Chapter 7

Zebrafish or *Danio rerio*: A New Model in Nanotoxicology Study

Maria Violetta Brundo and Antonio Salvaggio

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Abstract

Nanotoxicology represents a new research area in toxicology that allows to evaluate the toxicological properties of nanoparticles in order to determine whether and to what extent they represent an environmental threat. Behavior, fate, transport, and toxicity of nanoparticles are influenced to their particular properties and of several environmental factors. The mechanisms underlying the toxicity of nanomaterials have recently been studied specially in aquatic organisms. In particular, in recent years, the use of *Danio rerio* or zebrafish as an animal model system for nanoparticle toxicity assay increased exponentially. In this review, we compare the recent researches employing zebrafish, adults or embryos, for different nanoparticles' toxicity assessment.

Keywords: *Danio rerio*, nanoparticles, nanomaterials, biomarkers, ZFET

1. Introduction

Nanotechnology has advanced exponentially over the past decade, and nanoscale materials are being exploited in several applications [1]. Between 2011 and 2015, there has been a 30-fold increase in the production of nanoproducts [2].

Engineered nanodevices are finding a new range of applications for the possibility of modifications of their shape, size, surface, and chemical properties. These characteristics are not present in their bulk counterparts [1]. For example, they have very high specific surface areas that give rise to enhanced reactivity and solubility, reduced melting and sintering temperatures, as well as altered crystal structures [1].

Nowadays, we are using a wide variety of commercially available nanoparticles [1]. Metal and carbon nanoparticles (NPs) represent the largest and fastest growing group of NPs [2].

Hence, environmental contamination is already occurring and is predicted to increase dramatically. This growth of nanotechnology has not advanced without concerns regarding their potential adverse environmental impacts. Several studies of nanotoxicology have been made in fact to evaluate the toxicity of various NPs [3–5]. However, there is much to do for evaluating whether the NPs may be an environmental threat. In fact, there are an increasing number of studies where the toxicity of a several engineered nanoparticles or nanomaterials such as fullerenes, graphene metal nanoparticles, metal oxides nanoparticles, crystalline materials, amorphous materials, and nano-sized polymers has been evaluated [6]. For toxicity assays several model organisms are used, such as *Daphnia magna* [7–9], *Paracentrotus lividus* [10, 11], *Mytilus galloprovincialis* [12, 13] or *Mytilus edulis* [14, 15], *Artemia salina* [16, 17], *Danio rerio* [18–21], and other animal models like mice [22, 23]. Each and every year, the number of engineered nanomaterials and their products is continuously increasing, which is necessary to have a representative model organism, able to assess nanotoxicity accurately. In this regard, zebrafish as model organism has attracted scientific interest. In this review, we focused on nanotoxicological studies conducted using as model zebrafish, adults or embryos, with different assessment methods

2. Zebrafish: a perfect experimental animal model

Danio rerio (Hamilton and Buchanan, 1822), commonly known as zebrafish, is a small tropical freshwater fish which lives in river basins of India, Northern Pakistan, Nepal, Bhutan, and South Asia. It belongs to the family of Cyprinidae, within the order of the Cypriniformes. Zebrafish adults are 4–5 cm long, and so they can be easily managed in large numbers in the laboratory.

Zebrafish can tolerate a temperature range of 24.5–28.5°C [24]; however, the growth speed of zebrafish embryos varies according to temperature [25].

One of the reasons why zebrafish is an excellent laboratory model is for its ability to spawn huge amounts of eggs the whole year.

Zebrafish embryonic development has been well characterized to [25]. The embryos themselves are transparent during the first few days of their lives because chorion is transparent. Pigmentation in the embryos starts about 30–72 h post fertilization [26]. Fertilization activates cytoplasmic movements, easily evident within about 10 min. The first cleavage of the newly fertilized egg occurs about 40 min after fertilization. The cytoplasmic divisions are meroblastic, and at the end of them, a blastodisc forms.

Blastula of zebrafish is a "stereoblastula" because blastocoel is not present. The blastula and gastrula stage of zebrafish at 28°C is equivalent to 2.25–5.25 h post fertilization (hpf) and 5.25–10 hpf, respectively [25].

Ballard [27] coined the term "pharyngula" (24–48 h) to refer to the embryo that has developed to the phylotypic stage, when it possesses the classic vertebrate bauplan. This is the time of development when one can most readily compare the morphologies of embryos of diverse vertebrates.

During hatching period (48–72 h), they are called "embryos" until the end of the third day and afterward "larvae," whether they have hatched or not [25].

