loading ionic liquids (ILs/PDA) as microwave susceptible agent for enhancing the selectivity and targeting of tumor microwave thermal therapy that showed good biocompatibility and lack of toxicity in acute studies $^4$. Other NPs such as poly (β$_\text{L}$-malic acid) (PMLA) nanoparticles have been shown to be safe in acute toxicity testing $^5$. Moreover, the influence of surface functionalization and size on PAMAM dendrimers (G3.5-COOH, G4-NH$_2$, G4-OH, G6.5-COOH, G7- NH$_2$ and G7-OH) acute toxicity was evaluated.
Dendrimers were administered *per os* to CD-1 mice and the animals followed for 10 days. No toxicity was observed for all dendrimers tested but G7-OH and G7-NH$_2$. Studies of acute toxicity help to establish a correlation between the magnitude of the toxic effect and the administered dose. In addition, these studies also provide statistical information about the dose causing death in 50% of treated animals (LD$_{50}$). For instance, it has been reported that PAMAM-NH$_2$ dendrimers have antibacterial activities and this action might be useful for treating septicemia induced by resistant bacteria. To distinguish therapeutic from toxic doses, BALB/c mice were intraperitoneally injected with G2.0 PAMAM-NH$_2$ at increasing doses to determine nanoparticle LD$_{50}$. This acute toxicity assay revealed that LD$_{50}$ for G2.0 PAMAM-NH$_2$ was 80 mg/kg while G2.0 PAMAM-NH$_2$ at 10 mg/kg was effective against bacteria with relatively low toxicity. Finally, acute toxicity studies can be used to set the basis for the dose range to be used in longer toxicity studies.

**b) Subchronic toxicity studies.**

The objective of this kind of studies consists in recognizing and evaluating the toxic effects of a xenobiotic that appear after continuous exposure to the chemical compound. Subchronic toxicity studies range from 28 days to 90 days and provide information about: i) the main toxic effects of compound; ii) the target organs involved; iii) kinetics of biotransformation of the xenobiotic; iv) the accumulative nature of toxic effects, v) the possible reversibility of observed effects; and vi) the dose to be used in chronic toxicity studies. There are not many examples of sub-chronic studies on toxicity of nanoparticles since these studies are expensive and time-consuming, being carried out for nanoparticles with a clear potential for reaching the clinical stage. Thus, monomethoxy(polyethylene glycol)-poly (D, L-lactic-co-glycolic acid)-monomethoxy (PELGE) nanoparticles were studied for subchronic toxicity in rats. No clear signs of subchronic toxicity were observed following 28 days of continuous intravenous administration based on clinical observation, body weight, hematology parameters and histopathology analysis. However, PELGE nanoparticles were associated with histopathological changes in the spleen, increased serum IgM and IgG levels, changes in blood lymphocyte subpopulations and enhanced expression of pro-inflammatory cytokines. Therefore, these results showed that PELGE nanoparticles produced low subchronic toxicity but substantial immunotoxicity. Subchronic toxicity in mice of graphene quantum dots (GQD) has been also studied. Mice were intravenously (i.v.) or
intraperitoneally (i.p.) injected with 20 mg/kg of pegylated GQD (GQD-PEG) every other day for 14 days, and then monitored for up to 40 days. GQD-PEG did not show any toxicity in hematological, biochemical, and histopathology studies. On the contrary, the liver and the spleen of treated mice looked darker than controls, consistent with aggregation of the nanoparticle, and suggesting chronic damage due to slow clearance of the nanoparticle. Oral subchronic exposure to silver nanoparticles (AgNPs) in rats daily treated by gavage during 90 days did not produce any toxicity.

c) Chronic toxicity studies.

The objective of this type of study is to identify toxic effects that appear following daily administration of the xenobiotic for a long time. Tests used in chronic toxicity studies are designed to identify those toxic effects with long latency time, such as carcinogenic effects or those produced by accumulative mechanisms caused by the xenobiotic compound. For this purpose, they are conducted using a minimum of one rodent and one non-rodent species being the use of the later considered when available data suggest that the data obtained in the later species are more relevant for the prediction of toxic effects on human health. The preferred rodent species is rat, although other rodent species, e.g., mouse, may be used. Although the use of mice in carcinogenicity testing may have limited utility, under some current regulatory programs carcinogenicity testing in mice is still required. Rats and mice have been the preferred experimental models because of their relatively short life span, their widespread use in pharmacological and toxicological studies, their susceptibility to tumor induction, and the availability of sufficiently characterized strains.

Chronic toxicity study design consists of two parallel phases, a chronic phase and a carcinogenicity phase. The xenobiotic is generally administered orally although testing by inhalation or through the dermis may also be appropriate. For the chronic phase, the compound is administered daily at different doses to several groups of test animals, one dose per group, generally for a 12 months period, although longer or shorter times may also be chosen depending on regulatory requirements. In the majority of cases, one or more doses are tested, between 25% and 50% of the Maximum Tolerated Dose (MTD) which is the highest dose that can be tolerated without significant lethality from causes other than tumors. The length of the toxicological study is chosen to ensure sufficient time to allow any effect of cumulative toxicity to become manifest, without the
confounding effects of changes associated to aging. The study design may also include one or more interim animal sacrifice, e.g. at 3 and 6 months, and additional groups of animals may be included to accommodate this experimental design. For the carcinogenicity phase, the test substance is administered daily to several groups of test animals during a major portion of their life span. The animals in both phases are observed closely for signs of toxicity and for the development of tumoral lesions. Animals which die or are euthanized during the test are necropsied and, at the conclusion of the test, surviving animals are euthanized and necropsied to look for signs of tumors.

