Embryonic Toxicity of Nanoparticles

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Key Words

Development · Nanotechnology · Toxicology · Chicken · Mouse

Abstract

Applications of nanoparticles (NP) in medicine, industry and other branches of human activities undoubtedly contribute to technology development and well-being. However, as NP are very small units in a wide range of materials, there is a lack of information related to possible side effects potentially affecting the health of organisms. There is increasing experimental interest in the determination of environmental effects on humans exposed to NP. Most such experimental studies focus on adult populations or adult experimental animals. However, embryos can be more sensitive to pollutants and environmental impacts in some species. Therefore, some investigations dealing particularly with the effects of NP on embryonic development have appeared recently and this issue is becoming of great concern. The aim of this review is to summarize the knowledge on the effects of various nanomaterials on embryonic development. A comprehensive collection of significant experimental nanotoxicity data is presented, which also indicate how the toxic effect of NP can be mediated and modulated with respect to possible effective protection strategies.

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Introduction

Nanoparticles (NP) are defined as particulate matter with at least one dimension that is less than 100 nm. Due to their small size, the total surface area of the particles is maximized resulting in the highest activity per unit of weight. The developing field of nanotechnology has led to extensive use of NP in technical products, human and veterinary medicine, and the building and cosmetics industries. Various types of metals are most commonly used for NP production, including silver, gold, copper and platinum. NP of metals can be easily conjugated with different functional groups, such as polylysine, polyethylene glycol (PEG) or bovine serum albumin [Asharani et al., 2008a; King-Heiden et al., 2009]. On the other hand, quantum dots (QD) are small semiconductor nanocrystals composed of a semiconductor core, which is often

Abbreviations used in this paper

cAg	colloidal silver nanoparticles
cAu	colloidal gold nanoparticles
DT	2,3-dimercaptopropanesulfonate
hpf	hours post-fertilization
MWCNT	multiple-wall carbon nanotube
NP	nanoparticle
PEG	polyethylene glycol
QD	quantum dot
SWCNT	single-wall carbon nanotube

Fig. 1. Scheme of factors influencing the embryonic toxicity of NP. Agglomeration of manganese NP observed in the scanning electron microscope.

encapsulated by a shell or coated with organic molecules. They are only a few nanometers in diameter (2–10 nm) and are commonly used as fluorophores in vital bioimaging of developmental processes [Bruchez et al., 1998; Michalet et al., 2005; Rieger et al., 2005].

During recent years, NP have been widely applied in biomedicine for different purposes. NP can be used in vaccines [Adair, 2009], in personalized cancer therapy [Stegh, 2013], drug delivery [De Jong and Borm, 2008] or in diagnostic methods [Bentolila et al., 2009]. The use of NP in veterinary medicine has recently been reviewed [Underwood and van Eps, 2012]. Presently, there is also much preclinical- or clinical-phase testing underway regarding various applications of nanomaterials in human medicine [Wagner et al., 2006; Cheng et al., 2012].

The large number of nanoproducts surrounding us can cause the release of NP into the environment, which increases animal and human exposure to these materials. Passing the threshold dose of toxicity during the sensitive part of the embryonic and fetal periods could then be a source of different developmental malformations with fatal impact on exposed animals and their offspring. Therefore, knowledge of possible effects on embryonic development is essential. Recently, an expanding number of studies has focused on embryonic stages; however, comparison between these experiments is difficult as different types of particles, sizes or concentrations were tested.

Here, we summarize the main factors influencing the effects of NP on embryos and review their impact on different model organisms.

Factors Influencing the Embryonic Toxicity of NP

Chemical composition, particle size, shape, surface modification and degree of agglomeration can influence bioaccumulation and the type of developmental defects during embryogenesis (fig. 1). Furthermore, a superficial coating effect, concentration of particles, surface charge, Zeta potential and crystal form influence toxicity in embryos. There are also many parameters that can disrupt the biological activity of NP, such as the pH of the solution, salt concentration or temperature [Rogers et al., 2007]. All these factors have to be monitored during experiments to provide consistent conditions.