There are several advantages for using zebrafish as a model species in nanotoxicological studies. Main benefit regards its size. Zebrafish adult is approximately 5 cm long, so it can be handled without any difficulty and reduces housing space and husbandry costs.

The tiny size of the larval and adult zebrafish allows to reduce quantities the dosing of experimental solutions and thereby creates limited volumes of waste to disposal and minimizes quantities of lab ware and chemicals.

Small embryos allow reasonable sample sizes to be tested together using a multiwell plate or series of Petri dishes to provide several experimental replicates at one time. From the egg stage, zebrafish embryos can survive for several days through the absorption of yolk and can be visually assessed for malformation [26].

The rapid maturation of zebrafish (sexual maturation occurs around 100 days) allows easy experimentation for transgenerational endpoints required for mutagenesis screening and assessing chemicals for teratogenicity.

This species shows high fecundity (single female can lay up to 200 eggs per week) and transparent embryos. The eggs hatch rapidly and organogenesis occurs quickly. As a result, the major organs are developed within few days post fertilization (dpf) in larvae.

Zebrafish eggs remain transparent from fertilization to when the tissues become dense and pigmentation is initiated (at approximately 30–72 h post fertilization (hpf)); this allows unobstructed observations of the main morphological changes up to and beyond pharyngulation. Therefore, using little magnification, adverse effects of chemical exposure on development of the brain, notochord, heart, jaw, trunk segmentation, and measurements of size can be assessed quantitatively.

Their optical clarity allows for identification of phenotypic traits during mutagenesis screening and assessment of endpoints of toxicity during toxicity testing. This proves even more valuable when immunochemistry (IHC) techniques are used. There are a vast amount of immunohistochemical markers available, allowing assessments of aberrant morphology or activation of certain signaling pathways by toxicants through the staining of specific tissues and cells types.

The zebrafish research community has developed a range of resources very useful to the toxicologists, including mutant strains, cDNA clone collections, and whole genome that has been sequenced a few years ago. Highly conserved signaling pathways are found both in zebrafish and mammals with a high level of genomic homology [28].

In recent years, the use of zebrafish as an established animal model system for nanoparticle toxicity assay is growing exponentially. Different types of parameters are used to evaluate nanoparticle toxicity such as hatching achievement rate, developmental malformation of organs, damage in gills and skin, abnormal behavior (movement impairment), reproduction toxicity, and finally mortality. In fact, there are an increasing number of literatures that document the concern over toxicity for broad range of engineered nanoparticles or nanomaterials. In this regard, zebrafish as an in vivo model organism has attracted scientific interest because of its unique features abovementioned.

Interestingly, zebrafish behavioral response is also a sensitive indicator for abnormal change in toxicity. An experiment performed by [29] has also shown that TiO_2 nanoparticles affect larval swimming parameters, including velocity and activity level.

The disruption of gills, skin, and endocrine system by nanoparticles is another parameter to understand nanoparticle-induced toxicity. It has been reported that silver ions (Ag^+) generated by AgNPs exert acute toxicity, mainly due to their interaction with the gills. In the gills, ionic Ag^+ inhibits Na^+/K^+-ATPase action and the enzymes related to Na^+ and Cl^- uptake, finally affecting osmoregulation [30].

Nanoparticle affects male and female reproductivity and fetal development. Wang et al. [31] assessed the disturbance in zebrafish reproduction after the chronic exposure of TiO_2 nanoparticles.

Using this model organism, several specific protocols have been used for the toxicity screening. The correlation of successful hatching efficiency and embryo toxicity is an important parameter to evaluate the nanotoxicity.

2.1. Hatching analysis

The hatching-related parameters may be one of the endpoints that have been underestimated in the several studies. There are conflicting results about the endpoint in the same and different species [32, 33], because the results are not easy to interpret. Consequently, hatching-related parameters do not seem to be able to show the toxicity of a nanoparticle especially at environmental-relevant concentrations. In fact, many papers associate different endpoints related to hatching and embryo development [34].

Paatero et al. [35] have used *Danio rerio* embryos to study toxicity profiles of differently surface-functionalized mesoporous silica nanoparticles (MSNs). Embryos with the chorion membrane intact, or dechorionated embryos, were incubated or microinjected with amino (NH2-MSNs), polyethyleneimine (PEI-MSNs), succinic acid (SUCC-MSNs), or polyethylene glycol (PEG-MSNs)-functionalized MSNs. Toxicity was assessed by viability and cardiovascular function. Typically cardiovascular toxicity was evident prior to lethality. Confocal microscopy revealed that PEI-MSNs penetrated into the embryos, whereas PEG^-, NH_2^-, and SUCC-MSNs remained aggregated on the skin surface. Direct exposure of inner organs by microinjecting NH_2-MSNs and PEI-MSNs demonstrated that the particles displayed similar

toxicity indicating that functionalization affects the toxicity profile by influencing penetrance through biological barriers.

