Toxicogenomics Study of Nanomaterials on the Model Organism Zebrafish

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ABSTRACT

We have adapted an "in vitro" toxicity test using zebrafish embryos for a toxicogenomics approach. Our goal is to develop a rapid toxicity assay for nanoparticles to be used in food and other applications. We incubated fertilized fish eggs for 48 h in the presence of a solution of purified MWCNT (1 ppb to 10 ppm) or with nanosilver solutions (1 ppb to 5 ppm). We could not detect effects on development or timing. We have used quantitative RT-PCR to analyze the expression patterns of *Cyp1A1*, *Ahr2*, *Cpt1*, *iNOS*, *Ncf1*, *NudT1*, *c/EBP-α* and *MafT*. These genes are involved in detoxification patterns, in the protection against oxidative stress or code for regulatory factors involved in the immune response. At these concentrations, nanosilver had clear effects on the expression of most of the genes in a dose dependant manner. Induction was maximum in the case of regulatory factors. On the contrary, changes in gene expression induced by MWCNTs were much lower, suggesting a reduced toxicity at the tested concentrations.

Keywords: MWCNT, nanosilver, real-time PCR, toxicogenomics, zebrafish

1 INTRODUCTION

Nanomaterials are increasingly incorporating into our daily lives. The present and future applications of nanomaterials include fields as biomedicine, cosmetics or food science, raising a concern about the need to determine their toxic effects. Recently, a survey has identified at least 212 consumer goods which had nanomaterials incorporated in them. More than half were regarded as "Health and fitness" products [1]. Still, there is an ongoing debate around the open question on toxicological and environmental risks of nanoparticles and nanotubes.[2, 3]. Different studies have been carried out to address these fundamental questions [4, 5] but, still, clearly more information is needed.

Acute and chronic fish tests are frequently used for the investigation of the toxic properties of chemicals and are performed as part of European regulations for chemicals and pesticides. For acute fish tests, an alternative assay based on the study of the effects of toxic substances on zebrafish embryos has been developed [6]. Traditionally, toxicology has studied the possible deleterious effects of a molecule through the analysis of cytological, physiological or morphological endpoints, commonly established on an empirical basis. Unfortunately, often changes only become apparent after a prolonged exposure or as the result of exposure to high doses of the compound. In that sense, it is highly desirable to develop rapid and general methods to score the possible toxic effects of a wide range of products.

Evaluation of risks in humans is derived from results taken from experiments on toxic effects on other species, basically on rodents. Ethical issues and increasing pressure against animal experimentation have lead towards the development of "in vitro" studies. Although tests using cell cultures are rapid and cost-effective, their validity is hampered by the inherent inability to discriminate the effects on a specific target organ.

We have chosen a toxicogenomics approach to study the possible changes in gene expression as a result of the exposure to different nanomaterials and to determine their eventual modes of toxicity. Here we present the results of toxicological studies of the effects of different concentrations of MWCNTs or colloidal silver on the development and gene expression of zebrafish embryos. The test is legally considered as "in vitro", although the results are screened on a complete animal. This strategy is widely used in toxicology and ecotoxicology but such studies using nanomaterials are still missing.

2 MATERIALS AND METHODS

2.1 Fish

Adult zebrafish strain WIK were kept in 30- or 60- liter tanks at 28 ºC under a 12 h/12 h light/dark cycle. They were fed dry flakes twice a day and *Artemia salina* nauplii once a day. Every morning, eggs were collected in a custom cage. Typically, several hundred eggs can be collected daily.

2.2 Nanomaterials

MWCNT were prepared in a quartz reactor at 650 ºC by CVP, with methane as the precursor gas. Nanotubes were purified by treatment with nitric and chlorhydric acid. The sample was analyzed by ICP, ATM and a thermogravimetric assay. No remains of the catalyzer were

detected. For the toxicity experiments, MWCNTs were ground, suspended in an aqueous solution and ultrasonicated for 6 hours.

Colloidal nanosilver (5-20 nm diameter) was purchased from Polytech & Net Gmbh (Schwalbach, Germany) as a 10,000 ppm solution.