The impact of NP size was shown in zebrafish embryos [Ispas et al., 2009]. The exposure to differently sized nickel NP as well as larger particle clusters of aggregated entities with a dendritic structure revealed a higher toxicity level than any of the other particle forms or sizes (table 1). As there were no significant differences in toxicities of nickel NP of different size, the spatial configuration of the NP probably affected toxicity more than their size [Ispas et al., 2009]. On the other hand, a size-dependent

Table 1. Effect of NP on fish embryos

Species	Embryonic stage	Duration of exposure	Type of exposure	NP	NP size	Concentration	Effect on embryos	Reference
Zebrafish	cleavage (8-cell) stage to larvae	120 hpf	suspension in egg water	Ag	11.6±3.5 nm	0–0.07 nM	most embryos normal, occasional finfold abnormalities and spinal cord flexure (>0.04 nM)	Lee et al. [2007]
						0.07–0.19 nM	the number of deformed embryos increased with concentration, cardiac malformations (>0.07 nM), yolk sac edema (>0.07 nM)	
						0.19–0.44 nM	dead or abnormal embryos, multiple morphological deformities (>0.38 nM)	
						0.44–0.71 nM	dead or abnormal embryos, head edema, eye deformities (undeveloped optic cups, no retina or lens)	
Zebrafish	embryos	72 hpf	suspension in embryo water	Ag	5–20 nm	5 µg/ml	white opaque appearance, slimy coating, brown flakes inside the chorion, other structures normal	Asharani et al. [2008a]
						10 µg/ml	slight increase in lethality (about 20% embryos), embryos with slimy coating and brown specks	
						25 µg/ml	increase in lethality (about 40% embryos)	
						50 µg/ml	increase in lethality (about 70%), hatching delay, abnormal body axes and twisted notochord (>50 µg/ml), slow blood flow, yolk sac distorted, heart rate decreased and apoptosis in the skin (>50 µg/ml)	
Zebrafish	64- to 256-cell stages (2.5 hpf)	72 hpf	suspension in water	Ag (supported by Ag ₂ O, Ag ₂ H, TiO)	10–20 nm	100 µg/ml	increase in lethality (about 85%), hatching delay (only 15–33% embryos hatched), other phenotype similar to concentration of 50 µg/ml	Yeo and Kang [2008]
						10, 20 ppt	hatching rate decreased, abnormal notochord, weak heartbeats, edema and abnormal vertebra, no eyes or smaller	
						≤0.02 nM	most embryos normal	
						0.02–0.2 nM	cardiac and eye deformities, yolk sac edema, finfold and tail abnormalities, skeletal flexure, head edema	
Zebrafish	cleavage (8-cell) stage to larvae	120 hpf	suspension in egg water	Ag	41.6±9.1 nm	≥0.2 nM	100% lethality	Lee et al. [2012a]
						≥0.2 nM	100% lethality	
						≥0.02 pM	finfold abnormalities and spinal cord flexure, rare eye abnormalities, rare abnormal tail	
						1.0 pM	finfold abnormalities and spinal cord flexure, eye abnormalities, cardiac malformation, yolk sac edema, rare abnormal tail	
Zebrafish	cleavage (8-cell) stage to larvae	120 hpf	suspension in egg water	Ag	95.4±16 nm	2.0 pM	finfold abnormalities and spinal cord flexure, eye abnormalities, often cardiac malformation, yolk sac edema, often abnormal tail	Lee et al. [2012b]
						2.0 pM	finfold abnormalities and spinal cord flexure, eye abnormalities, often cardiac malformation, yolk sac edema, often abnormal tail	
						2.0 pM	finfold abnormalities and spinal cord flexure, eye abnormalities, often cardiac malformation, yolk sac edema, often abnormal tail	
						2.0 pM	finfold abnormalities and spinal cord flexure, eye abnormalities, often cardiac malformation, yolk sac edema, often abnormal tail	

Table 1 (continued)