Chronic toxicity studies determine the possible carcinogenicity, genotoxicity, or embryotoxicity of the xenobiotic. In addition, different parameters of hematological and clinical biochemistry, phototoxicity, allergic reactions, as well as behavior studies and weight control of treated animals are also determined. Histopathological tests of main target organs are also performed being the localization and distribution of toxics or its metabolites evaluated in those samples. These studies also provide information about toxic effects latency, possible reversibility of observed effects and threshold dose, meaning the maximum dose that not produce toxic effects. The threshold dose is very useful as it provides the bases for identifying important parameters like daily admissible dose and tolerance limits.

Although some chronic toxicity studies for nanoparticles have been described in the literature, they do not fulfil strictly the above criteria to be considered chronic studies since those studies do not cover the 12 month study time established in the OECD and ICH guidelines for chronic studies. Therefore, they have been considered in this review as subchronic toxicity studies.

2. Methods used in in vivo toxicological testing of xenobiotics

The most commonly used tests to identify possible in vivo toxic actions of xenobiotics are described in the following paragraphs.

2.1 Pyrogenic test

Pyrogens are substances that cause fever. An important initial step in the preclinical characterization of nanoparticles is testing for sterility and pyrogenicity. Unites States
Food and Drug Administration (FDA) recommends two methods for pyrogenicity evaluation: the lumilus amebocyte lysate (LAL)-based assay and the rabbit pyrogen test. However, it has been described that certain nanoparticles may interfere with one or more formats of this test (i.e., PMLA, PAMAM NPs, gold colloids). So, the *in vivo* rabbit pyrogen test might not be used with these type of nanoparticles.

To perform the *in vivo* rabbit pyrogen test, the USP 151 protocol is generally followed. This test involves measuring the rise in temperature of rabbits following the intravenous injection of a xenobiotic test solution. The procedure is the following: within 30 minutes prior to the injection of the test dose, the temperature for each rabbit is determined. This will be considered the baseline for determining any temperature increase resulting from the injection of the test solution. Every animal group must be very homogeneous and the intragroup variation should not exceed one degree Celsius and no animal should exceed a temperature of 39.8°C. Unless otherwise specified, 10 mL of the test solution containing the xenobiotic (at the test concentration) per kg of body weight are injected into an ear vein of each of three rabbits, completing each injection within 10 minutes. The solution containing the xenobiotic is warmed to 37 ± 2°C before the injection and the animal temperature is recorded every 30 minutes between one and 3 hours after the injection. If no rabbit shows a rise in temperature of 0.5°C or more above its respective baseline, the product meets the requirements for the absence of pyrogens. If any rabbit shows an individual temperature rise of 0.5°C or more, further testing on 5 additional rabbits should be performed. Then, if no more than three of the eight rabbits show individual rises in temperature of 0.5°C or more and if the sum of the eight individual maximum temperature rises does not exceed 3.3°C, the material under examination meets the requirements for the absence of pyrogens. The procedure is represented in Figure 2.

Pyrogenic test is designed to predict whether the parenteral administration of a xenobiotic will have an acceptable risk level of a febrile reaction in the future patient. Although fever, within reasonable limits, is not considered a serious side-effect, pyrogenic tests might provide a warning on other possible side-effects, that might appear during widespread use of a drug, including in this category nanoparticles. The test is very simple to perform and it should be done, at an early stage, as part of the toxicity studies for any nanoparticle aimed to reach clinical setting.
2.2 Genotoxicity studies

Genotoxicity studies are essential to evaluate effects of a xenobiotic on genetic material. These type of studies are important because the induction and accumulation of genetic damage can cause genomic instability leading to cancer.\textsuperscript{20,21} There are several \textit{in vitro} and \textit{in vivo} tests that explore the possible genotoxic potential of a compound. Before explaining the different tests used to study \textit{in vivo} genotoxicity it is necessary to define two basic concepts: a) clastogenic agent is the physical or chemical agent able to produce chromosome breaks, and b) aneugenic agent is the chemical compound that affects cell division and the components involved in the cellular mitosis producing gain or loss of a full chromosome leading to aneuploidy.\textsuperscript{22}

The mechanisms of NP-induced genotoxicity are still not well understood and it is often difficult to identify whether a toxic action on DNA is specific for the presence of the nanoparticle. It has been described that genotoxicity may be produced by the direct interaction of NPs with the genetic material (DNA)\textsuperscript{23-25}. However, NPs can potentially interact not only with DNA but also with proteins involved in DNA replication, transcription or repair (e.g C60 fullerene binds to human DNA topoisomerase II α in the ATP binding domain, which might inhibit the enzyme activity)\textsuperscript{26}. Moreover, NPs can also cause indirect DNA damage by NP-induced reactive oxygen species (ROS), or by toxic ions released from soluble NPs\textsuperscript{21,23,27,28}.