2.3 Embryo Test

Embryos were collected in the early morning, cleansed and incubated in the presence of a dilution of the nanomaterial of choice. Fertilized eggs were identified on a binocular microscope and unfertilized eggs were discarded. Five dilutions each of MWCNTs (0.001 to 10 ppm in a 10-fold dilution series) and colloidal nanosiver were used (1 ppb, 10 ppb, 0.1 ppm, 1 ppm and 5 ppm). 30 eggs were incubated for 48 hours in 60-mm Petri dish containing an appropriate dilution of the sampled nanomaterial. Control samples were equally handled and incubated on water. All experiments were carried out by triplicate.

The embryos were incubated at 26 ºC for 48 hours. At 8, 24 and 48 hours after fertilization, the embryos were checked on a microscope to monitor possible malformations or delays in growth.

2.4 RNA extraction

At 24 and 48 haf, embryos were collected and their RNA was extracted using an ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems), according to the manufacturers' instructions.

2.5 Real time RT-PCR

1 µg RNA was treated with DNAase I, reverse transcribed into cDNA and resuspended in 25 µl of water. 1 µl cDNA was used as template in each amplification reaction. Specific oligonucleotides were synthesized for the zebrafish genes *Cyp1A1*, *Ahr2*, *Cpt1*, *iNOS*, *Ncf1*, *NudT1* and *c/EBP-α*, using sequences stored in NCBI and TIGR databases.

Real-time PCR was performed using an ABI Prism 7000 Sequence Detection System, with a SYBR Green PCR master mix (Applied Biosystems). 40 ng cDNA were used as a template. Reaction conditions consisted of 50 ºC for 2 min and 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. A dissociation step was added at the end of the PCR.

The β-actin gene was used as a constitutive control, to normalize all the samples. The threshold cycles were calculated by the 7000 system software and expression levels of the RNAs were calculated by the $2^{-\Delta\Delta Ct}$ method [7].

3 RESULTS AND DISCUSSION

Our aim was to determine the possible toxic effects of nanomaterials currently in use using an "in vitro" test on developing zebrafish embryos.

Carbon nanotubes are vastly used in the nanotechnological industry. They possess unique electrical, mechanical and thermal properties, with potential applications in the electronics, computer, biosensing and other industries [8].

Nanosilver was chosen considering its current use in the food and food packaging industries, mainly due to its well-known microbicidal and bacteriostatic properties [9]. For example, it has been employed in food containers and other devices (for example, in FresherLonger™ Miracle Food Storage by Sharper Image® or in the coating of Samsung refrigerators to suppress the spread of bacteria and other microbes).

There are different potential routes of human exposure to nanoparticles, lungs, skin and the gastrointestinal tract being the most relevant. With the use of a living chordate and the monitoring of the expression of relevant genes, we aimed to simultaneously address different modes of toxicity.

Developing embryos collected 2 hours after fertilization were incubated in the presence of dilution series of either nanosilver or MWCNTs (Figure 1). At 8, 24 or 48 hours after fertilization, zebrafish embryos were monitored at the binocular microscope. The mortality rate, normal gastrulation, apparition of somites and tail detachment were recorded. We also screened for any malformations or delayed embryo development.

When the data from the analyzed groups were compared, no differences were found among them. When post-hatching larvae were fed and allowed to grow in the solutions, they developed normally. We could not detect differences in development or survival in the first 2 weeks. At this point the experiment was brought to an end..

To identify more subtle toxic effects, we decided on the monitoring of different genes with known function in biological processes as detoxification, protection against oxidative burst of regulation. *Cyp1A1*, *Ahr2*, *Cpt1*, *iNOS*, *Ncf1*, *NudT1 c/EBP-α* and *MafT* were the genes of choice.

Cyp1A1 is a member of the cytochrome P450 isozyme family and has been used for decades as a biomarker for polycyclic aromatic hydrocarbon exposure. Its involvement in detoxification processes against other chemicals is also known.

Ahr2, the aryl hydrocarbon receptor, is a cytosolic receptor able to bind dioxins and other chemicals. As a result it dimerizes with the Aryl Hydrocarbon Nuclear Translocator and the complex associates with specific DNA sequences, altering expression of downstream genes..

Cpt1, carnitine palmitoyl transferase, is a key enzyme in the β-oxidation of fatty acids by enabling their transport across the mitochondrial membrane and it is involved in nutritional redox homeostasis.

iNOS encodes for the inducible nitric oxide (NO) synthase. NO is a messenger molecule with diverse

functions throughout the body. In macrophages, NO mediates tumoricidal and bactericidal actions In acute inflammatory conditions associated with cytokine exposure, nitric oxide acts as a potent inhibitor of apoptosis in the liver.