Species	Embryonic stage	Duration of exposure	Type of exposure	NP	NP size	Concentration	Effect on embryos	Reference
Zebrafish	cleavage (8-cell) stage to larvae	120 hpf	suspension in egg water	Ag-CALNNK NP ⁴⁻⁶	11.7 ± 2.7 nm	0–0.1 nM 0.2–0.4 nM	all embryos normal increase in dead embryos with concentration, finfold abnormalities, tail/spinal cord flexure, yolk sac edema, cardiac malformation, no eye	Lee et al. [2013]
				Ag-CALNNS NP ⁴⁻⁶	11.7 ± 2.7 nm	0–0.2 nM	most embryos normal, third of embryos dead, no deformed embryos embryonic lethality about 40%, it did not increase with concentration, rare finfold abnormalities, tail/spinal cord flexure, rare yolk sac edema and cardiac malformation	
						0.4–0.6 nM	embryonic lethality about 40%, it did not increase with concentration, finfold abnormalities, tail/spinal cord flexure, yolk sac edema, cardiac malformation, small eye	
				Ag-CALNNE NP ⁴⁻⁶	11.7 ± 2.7 nm	0–0.2 nM	most embryos normal, few embryos dead, no deformed embryos	
						0.4–0.6 nM	embryonic lethality increased with concentration, most embryos dead at 0.6 nM, finfold abnormalities, tail/spinal cord flexure, yolk sac edema, cardiac malformation, small eye	
Zebrafish	eggs (2–4 hpf)	120 hpf	suspension in egg water	Ag	3, 10, 50, 100 nm	0.25, 2.5, 25, 100, 250 µM	high mortality at 250 µM, sublethal toxic effect at concentrations of 25 and 100 µM with several larval morphological malformations (yolk sac edema, jaw and snout anomalies, small head, tail and body degradations) with some differences between smaller and larger NP	Bar-Ilan et al. [2009]
				Au			normal (mortality <3%)	
Zebrafish	cleavage stages of embryo	120 hpf	suspension in egg water	Au	11.6 ± 0.9 nm	0, 0.025, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80, 1.0, 1.2 nM – corresponding to concentrations of 0, 0.26, 0.53, 1.1, 2.1, 4.2, 6.3 µg/ml	depending on concentration: – majority of embryos normal (81 ± 13 to 63 ± 20%) – some embryos dead (17 ± 11 to 31 ± 18%) – just few abnormal (0 to 6.6 ± 5.5%) – no tail or its flexure, finfold abnormality, acephaly, cardiac malformation, yolk sac edema	Browning et al. [2009]
Zebrafish	embryos (8-cell stage)	72 hpf	suspension in egg water	Ag	5–35 nm	10, 25, 50, 75, 100 µg/ml	hatching delay, concentration-dependent drop in heart rate, spinal cord flexure, pericardial effusion, cardiac malformations, circulatory defects, absence or malformation of eyes, concentration-dependent increase in mortality (about 50% embryos for 100 µg/ml)	Asharani et al. [2011]
				Au	15–35 nm	10, 25, 50, 75, 100 µg/ml	normal (no increase in mortality)	
				Pt	3–10 nm	10, 25, 50, 75, 100 µg/ml	hatching delay, concentration-dependent drop in heart rate, spinal cord flexure, concentration-dependent increase in mortality (about 30% embryos for 100 µg/ml)	

Table 1 (continued)

Species	Embryonic stage	Duration of exposure	Type of exposure	NP	NP size	Concentration	Effect on embryos	Reference
Zebrafish	embryos	24–72 hpf	suspension in water	carbon nanotubes	N/A	40 µg/ml ≥60 µg/ml	no phenotype delayed hatching, abnormal spinal cord, slimy mucous-like coating around embryos	Asharani et al. [2008b]
Zebrafish	4 hpf	4–96 hpf	suspension in water	SWCNT	average diameter 11 nm; length 0.5–100 µm	20, 40, 60, 120, 240 and 360 mg/l	no embryo mortality, hatching delay when using high concentrations >120 mg/l, no effect on embryonic development up to 96 hpf (head–trunk angle and body length normal)	Cheng et al. [2007]
				double-walled carbon nanotubes	N/A	120 and 240 mg/l	hatching delay at concentrations >240 mg/l	
				carbon black	0.01–1.0 µm	120 and 240 mg/l	no effect on hatching	
Zebrafish	1-cell stage	to the larval stage	microinjection	SWCNT	average diameter 1–2 nm; length 100–800 nm	2.4 ng (2 nl)	no negative effect	Cheng et al. [2011]
Zebrafish	1-cell stage	80 hpf	microinjection	MWCNT	diameter 19.9±8.25 nm; length 0.8–0.1 µm	0.4 pg to 2 ng	developmental arrest until death, not initiated epiboly, lower dose (<5 pg) produced abnormal notochord, small eyes, delayed yolk sac and yolk extension resorption, edema, shortened body length	Cheng and Cheng [2012]
Zebrafish	blastula stage (3–3.5 hpf)	24–192 hpf	water suspension (E3 medium)	Cu	50–100 nm	0.01, 0.1, 1 or 10 mg/l	delayed hatching of embryos, morphological malformations, concentration >0.1 mg/l is lethal	Bai et al. [2010a]
Zebrafish	blastula stage (3–3.5 hpf)	96 hpf	water suspension (E3 medium)	ZnO	47.3±12.9 up to 1,002.0±259.2 nm	0 mg/l 5 mg/l 10 mg/l	normal body length normal body length, lower hatching rate shortened body length of larvae, tail malformation, low hatching rates	Bai et al. [2010b]
					25 mg/l		shortened body length of larvae, tail malformation, no hatching	
					50 and 100 mg/l		no hatching	
Zebrafish	24 eggs (blastula stage)	96 hpf	aqueous suspensions	ZnO	50–360 nm	50, 10, 5, 1, 0.5, 0.1, 0 mg/l	dose-dependent toxicity, calculated LC ₅₀ was 1.793 mg/l, no survivals at 50 mg/l, tissue ulceration	Zhu et al. [2008]
				TiO ₂	100–550 nm	500, 100, 50, 10, 1, 0 mg/l	not toxic to embryos	
				Al ₂ O ₃	285–2,450 nm	1,000, 100, 10, 1, 0 mg/l	not toxic to embryos	
Zebrafish	48 hpf	96 hpf	suspension in Hanks' balanced salt solution	TiO ₂	86 nm	170 ng/ml	microinjection in the otic vesicle resulted in gene expression changes in circadian rhythm, kinase-related activities, exocytosis and trafficking of Golgi organs	Jovanovic et al. [2011b]