The most used \textit{in vivo} genotoxicity tests are described in the following paragraphs.

2.2.1 Mammalian \textit{in vivo} chromosomal aberrations assay (CA)

The mammalian \textit{in vivo} chromosome aberration test is used for the detection of structural chromosome aberrations induced by xenobiotics in bone marrow cells obtained from animals, usually rodents. Structural chromosomal aberrations may affect two structures: chromosomes or chromatids. While the majority of genotoxic chemical-induced aberrations affect chromatids, chromosome aberrations might also occur. Chromosomal damage and related events are the cause of many human genetic diseases and there is substantial evidence that, when these lesions and related events cause alterations in oncogenes and tumor suppressor genes, they are involved in the genesis of cancer both in humans and animals. This test is performed following the standardized protocol OECD 475.\textsuperscript{29} The general procedure is shown in Figure 3.
Animals are exposed to the xenobiotic using the proper route of exposure and are euthanized. Prior to sacrifice, animals are injected intraperitoneally with an appropriate dose of metaphase arresting agent (e.g. colchicine). Immediately after sacrifice, bone marrow is obtained, exposed to hypotonic solution and fixed. Cells are then spread on slides and stained. Cells in metaphase are analyzed to detect chromosomal aberrations. Both treated and control groups must include at least 5 animals per sex. Test substances should be preferably administered as a single dose. At least 3 subtoxic concentrations of the compound, with corresponding positive and negative controls should be used. The mitotic index, taken as a marker for cytotoxicity, should be determined as a measure of cytotoxicity in at least 1,000 cells per each animal including positive controls, and untreated or vehicle/solvent-treated animals taken as negative controls. Since slide preparation procedures often result in breakage of a proportion of metaphases with the resulting loss of chromosomes, to be considered a valid test the cell score should, therefore, contain a number of centromeres not less than $2n\pm2$, where $n$ is the haploid number of chromosomes for that species.

There are several criteria for considering a result positive for this test such as: i) a dose-related increase in the relative number of cells with chromosome aberrations; ii) a clear increase in the number of cells with aberrations in a single dose group at a single sampling time; iii) an increase in polyploidy that may indicate that the test substance has the potential to induce numerical chromosome aberrations; and iv) an increase in endoreduplication that may indicate that the test substance has the potential to inhibit cell cycle progression. For more in-depth information on the possible genotoxic actions of the xenobiotic, other techniques such as, fluorescence in situ hybridization (FISH) might be used. FISH uses specific molecular probes for specific chromosomal regions to facilitate the detection of break points or fragile regions within that chromosome. However, when the objective consists only in detecting possible clastogenic activity, it is not necessary to apply a technique like FISH.

As mentioned above, genotoxicity may be induced by ROS generation. ZnO-NP has immense potential and application in the health care sector. In vivo results demonstrated that ZnO-NP induced ROS production and genotoxic response in mice bone marrow. The CAs scored were mainly found to be chromatid breaks and the number of breaks per cell to be significantly higher than the control. About 10-fold increase in CAs was observed at the highest dose administered (25 mg/kg body weight).
2.2.2 The Micronucleus Test

The micronucleus test in vivo is a method devised primarily for screening chemicals with potentials to cause chromosome abnormalities. These abnormalities can be structural or numeric. This test is performed in rodents and, therefore, like other in vivo tests, it has the advantage of taking into account the pharmacokinetic properties of the xenobiotic.

Micronucleus is the name given to the small nucleus that is formed when a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division. Micronuclei are generated during anaphase from lagging acentric chromosome or chromatid fragments caused by incorrectly repaired or unrepaired DNA breaks or by nondisjunction of chromosomes. This incorrect segregation of chromosomes may result from hypomethylation of repeat sequences present in pericentromeric DNA, irregularities in kinetochore proteins or their assembly, dysfunctional spindle apparatus, or flawed anaphase checkpoint genes. Micronuclei can be formed in all kind of cells from any proliferative tissue, but it can be detected easily in those cells without nucleus, such as erythrocytes. A restricted analysis to nucleated bone-marrow cells is rather tedious and unreliable if the xenobiotic toxic effects are small. For this reason, micronucleus test is performed most often in rat or mouse bone-marrow erythrocytes, although it is possible to analyze others tissues. Specifically, immature erythrocytes are used in this method, when they expel their nucleus but still preserve stain properties different from older, more mature, forms. In addition, the number of polychromatic erythrocytes is almost unlimited and the spontaneous rate of micronucleated cells is low and consistent. The test is very sensitive since it can identify the effect of very low dosages of standard chromosome-breaking mutagens.