Ncf1, p47phox, neutrophil cytosolic factor, is a subunit of NADPH oxidase, which plays an essential role in the immune systems. NADPH oxidase is active in phagocytes, cells of the immune system that engulf and destroy foreign invaders such as viruses, bacteria and fungi. Our goal was to determine if there is an induction of phagocyte activity upon exposure against nanoparticles.

NudT1, nucleoside diphosphate linked moiety X is involved in cleansing the cell of potentially deleterious endogenous metabolites and to modulate the accumulation of intermediates in biochemical pathways.

c/EBP-α, CCAAT/enhancer binding protein, is a bZIP transcription factor which can bind as a homodimer or heterodimer to certain promoters and enhancers. The encoded protein has been shown to bind to modulate the expression of the gene encoding leptin, a protein that plays an important role in body weight homeostasis. Also, the encoded protein can interact with CDK2 and CDK4 kinases, causing growth arrest in cultured cells.

MafT, is a member of the Small Maf protein family transcription factor that is involved in the regulation of the cellular defense system. Acting as a homo- or heterodimer, it binds specific regulatory *cis*-acting DNA elements.

In our experiments, RNA was extracted at 48 hours after fertilization from zebrafish embryos incubated in the presence of 5, 1, 0.1, 0.01 or 0.001 ppm nanosilver and from embryos of the same age incubated in the presence of 10, 1, 0.1, 0.01 or 0.001 ppm MWCNT.

RT-PCR amplifications were carried out using specific primers (Figure 2). Different patterns of expression were found for the genes tested. At the highest nanosilver concentrations used, we found a clear effect on gene expression in most of the cases. On the contrary, changes in expression observed as a result of the incubation in the presence of the highest doses of MWCNT were much less pronounced. The dramatic effect on gene induction shown by colloidal silver is in agreement with its known microbiocidal activity and probably at 5 ppm its toxic effects on developing fish embryos are evident at the biochemical level. Most of the analyzed genes showed an increase in expression after silver treatment, and the response seemed to be dose-responsive. In particular, incubation in 5 ppm nanosilver resulted in an increase of expression for *Ahr2*, *NudT1*, *c/EBP-α*, *MafT* and, although at a lesser extent, *iNOS*. *Cyp1A1* reached a peak in expression at 1 ppm nanosilver.

The coordinate expression of the different genes may reveal general toxic effects of silver at these concentrations. As pointed before, no effect on developing embryos or larvae could be detected but it's interesting to note the simultaneous induction of expression of genes coding for

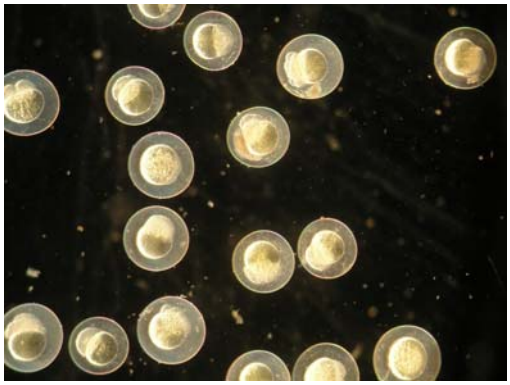

Figure 1. Zebrafish embryos at 2 hours after fertilization, showing the first divisions. Incubation experiments start at this stage.

transcription and regulatory factors that may, in turn, modulate the transcription of a set of responsive genes.

Although some changes in gene expression (*Ncf1*, *MafT*) were found in embryos incubated in the presence of MWCNT these changes never reached the extent found in the case of nanosilver. In our hands, MWCNT had much milder effects on the expression of the analyzed genes.

However, in previous experiments, inflammation, granulomas and fibrosis were detected in rat lungs 60 days after a single intratracheal instillation of 2 mg MWCNTs [10]. Also, proinflammatory reactions were detected after a 48-hour incubation of a culture of human epidermal keratinocytes with 0.4 mg/ml MWCNT [11].

At the doses used, our results may reflect a lower toxicity when compared with silver, although we could not completely discard that MWCNTs could not efficiently penetrate through the eggs' chorion. Additional experiments are being carried out in our laboratory to address this point.

Finally, we are developing an experiment using microarray hybridization of cDNA collected from embryos incubated in the presence of nanosilver and MWCNT. Recently a paper taking advantage of the zebrafish embryo test to study 3,4-dichloroaniline effects through a toxicogenomics approach has been published [12]. This strategy will be used for the study of the effects of nanomaterials on developing embryos. Results will be presented at the conference.