Samaee et al. [36] have studied the nano-TiO_2 toxicity to zebrafish embryos through evaluating the success in hatching in relationship with hours postexposure. Zebrafish embryos 4 h post fertilization were exposed to $nTiO_2$ (0, 0.01, 10, and 1000 μg mL(−1)) for 130 h. The hatching rate (HR) was calculated for each concentration tested. It was observed that TiO_2 nanoparticles can cause premature hatching in zebrafish embryos, dose dependently.

Ong et al. [37] have used silicon, cadmium selenide, silver, and zinc NPs as well as single-walled carbon nanotubes to assess NP effects on zebrafish hatch. They have reported complete inhibition of hatching and embryo death within chorion upon nanoparticle exposure, because the nanoparticles interact with the hatching enzymes, and they concluded that the observed effects arose from the NPs themselves and not their dissolved metal components.

2.2. Developmental disorder analysis of zebrafish embryos: zebrafish embryo toxicity test

Zebrafish embryo toxicity test (ZFET) is a modern nonanimal test, and it represents an alternative approach to acute toxicity testing, since with the same sensitivity and specificity, it is possible to find more simplification, economicity, and speedy of execution, as well as suggested by the European Community in order to decrease the impact of the experimental tests on live animals [20, 38]. Fish Embryo Toxicity Test is included in the guidelines to perform toxicity test about FDA and ICH for the pharmaceutical products and about EPA and OECD for the chemical substances [39].

Fish embryo-larval assays provide a screening and investigative tool able of testing a larger number of nanoparticles, and this model has become increasingly common for investigation of developmental toxicity mechanisms [18–20, 40, 41]. ZFET is not a suitable test if you want to evaluate the developmental malformations after the 96 hours post fertilization (hpf), such as the skeletal anomalies, because calcification process in zebrafish starts the seventh day of development.

Usenko et al. [42] have evaluated carbon fullerene (C_{60}, C_{70}, and $C_{60}(OH)_{24}$) toxicity in zebrafish embryos. They observed caudal fin malformation at the concentrations of 200 ppb of C_{60} and C_{70} and yolk sac edema, pericardial edema, and pectoral fin malformations over the concentrations of 2500 ppb of $C_{60}(OH)_{24}$. Additionally, they also observed swelling of zebrafish embryos and delay in development upon exposure to 5000 ppb of $C_{60}(OH)_{24}$.

Brundo et al. [18] tested the nanomaterials that were synthesized proposing a groundbreaking approach by an upside-down vision of the Au/TiO_2 nano-system to avoid the release of nanoparticles. The system was synthesized by wrapping Au nanoparticles with a thin layer of TiO_2. The nontoxicity of the nano-system was established by testing the effect of the material on *Danio rerio* larvae. Zebrafish larvae were exposed to different concentrations of nanoparticles of TiO_2 and Au and to new nanomaterials. Authors evaluated as biomarkers of exposure the expression of inducible metallothioneins. The results obtained by toxicity test showed

that neither mortality nor sublethal effects were induced by the different nanomaterials and free nanoparticles tested. However, only zebrafish larvae exposed to free Au nanoparticles showed a different response to anti-MT antibody. In fact, the immunolocalization analysis highlighted an increase synthesis of the inducible metallothioneins.

Xu et al. [41] evaluated the effect of CuO-NPs on early zebrafish development. The results reveal that CuO-NPs can induce abnormal phenotypes of a smaller head and eyes and delayed epiboly. The gene expression pattern shows that CuO-NPs spatially narrow the expression of dorsal genes chordin and goosecoid and alter the expression of dlx3, ntl, and hgg which are related to the cell migration of gastrulation. The decreased expression of pax2 and pax6 involved in neural differentiation was accordant with the decreased sizes of neural structures. $Cmlc_2$ expression suggests that CuO-NPs prevented looping of the heart tube during cardiogenesis. Furthermore, quantitative RT-PCR results suggest that the CuO-NPs could increase the canonical Wnt signaling pathway to narrow the expression of chordin and goosecoid in dorsoventral patterning as well as decrease the transcription of Wnt5 and Wnt11 to result in slower, less directed movements and an abnormal cell shape. These findings indicated the CuO-NPs exert developmental toxicity.