This test is performed following the standardized procedure indicated in protocol OECD 474. When compounds of unknown mutagenic activity are tested, a subacute treatment over 30 hours should be chosen. The intention is to expose a large proportion of the cell population to the compound to be tested during two cell cycles. The dose administered should always cover a wide range, from the likely therapeutic dose up to the highest tolerable dose. The route of administration should be the same that it is anticipated to be used in humans. Animals are euthanized between 24 and 48 hours after treatment. In this test, animals are not treated previously with metaphase arresting
agents. Then cells are stained and are analyzed by microscopy. A perfect morphology of the nucleated cells serves as criterion of good quality for the procedure, even though the nucleated cells are not evaluated in the test. Their staining has to be vigorous, red in mature erythrocytes and with a strong bluish tint in the immature forms (polychromatic erythrocytes). Generally, around 1,000 polychromatic erythrocytes are evaluated per animal and the total number of mature micronucleated erythrocytes is used as control. The result is considered positive when in a xenobiotic-treated group shows a statistically significant increase in the number of micronucleus number respect to a negative control.

This testing procedure has a number of important advantages over the analysis of bone-marrow metaphase chromosomes since it is simpler and faster than chromosome analysis for the same material, but not at the expense of accuracy. The test is, at least, as sensitive as the metaphase method for monitoring chromosome breakage. In addition, it allows identification of toxic effects on the spindle apparatus. All these properties render this method highly suitable for routine toxicological screening.

This test has been used to test nanoparticle genotoxicity. Thus, single-wall carbon nanotubes (SWCNTs) genotoxicity was evaluated using a bone marrow micronucleus test. No effect of SWCNTs on the number of micronuclei in the immature erythrocytes of mice was observed. The genotoxicity of gold nanoparticles (AuNPs) of different sizes (2, 20 and 200 nm) was also studied in the lungs of rats and no toxicity was found. However, in other study AuNPs (5, 20, 50 nm) were evaluated in mice. While the standard in vivo micronucleus test, showed no toxicity after 4 days exposure to NPs, exposure for 14 days, indicated that the 5 nm AuNPs presented significant clastogenic damage, with a dose-dependent increase in the number of micronuclei observed.

2.2.3 Comet assay

Comet assay identifies DNA chain breaks in eukaryotic cells. This method can be used to identify possible mutagens and carcinogens for humans. In addition, it has high sensibility and reproducibility. This test is performed following the normalized procedure OECD 489.

After xenobiotic administration, cells suspensions are obtained from different organs by incubation with collagenase or trypsin. Then, cells are placed in agarose on a slide and finally are lysed. Only a few cells per visual field are typically used since higher cell
densities might result in a significant proportion of overlapping comets. After the agarose gel has solidified, slides are placed, generally for at least one hour, in a lysis solution containing high salt concentration and detergents. There is a minimal time needed to appropriately release the DNA and this time might vary depending on the cell type. At the next stage, prior to electrophoresis, the slides are incubated in a strong alkaline (pH > 13) electrophoresis buffer to produce single stranded DNA. After DNA unwinding, the single-stranded DNA is electrophoresed under alkaline conditions to produce comets. After electrophoresis, the alkaline buffer in the gels is neutralized by rinsing the slides with a suitable buffer. After neutralization, slides can be stained and the comets scored. The tail intensity of the comet compared to their head intensity is proportional to the number of DNA chain breaks. This test has been used to study genotoxicity of AgNPs with a size ranging from 90 to 180 nm in Swiss Albino male mice. Single intraperitoneal administration of AgNPs gave a significant increase in tail DNA intensity at doses of 10 mg/kg body weight. Moreover, cerium oxide nanoparticles have also shown toxicity in comet assays following oral administration to Wistar rats.

2.3. Carcinogenetic studies

Currently, the available methods for evaluating in vivo the carcinogenic potential for different xenobiotics are limited. There are both in vitro and in vivo tests available to evaluate carcinogenesis. In in vitro tests, the ability of xenobiotics to transform healthy cells into tumoral cells is observed. However, it must be noted that this type of test is able to detect carcinogenicity, but not genotoxicity. In addition, in vitro tests overlook the chemical biotransformation of the xenobiotic that takes place in the organism and, for this reason, the conclusions drawn from them, are somehow limited.

In vivo, the most commonly used carcinogenic test is based on the observation of the xenobiotic effect in rodents for two years. Since carrying on this test might involve the life span of the animals, it is expensive and complex. This test is performed following normalized protocol OECD 451. The maximum tolerated dose is administrated chronically to the animals. So, it is possible to evaluate the tumoral incidence for each organ. To perform this test, there are different groups of animals: control group, test group and satellite group being this last group used for determining whether the
xenobiotic effects are reversible or not. In this type of study, food intake and the weight loss is thoroughly controlled, as well as different parameters of hematological and clinical biochemistry. In addition, when the animals are euthanized, histopathological studies of the main target organs are performed \(^\text{13}\). Positron emission tomography (PET) might be used to identify the appearance of early abnormal growth. PET imaging has unique advantages combining high sensitivity and the ability to conduct quantitative analysis of whole-body imaging. \(^\text{43}\).

However, despite the importance of conducting this type of studies, due to the long time and the high costs, no studies of carcinogenesis for nanoparticles have been described so far.