4 CONCLUSIONS

Here we present a valid approach for a rapid and cost effective nanotoxicological analysis. Also, the possibility of producing large numbers of genetically uniform organisms facilitates the obtention of statistically sound results.

Developing zebrafish embryos have been challenged with nanosilver and MWCNT to check for the validity of the test. The study of changes in gene expression patterns as the result of incubation with nanomaterial shows that this is a promising approach for the study of their toxic effects at

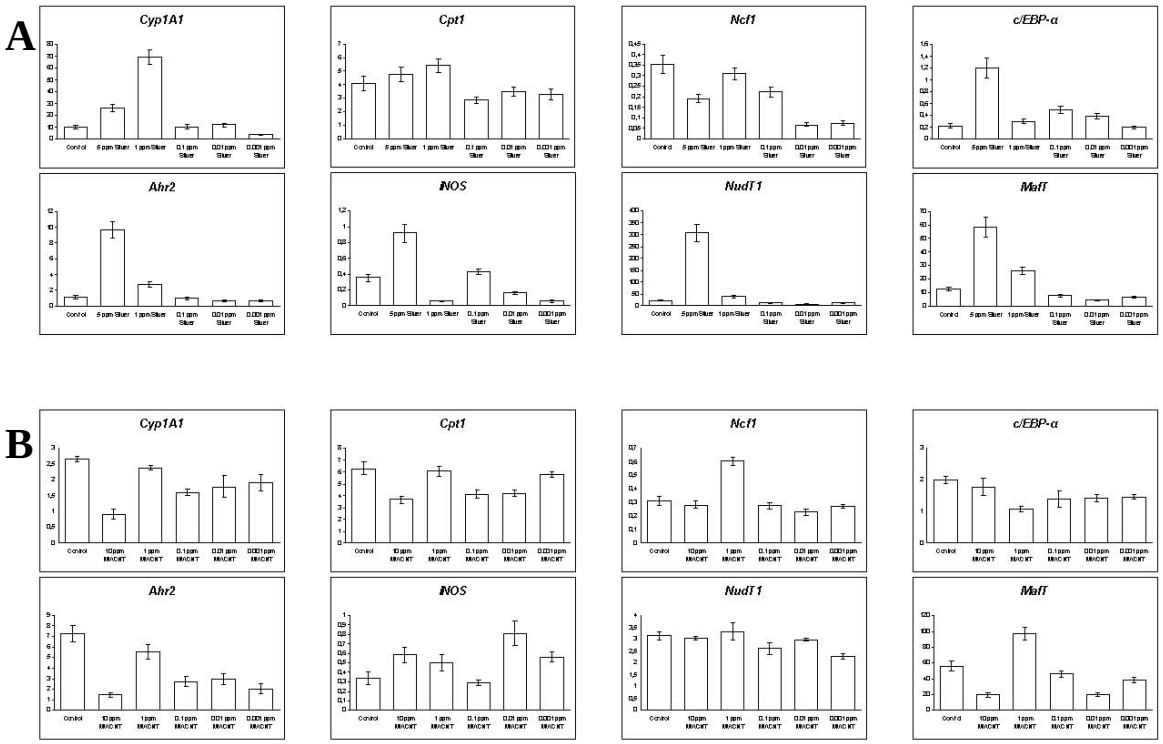

Figure 2: Gene expression of *Cyp1A1*, *Ahr2*, *Cpt1*, *iNOS*, *Ncf1*, *NudT1 c/EBP-α* and *MafT* in zebrafish exposed from 2 h post fertilization for 48 hours to **A**.- Different concentrations of nanosilver or **B**.- Different concentrations of MWCNTs. Gene expression was measured with quantitative real time RT-PCR by triplicate. Gene expression is related to β-actin expression.

the molecular level. We have found changes in genes relevant in detoxification, regulation or the response against oxidative damage.

As an extension of the method, we are using zebrafish microarrays. These data will be presented at the conference. The discovery of genes showing similar patterns of expression may reveal a coordinate expression and may help to elucidate the underlying mechanisms of toxicity. Although it might be difficult to identify specific biomarkers or common cellular or biochemical responses, the possibility of identifying biomarkers related to a toxic response is a promising field for the early detection of the effects on human health associated to nanomaterials [13].

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