Nanotoxicology: challenges and future

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Introduction

The current expansion of nanotechnology and the steady broadening of the range of its practical applications allow to predict, for the coming years, a very substantial increase in the levels of exposure (accidental or intentional) to nanoparticles of anthropogenic origin for humans (including especially sensitive population groups) and for the environment. It is, therefore, imperative to put the basis to be able to perform a risk assessment, preferably integrated, in both areas. This includes having in each case the relevant data of both exposure and toxicity (hazard).

Particles smaller than 100 nm have certain characteristics which distinguish their toxic potential compared to the material (of the same chemical composition) from which they originate. Among other features, these small particles have an increased absorption, especially by unusual ways such as inhalation or skin, are capable of crossing barriers impassable in other conditions (such as blood-brain or retinal), penetrate in organs or tissues hard to reach for other compounds (such as the prostate), or interact with subcellular organelles in special and poorly characterized conditions.

On the other hand, the same considerations apply when nanotechnology is used to vectorize and deliver drugs, despite these being, often, previously known and duly characterized products. The presentation of the same compound in a nano scale can modulate its toxicity in a way that we cannot predict a priori. While it is expected that nanotechnology will enable us to achieve lower equipotent doses, and therefore obtain lower toxicity levels (as well as a more accurate vectorization should also help to reduce the deleterious effects), we cannot ignore the fact that the drug may reach new tissular, cellular or molecular targets, and it will do so in conditions that make their behavior difficult to predict.

There is a relatively abundant literature on nanotoxicology, especially in recent years. However, certain aspects are identified that still require significant efforts of reflection and experimentation, both in regard to methodological and theoretical considerations or to specific aspects of the toxicological evaluation which have been unsatisfactory or insufficiently addressed. Among the latter are, for example, the need for standardization of procedures in vitro, the lack of repeated dose toxicological data in vivo, and of genotoxicity or teratogenicity data. Because of the limited knowledge we have about the behavior of these materials, the application of broad spectrum techniques such as toxicogenomics would be also of special interest. Equally important is research to adapt the ecotoxicity tests, especially in regard to forms of exposure.

It appears also as an issue of utmost importance to study the distribution of nanoparticles in the body and the dynamics of internalization into cells.

Toxicity profile of gold and cobalt ferrite nanoparticles

Within the frame of a larger project (NANOSOST), we have tested several aspects of nanoparticles toxicity.

The acute toxicity was tested in rats by intraperitoneal (IP) administration of 3 kinds of nanoparticles (NPs): gold (AuNP), gold coated by hyaluronic acid (AuNP coated by HA) and cobalt ferrite (CoFe₂O₄ NP). The mean diameters of these NPs were 10,45 nm, 30 nm and 17 nm, respectively. The specific aim of the study was to determine a DL₅₀ by and Up&Down OECD 425 protocol and compare these results with the values obtained in parallel for gold (III) chloride (HAuCl₄) and CoFe₂O₄ in solution. Toxicity was studied by clinical signs, necropsy, hematological parameters and histopathological exam of kidney, liver, spleen and lung. At the same time, biodistribution by
TEM (liver, spleen, kidney and lung) and ICP-MS of different tissues (liver, spleen, pancreas, lymph node, kidney, lung and brain) at 14 days from the administration was assessed. A kinetic study by ICP-MS was performed by analyzing blood samples obtained at different times ranging from the moment of administration to 3 days later.

In this study, it was not possible to determine an exact DL₅₀ for NPs, since the maximum concentration tested (limited by the aggregation of NPs or the needed volume for higher concentrations) showed no mortality. In contrast this parameter was determined for gold (III) chloride (HAuCl₄) (DL₅₀ = 106,563 mg/kg) and CoFe₂O₄ (DL₅₀ = 116,942 mg/kg) in solution.

We did not find signs of toxicity in the necropsy of rats treated with nanoparticles, but we observed evidence of toxicity in rats treated with HAuCl₄ and CoFe₂O₄ solutions. Before the euthanasia of the animals, at 14 days after administration, we obtained blood sample for hematology determination. All values were normal for rat and there was no significant difference between them with a 95 % of confidence by ANOVA or Kruskal-Wallis (depending if the data follows a normal distribution or not, respectively).

Tissue biodistribution was also studied. 14 days after the IP administration we collected samples from different tissues to determine gold and cobalt concentration by ICP-MS. The differences in concentration and distribution shown between the groups that received a same dose (the NPs groups) are possibly due to different absorption between NPs: AuNP coated by HA concentrations were greater than CoFe₂O₄ NP.

AuNP with and without coating are mainly accumulated in liver and spleen. In contrast, 50 % of HAuCl₄ and CoFe₂O₄ NP are accumulated in kidney. In all cases the tissue that accumulated less gold or cobalt was the brain, as it was expected, but in all cases gold or cobalt was detected. So NPs and solutions were able to cross the blood-brain barrier. At the same time biodistribution by TEM in liver, spleen, kidney and lung was also assessed. Observations showed that the 3 types of nanoparticles were always inside vesicles or lysosomes.