2.4. Embryotoxicity studies

Knowledge of xenobiotic effects on embryonic development is very important in the field of public health and critical for new drug approval. After thalidomide disaster, reproduction toxicity testing has been routinely performed prior to a drug being released into the market \(^\text{1}\). According to International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) \(^\text{44}\) and normalized protocol OECD 422 \(^\text{45}\), most commonly used tests are: i) fertility and reproduction test, evaluating reproductive ability in males and females (gametes development, fertility, pre- and post-implantation viability, delivery and lactation); ii) teratogenicity test evaluating morphology and viability (external, visceral and skeletal) of fetus before birth; and iii) perinatal test to evaluate postnatal survival, external morphology and development \(^\text{44}\). There is not \textit{a priori} preferred animal model to study the risk of the use of a xenobiotic on human embryology. Accordingly, different species such as rats, mice, guinea pigs, cats, dogs and monkeys have been used for this purpose.

A significant number of embryotoxicity studies have been performed using different types of NPs. Covering all of them is beyond the scope of this short review. Here, we present some examples on rodents and also several embryotoxicity studies in zebra fish, an animal model that has revolutionized initial screening for embryological toxicity studies due to the short developmental period and the easy observation of possible embryological defects in this animal. The biodistribution and potential toxic effects of AuNPs of different size (ranging from 1.5 to 70 nm in diameter) was studied in non-
pregnant and pregnant mice at different gestational ages (E5.5, 7.5, 9.5, 11.5, and 13.5). No apparent toxic effects were observed in pregnant mice. On the other hand, the possible embryotoxic effect of a single low (10 mg/kg) or high (100 mg/kg) dose of positively-charged polyethyleneimine-Fe$_2$O$_3$-NPs (PEI-NPs), or negatively-charged poly (acrylic acid)-Fe$_2$O$_3$-NPs (PAAMNPs) during critical windows of organogenesis (gestational day 8 to 10) was studied. While the low dose of NPs, regardless of charge, did not induce toxicity, the larger dose led to charge-dependent fetal loss as well as to morphological alterations in the uterus (caused by both positively and negatively charged NPs) and testes (only produced by positively charged NPs) of surviving offspring. In addition, positively-charged PEI-NPs given later in organogenesis resulted in a combination of short-term fetal loss (42%) and long-term alterations in reproduction, including increased fetal loss for second generation offspring (mice exposed in utero). In addition, CdTe quantum dots enter and accumulate in mouse testis causing dose-dependent toxicity.

Recently, zebrafish embryos (Danio rerio) have been used for in vivo development security tests because they have several advantages over other species such as: i) it is possible to generate and maintain large numbers of them in an animal house due to its small size; ii) they have a quick embryonic development (72 hours post-fecundation) markedly shortening the times to identify potential effects on embryonic development; iii) they only need three months to reach sexual maturity; iv) they produce large amounts of eggs (300-500 per female); and v) the embryos are transparent allowing a real time study of the toxic effects as well as recording images of in vivo pathological phenotypes without requiring sacrifice of the animal. In addition, sequencing of the zebrafish genome has revealed a similarity of about 70% with humans suggesting that many of the molecular and cellular mechanisms relevant for pathogenesis of certain human diseases might be present in the zebrafish.

Embriotoxicity tests in zebrafish are performed according to the normalized protocol (OECD 236). The procedure is schematically presented in Figure 4. Newly fertilized zebrafish eggs are exposed to the xenobiotic for a period of 96 hours. Every 24 hours, the eggs are observed for indicators of lethality such as: i) coagulation of fertilized eggs, ii) lack of somite formation, iii) lack of detachment of the tail-bud from the yolk sac, and iv) lack of heartbeat. At the end of the exposure period, acute toxicity is determined based on positive scores in any of the four observations recorded, and the LD50 is
calculated. Concerning substances that may be metabolically activated, there is evidence that zebrafish embryos do have biotransformation capacities. However, the metabolic capacity of embryonic fish is not always similar to that of juvenile or adult fish. Therefore, if there are any indications that metabolites or other transformation products may be more toxic than the parent compound, it is also recommended to perform the test with those metabolites/transformation products and taking also into account those results when drawing conclusions from the toxicity tests. Zebrafish embryos are not expected to be sensitive to substances with a very bulky molecular structure or to substances causing delayed hatch which might preclude or reduce the post-hatch exposure due of limited bioavailability of the substance. Under these conditions, other toxicity tests might be more appropriate.

Zebrafish is becoming a very popular model for NP toxicity testing. Toxicity of silica nanoparticles with diameters 20, 50 and 80nm was evaluated using an in vivo zebrafish platform that analyzes multiple endpoints related to developmental, cardio-, hepato-, and neurotoxicity. Results showed that except for an acceleration in hatching time and alterations in the behavior of zebrafish embryos/larvae, silica NPs did not elicit any developmental defects, or any cardiac or hepatotoxicity. On the other hand, embryotoxicity studies in zebrafish of Poly (ethylene glycol) methyl ether-block-poly (lactide-co-glycolide) (mPEGPLGA) nanoparticles carrying acetyltanshinone IIA (ATA), a novel anti-breast cancer agent, displayed much lower toxicity than free ATA.