Pecoraro et al. [20] have tested nanocomposite membranes prepared using Nafion polymer combined with various fillers, such as anatase-type TiO_2 nanoparticles and graphene oxide. The nontoxicity of these nanocomposites, already shown to be effective for water purification applications [19], was recognized by testing the effect of these different materials on zebrafish embryos. They evaluated as biomarkers of exposure the expression of heme-oxygenase 1 and inducible nitric oxide synthases. Embryo toxicity test showed that neither mortality nor sublethal effects were caused by the different nanoparticles and nanosystems tested. Only zebrafish larvae exposed to free nanoparticles have shown a different response to antibodies anti-heme-oxygenase 1 and anti-inducible nitric oxide synthases. The immunolocalization analysis in fact has highlighted an increase in the synthesis of these biomarkers.

2.3. Pathologies analysis in organs of zebrafish embryos and adults

As no authorization is required, many authors prefer the ZFET, and few toxicity studies on nanoparticles are conducted with embryos after 96 hpf. Developmental malformation of zebrafish embryos was studied to several authors, and they can relate incomplete organ development, deformity of body parts, or lack of pigmentation. Zhu et al. [43] did one of the first studies on developmental toxicity in fish caused by iron oxide nanoparticles in aquatic environments. To study the ecological effects of iron oxide nanoparticles on aquatic organisms, they used early life stages of the *Danio rerio* to examine such effects on embryonic development in this species. The results showed that ≥10 mg/L of iron oxide nanoparticles instigated developmental toxicity in these embryos, causing mortality, hatching delay, and malformation. Moreover, an early life stage test using zebrafish embryos/larvae is also discussed and recommended in this study as an effective protocol for assessing the potential toxicity of nanoparticles.

Pecoraro et al. [21] did a study on adverse effects of AgNPs in adult of *Danio rerio*. Fishes exposed to increasing concentrations (8, 45, 70 μg/l) of silver nanoparticles (AgNPs, 25 nm in average diameter) and after treatment for 30 days were quickly euthanized in MS-222. Authors have evaluated bioaccumulation of AgNPs using ICP-MS and analyzed histological changes, biomarkers of oxidative damage, and gene expression in the gut, liver, and gill tissues of AgNPs-treated zebrafish. The histological analysis showed lesions of secondary lamellae of the gills with different degrees of toxicity such as hyperplasia, lamellar fusion, subepithelial edema, and even in some cases telangiectasia. Huge necrosis of the intestinal villi was found in the gut. No lesion was detected in the liver. The analysis revealed a high expression of metallothioneins 1 (MTs 1) in animals exposed to AgNPs compared to the control group. The ICP-MS analysis shows that the amount of particles absorbed in all treated samples is almost the same. They affirm that AgNP toxicity is linked more to their size and state of aggregation than to their concentrations. Silver nanoparticles can damage gills and gut because they are able to pass through the mucosal barrier thanks to their small size. However, the damage is still reversible because it is not documented injury to the basal membrane.

3. Discussion

The toxicology of engineered NMs is a relatively new and evolving field, and although their applications are increasing, there are many concerns about their environmental and health impacts [44]. A large number of studies carried out on several nanoparticles have produced conflicting results. In fact, despite continuous attempts to establish a correlation between structure of the particles and their interactions with biological systems, we are still far from elucidating with certainty the toxicological profile of NPs [45]. Among these investigations, a large numbers of authors, for example, have confirmed the nontoxicity of AuNPs [46–50]; conversely, others have observed the toxicity of AuNPs [51–53].

Despite some authors showed low toxicity of other particles such as TiO_2 NPs [54, 55], studies demonstrated that exposure to high concentrations of TiO_2 particles was able to induce lung tumor formation after 2 years in rats [56]. Moreover, the International Agency for Research on Cancer (IARC) has classified TiO_2 as a possibly carcinogenic to human (Group 2B carcinogen) [57].

For this reason, same researches are developing an innovative nanomaterial that could help to overcome problems related to the toxic effects of NPs, being able to exploit all their qualities [12, 18, 20].

The use of zebrafish as animal model is recommended in several of these researches because it is an inexpensive, quick, and easy model to assess the nanoparticle toxicity [58], and it can offer many advantages for toxicological research [59, 60]. In particular, ZFET, an alternative approach to acute toxicity testing, is important in order to decrease the impact of the experimental tests on live animals as well as suggested by the European Community. Therefore, the use of zebrafish model can be proposed for screening the toxicity profile of nanomaterials and their rapid feedback [61].

Author details

Maria Violetta Brundo[1]* and Antonio Salvaggio[2]

*Address all correspondence to: mvbrundo@unict.it

1 Department of Biological, Geological and Environmental Science, University of Catania, Catania, Italy

2 Experimental Zooprophylactic Institute of Sicily "A. Mirri", Catania, Italy

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