A subchronic toxicity study was carried out by exposure for 21 days through the respiratory tract of three solutions of gold nanoparticles, with a size of 12 nm, alone or coated with hyaluronic acid, and non-coated nanoparticles of 100 nm, as well as its control (sodium citrate buffer). The trial design was based on the OECD 412: for subacute inhalation toxicity study. Each group consists of 4 males and 4 females. We tested three exposure time for each substance tested, plus a control group that was exposed to vehicle (sodium citrate). The inhalation exposure was conducted with the Aeroneb Lab Nebulizer System.

Clinical signs, ponderal evolution, hematology, clinical biochemistry (GPT, GOT, total bilirubin, total protein, BUN and creatinine) and histopathology (intestines, kidney, liver, brain, lung and nasal mucosa) were assessed.

Coagulation (fibrinogen, prothrombin time (PT) and activated partial prothrombin time (APTT)), genotoxicity in blood and liver using Comet assay, MDA in plasma, lung and liver, glutathione-S-transferase (GST) in plasma, lung, liver and spleen, LDH in liver, lung and serum, apoptosis by TUNEL assay in lung and liver, CYP3A4 and CYP1A1expression in liver and biodistribution of nanoparticles in various organs, liver, kidney, spleen, pancreas, lymph nodes, lung, carina, testis and olfactory bulb (by ICP-MS and TEM), kinetics of absorption of NPs (concentration of nanoparticles by ICP-MS in blood at different times) were also assessed.

Some in vitro studies were also performed with the same NPs:

Cytotoxicity by WST-1 assay: Human dermal fibroblasts were exposed to different doses of 12 nm Au-HA ranging from 0 to 0,5mg/ml and tween was used as positive control. A very slight dose dependent reduction in cell viability was observed, but cell viability was always above 90 %. Accordingly, 12 nm Au-HA was no cytotoxic for the tested doses. Cell internalization was investigated by transmission electron microscopy (TEM) observation of 3T3 cells treated with 500 µg/mL at different exposure times ranging from 15 minutes to 24 hours (15 min, 30 min, 1 h, 4 h and 24 h). 500 µg/mL was a sub-toxic concentration of Au-HA nanoparticles (NPs) as we observed by WST-1 assay.
**Embryotoxicity.** The EST is an in vitro standard assay, which permits to classify substances as strongly, weakly or non-embryotoxic. Due to the particular physical–chemical nature of nanoparticles, we introduced a modification to the standard protocol exposing the Embryonic Stem Cells (ES-D3) to nanoparticles only during the first 5 days of the assay. Moreover, we proposed a method to discriminate and compare the embryotoxicity of the substances within the weakly embryotoxic range. Our ID50 results permit to classify cobalt ferrite nanoparticles coated with gold and silanes as non-embryotoxic. The remaining nanoparticles have been classified as weakly embryotoxic in this decreasing order: gold salt (HAuCl₄•3 H₂O) > cobalt ferrite salt (CoFe₂O₄) > cobalt ferrite nanoparticles coated with silanes (Si–CoFe) > gold nanoparticles coated with hyaluronic acid (HA–Au).

**Genotoxicity.** we analyzed induction of DNA damage by Comet Assay. Au NPs at a sub-toxic concentration induced a significant DNA damage of 40 % starting from 4 hours, while HA-Au NPs of 50 % from 24 hours. Gold Salts counterpart produced damage only after longer exposure (48 h). In all cases the percentage of DNA in tail was lower than the positive control, which induced 60 % of DNA in tail.

**Conclusions**

12 nm gold nanoparticles coated with 5 kDa MW Hyaluronan had no cytotoxicity by WST-1 assay at tested doses (below 0.5mg/mL), they were internalized by cells through endosomal/lysosomal pathway after 1 hour of exposition and remained inside vesicular structures in vivo after 14 days of the exposition, as was observed by TEM. ICP-MS showed that Au-HA NPs where mainly accumulated in liver and spleen (which are organs of the reticuloendothelial system), and in lesser amount in kidney, lymph nodes and lung. Very few, but some, nanoparticles were found in the brain, showing that, to a certain extent, Au-HA NPs are able to cross the blood-brain barrier.

Our experiments provide a first attempt aimed at evaluating nanomaterials behaviour in the context of in vitro embryotoxicity tests, based on the ECVAM-validated protocol for EST. We propose a modification of the protocol to make it suited for this kind of nanoparticles, in which the reduction of the exposure time allows the embryoid bodies to attach to the plate surface. This proposed modification brings also the important advantage of saving time on day 5, and, even more important, chemicals. The plotting of differences is also a crucial point that highly facilitates the interpretation of the results. Our results permit to classify HA–AuNPs as weakly embryotoxic. Concerning cobalt ferrite nanoparticles, they can be also classified as weakly embryotoxic if covered with silanes, but non-embryotoxic if covered with a shell of gold.

Gold nanoparticles, though being not cytotoxic induce DNA damage in the Comet Assay, probably through an indirect mechanism due to oxidative stress.

**This work has been partly published in:**