PAMAM dendrimer embryotoxicity has been evaluated at 24 and 120 hours postfecundation (hpf) on embryonic zebrafish mortality, development, and malformations. Neutral thiophosphoryl dendrimers did not produce malformations at any dose throughout the exposure. However, exposure to 50 ppm cationic PAMAM dendrimers G3-amine, G4-amine, G5-amine, and G6-amine caused 100% mortality by 24 hpf. Cationic PAMAM G4-amine and neutral PAMAM G6-amidoethanol caused significant cardiac effects (pericardial edema) at 10 ppm. However, negatively charged succinamic acid G5 and G6 dendrimers did not show any significant adverse effects even at the highest concentration tested. These results indicated that surface charge may be the best indicator of dendrimer embryotoxicity. In addition, dendrimer class and generation are other potential contributors to dendrimer embryotoxicity. One relevant application of dendrimers is their use as drug carriers to improve water solubility of...
some drugs to improve their bioavailability. In this direction, PAMAM dendrimer G4.5 (-COOH) was used as drug carrier for the antipsychotic drug risperidone and its toxicity tested on zebrafish by exposing embryos four days post fertilization (dpf) to increasing concentrations of risperidone and DG4.5-risperidone for 24h. The compounds produced a cellular disorganization in the raphe neuronal population being this effect larger in animals treated with free risperidone than those receiving the complex (DG4.5-risperidone). Therefore, in this case, the dendrimer decreased drug embryotoxicity.

Embriotoxicity studies represent a key part of data required for a new drug approval. The regulatory requirements on this subject are rather strict and are covered by both International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). In this direction, nanoparticles, although specific regulatory requirements for them is under development, should be subjected to the same strict requirements as small drugs. Initial testing on possible embryotoxic effects has been significantly accelerated by the use of animal models such as zebrafish where developmental processes require little time as compared to mammals and where possible developmental defects can be easily observed. However, data obtained in fish, and also in rodents, on possible embryotoxic effects of nanoparticles (and small drugs) cannot exclude embryotoxicity in humans as thalidomide dramatically showed. Thus, a continuous pharmacovigilance surveillance should be maintained in the case that some nanoparticles reach a clinical setting.

2.5. Immunotoxicity studies

Xenobiotics might produce significant effects on the immune system. In general, cationic (positively charged) particles are more likely to induce inflammatory reactions than anionic (negatively charged) and neutral species. Therefore, it is important to evaluate immunotoxicity caused by nanoparticles. According to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Safety Guideline (ICH S8) the evaluation of xenobiotic effects on immune system can be made in two stages: a) in the first stage, a general immunotoxicity study is performed to analyze different immune parameters. In this type of study, animals are not exposed to antigens and are not infected with bacteria or parasites, meaning that, at this stage, functional studies are not performed; and b) in a...
second stage, and depending on the results of the first stage, more specific and functional tests on immune system functionality can be performed.

General immunotoxicity tests have been developed to detect possible effects on the immune system, and these tests analyze the following parameters: i) blood cells count and formula; ii) plasma levels of IgM, IgG and IgA immunoglobulins; iii) weight and histopathological study of main lymphoid organs and tissues (thymus, spleen, lymph nodes, lymphoid tissue, and gut-associated lymphoid tissue); iv) bone-marrow morphological examination; and v) flow cytometry or immunohistochemistry studies to detect the possible variation of immune cells subpopulation. If the obtained results suggest an effect on the immune system, a functional test to determine humoral acquired immunity through colony-forming unit (CFU) analysis should be performed. This test determines host ability to trigger antibodies reaction against some specific antigens. This last process requires the coordinated action of several immunity cells such as macrophages, T cells and B cells. Thus, any problem in these cells can have an impact on B cells antibodies production ability. The in vivo CFU test is performed using serum obtained from immunized mice.

Nanoparticle effect on immune parameters might constitute a problem due to two possible pathways: a) immune reaction induced by surface components of the nanoparticles as it could be the case when nanoparticles are decorated with peptides to facilitate targeting or biological barriers (i.e. blood brain barrier) crossing or, more commonly, b) a direct effect of nanoparticles lacking peptide components on the immune system as is the case for metal-based nanoparticles. In this case, both immune system inhibition and an inflammatory response have been reported. For instance, silver nanoparticles (Ag-NPs) administered using a 28-day i.v. repeated-dose administration pattern, caused a strong reduction in NK cell activity, and reduced IFN-γ production suggesting suppression of the functionality of the immune system. Similarly, dextran-stabilized iron oxide nanoparticle showed significant suppression of proliferation of cultured T-lymphocytes stimulated with the mitogen phytohemagglutinin (PHA). On the other hand, mesoporous nanoparticles (MPS NPs) induced an inflammatory response characterized by an increase in liver and spleen weight and splenocyte proliferation in mice. In addition, mice treated with MPS NPs showed altered lymphocyte populations (CD3(+), CD45(+), CD4(+), and CD8(+)) in the spleen, and increased serum IgG and IgM levels,
It is important to note that it is estimated that about 20% of approved drugs withdrawal from market is due to immunological problems meaning either immune system blockade leading to deficient response to infection or immune stimulation leading to inflammation. Nanoparticles, as indicated above, reproduce such pattern in preclinical studies which indicates that nanoparticle immunotoxicity should be studied for nanoparticles aimed to reach clinical setting following the International Conference on Harmonization Safety Guidelines procedures \(^5^8\). However, it should be kept in mind that it is possible that, due to the complexity of nanoparticles, when used as carriers for different drugs or genetic material, the actual guidelines might fall short to identify all immunotoxic effects of such as compounds since the final compound, aimed to be therapeutic, might incorporate the mixed effects of nanoparticle, active pharmacological component, and NP coating.

2.6. Hematological studies.

For evaluation of xenobiotic toxic effects on blood tissue the following test are generally used: i) red blood cells count per blood volume (RBC); ii) hemoglobin concentration (Hb); iii) mean corpuscular hemoglobin (MCH) or hemoglobin amount in each red cell; iv) mean corpuscular hemoglobin concentration (MCHC) which is the hemoglobin concentration (not the amount) in each red cell; v) hematocrit value that measures the volume of red blood cells compared to total blood volume (red blood cells and plasma); vi) white blood cells count (WBC); vii) platelet count; viii) neutrophil count; ix) lymphocytes count; x) Coagulation tests such as prothrombin time and activated partial thromboplastin time; xi) reticulocytes count expressed in percentage respect to total mature red blood cells; and xii) complement activation that has been studied for PAMAM dendrimers (G5 PAMAM-NH\(_2\); G5 PAMAM-OH; G4.5 PAMAM-COOH) showing that PAMAM dendrimers with amine, hydroxyl or carboxyl terminal groups at doses ranging from 0.001 to 0.1 mg/mL inhibited C3a production \(^6^2\).

An additional test, hemolysis test, has been widely used when studying preliminary toxicity of nanoparticles. In this test, blood is obtained from healthy rodents or humans and it is anticoagulated with sodium heparin. Erythrocytes are separated from blood plasma by centrifugation at 4°C, then washed and finally resuspended in physiological saline. The RBC suspension is incubated with different concentrations of the xenobiotic for 2 hours at 37°C under agitation. As negative control, RBC suspension is incubated...
with physiological saline. As positive control the RBC suspension is incubated with Triton X-100 (10%, v/v) to obtain complete hemolysis. After the incubation period, the RBC suspension is centrifuged at 1,000× g for 10 minutes. The amount of released hemoglobin in the supernatants is determined spectrophotometrically at 540 nm. The degree of hemolysis is determined by the following equation:

\[
\% \text{Hemolysis} = 100 \times \frac{\text{Abs} - \text{Abs}_0}{\text{Abs}_{100} - \text{Abs}_0}
\]

where Abs, Abs₀, and Abs₁₀₀ are the absorbances of test samples, the suspension treated with physiological saline, and the suspension treated with Triton X-100 (10%, v/v), respectively.

Some NPs like polyethylenimine (PEI) enter systemic circulation and interact with various blood components such as RBC membrane structure and blood coagulation process. On the other hand, graphene quantum dots did not modify standard hematological markers, such as white and red blood cell count, hematocrit, mean corpuscular volume, total hemoglobin, platelet count, and mean corpuscular hemoglobin indicating that chemical composition of the NOPs plays a key role in hematological toxicity.

2.7. Biochemical parameters.

Biochemical parameters, both in urine and plasma, represent markers for internal organ dysfunction and so, represent early signs of possible xenobiotic toxicity. Generally, biochemical markers are grouped in profiles each one related to a major organ or pathological entity. The most relevant profiles used are the following:

2.7.1 General profile

The plasma concentration of different electrolytes such as calcium, potassium, chloride, sodium as well as glucose concentration are determined.

2.7.2 Lipid profile

This profile is related to identify a xenobiotic effect on lipid metabolism and related atherosclerosis. For that purpose, the levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, total lipids and triglycerides are determined in plasma.
2.7.3 Renal profile

Urea and creatinine plasma levels are good markers for a decrease in glomerular filtration rate (GFR). However, these markers only increase when there are GFR reductions in the range of 50 to 70%. For this reason, blood urea nitrogen is usually preferred as a marker for renal dysfunction. Changes in normal urine composition can reveal toxic renal effects caused by a xenobiotic. The most commonly used urine parameters to assess correct function of kidneys are: i) volume and osmolality; ii) the presence of proteins like Beta 2-Microglobulin suggesting a possible problem in tubules and albumin that might indicate a glomerular problem; and iii) glycosuria.

2.7.4 Hepatic profile

Alanine transaminase and aspartate transaminase are mainly distributed in liver cells, and their levels rise during liver cells necrosis. Levels of these enzymes correlate very well with the extent of liver cell damage, and are commonly used as indicators of liver function. Iron oxide magnetic nanoparticles (IOMNPs) have a great potential in a wide variety of biomedical applications such as contrast enhancement of magnetic resonance imaging, targeted drug delivery or gene delivery and tissue engineering. IOMNPs potential side-effects on liver, spleen and kidneys were evaluated in mice by monitoring various serum biochemical markers. The results showed no obvious hepatic and renal toxicity in the animals after IOMN treatment.

Biochemical parameters represent a good index for correct function of different internal organs. Modification of these parameters by xenobiotics, including nanoparticles, are good indicators of arising toxicity that appears, generally, well in advance of severe organ damage. Thus, it is very important that this type of test will be performed during preclinical testing of a nanoparticle. Since biochemical determinations of the different profiles indicated above only involve determination of plasma levels of ions (calcium, potassium, etc.) or chemical compounds (cholesterol, glucose, creatinine, etc.) or enzyme activity (transaminases), the situation does not differ significantly from that for small drugs and similar procedures might be followed.
2.8. Histopathological studies

If a possible damage pattern is observed by using one of the above methods, histopathological studies of the affected organs should be performed by using optic and electronic microscopy to determine the place and nature of the injuries.\(^{49,70}\)

Histopathological studies have been widely used to assess NP toxicity. Thus, a bioconjugate between duplex oligodeoxynucleotides (dODNs) and a PAMAM-succinamic acid dendrimer G4 used as a drug delivery system for the anticancer drug doxorubicine showed no severe tissue histological changes.\(^{71}\) Moreover, a G5 PAMAM dendrimer, modified with folic acid to increase targeting to tumor cells, was injected intravenously and heart, liver, spleen, lung, kidney, and brain tissues were collected and analyzed with no sign of tissue damage observed.\(^{72}\) In addition to dendrimers, other NPs have been studied for their tissue toxicity. Thus, O-Carboxymethyl chitosan encapsulated metformin (O-CMC) did not produce any structural alteration to organs such as brain, lung, heart, liver, spleen and kidney.\(^{73}\) Moreover, citrate-or polyvinylpyrrolidone-coated Ag NPs injected in mice did not cause any relevant change in the examined organs.\(^{74}\)

In conclusion, detailed histopathological studies are not routinely performed to determine nanoparticle toxicity. However, they must be performed whenever a possible macroscopic organ damage or clinical symptoms suggesting organ involvement are observed in order to identify the structural damage at cellular level. Generally, histological damaged will be heralded by alterations in one or several of plasma biochemical parameters indicated in section 2.7.

2.9. Behavioral studies

Many toxics can produce behavioral changes before histological damage can be observed, so behavioural studies provide a very efficient screening test for xenobiotic toxicity. For this kind of studies, rodents, followed by apes, are generally chosen as the animal species. Irwin test is the most commonly used test to determine adverse behavioural effects to a xenobiotic and to evaluate its possible neurotoxicity in rodents. In this test, behavioral profile is evaluated determining several parameters: i) alert level or stupor; ii) motor activity; iii) central excitation to determine neurological profile; iv) motor incoordination; v) muscle tone; vi) reflexes and; vii) autonomic profile though
ocular signs, secretions and general signs such as piloerection, hypothermia or colour skin \textsuperscript{75,76}.

Behavioral studies have been performed in mice for different NPs including recombinant human serum albumin with incorporated hydrophilic (NH\textsubscript{4})\textsubscript{2}Ce (IV) (NO\textsubscript{3})\textsubscript{6}γFe\textsubscript{2}O\textsubscript{3} particles for medical imaging, without adverse side effects suggesting a possible use as MRI contrasting agent \textsuperscript{77}. Lipid nanocapsules have been also tested for behavioral abnormalities in mice without signs of toxicity \textsuperscript{78}.

3. Conclusions

It is generally accepted that nanoparticle-based medicinal products will play a relevant role in the therapeutic armamentarium in a near future. In fact, several nanoparticle-based medicines including liposomes, lipid suspensions or nanocrystals have been already authorized by the European Union to reach the market under the present regulatory framework. The inexistence of specific guides for nanoparticle toxicity testing makes that nanoparticles are subject to the same regulatory processes as all other therapeutics. However, nanoparticles represent a clear challenge since they are much more complex than small drugs and, so, more specialized tests and experts to perform them will be likely needed as it has been already recognized by the European Medicine Agency (EMA).

The different procedures described in this review allow to obtain valuable information about the possible toxic effects \textit{in vivo} for a xenobiotic, including nanoparticles, with a possible therapeutic application under current regulatory rules. However, a negative result in these toxic tests do not provide absolute certainty that xenobiotic-induced human toxicity will not appear in the future when a more wide use of the compound in therapeutics will occur. For this reason, it is important to understand the key role that this kind studies play to minimize the possible risks for patients once the compound has been approved for therapeutic use.
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Figure legends

Figure 1. Types of nanoparticles.

Figure 2: General scheme of the pyrogenic test. See the text for explanation.

Figure 3: General scheme of in vivo chromosomal aberrations assay. See the text for explanation.

Figure 4: In vivo Zebrafish embryotoxicity studies.


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Figure 1
Figure 2
Figure 3
Figure 4