Table 1 (continued)

Species	Embryonic stage	Duration of exposure	Type of exposure	NP	NP size	Concentration	Effect on embryos	Reference
Zebrafish	sphere stage (4–6 hpf)	5 days after fertilization	suspension in culture media	CdSe/ZnS QD	9 nm 7 and 14 nm	38 μ M Cd 2–28 μ M Cd	coated with polylysine: normal coated with PEG: altered axial curvature, malformed tail, yolk sac malformations, opaque tissue	King-Heiden et al. [2009]
Zebrafish	6 hpf	6 h – 120 hpf	suspension in ocean salt	CdSe	3.5 nm	0, 0.05, 0.15 mg/l 0.45, 1.35 mg/l 4.05 mg/l 12.15 mg/l 31.25 mg/l	not toxic to embryos, normal hatching rate, no morphological malformations <35% lethality, slower swimming behavior at a concentration of 1.35 mg/ml, concentration-dependent increase in apoptosis about 72% embryos dead, oosperm coagulation, eyespots and melanin developmental inhibition, pericardial edema, bent spine, unhatched and tail without extension 91% embryos dead, oosperm coagulation, eyespots and melanin developmental inhibition, pericardial edema, bent spine, unhatched embryos and tail without extension no survivals	Zhang et al. [2012]
Zebrafish	64- to 256-cell stages (2.5 hpf)	2–72 hpf	suspension in city water	Cu _x TiO _y	30–50 nm	10 and 20 ppt (Cu: 1, 10, 15, 25 mol%)	decreased hatching rate, abnormal notochord formation, no tail, damaged eyes and abnormal heart development, lack of head development, edema, increase in glutathione and catalase activity, increase in glutathione S-transferase and glutathione reductase activity	Yeo and Kang [2009]
				TiO ₂	30–50 nm	10 and 20 ppt	decreased hatching rate, abnormal notochord formation, no tail, damaged eyes and abnormal heart development, lack of head development, edema, increase in catalase activity, increase in glutathione S-transferase activity	
Zebrafish	blastula stage (<6 hpf)	96 hpf	suspension in culture media	Ru@SiO ₂ Cy5.5@SiO ₂	200 nm 60 nm	0.0025–200 mg/l	hatching time and hatching rates were normal, no increase in mortality or morphological deformities, NP did not penetrate the chorion	Fent et al. [2010]

Table 1 (continued)

Species	Embryonic stage	Duration of exposure	Type of exposure	NP	NP size	Concentration	Effect on embryos	Reference
Zebrafish	24 hpf	5 days after fertilization	E3 embryo media	Ni	30 nm (spherical)	0–1,000 mg/l	LD ₁₀ = 187 mg/l, LD ₅₀ = 328 mg/l, intestinal defects with underdeveloped intestinal epithelial cells (LD ₁₀), skeletal muscle defects with the separation of trunk skeletal muscle fibers (LD ₅₀), jaw patterning defects (LD ₅₀)	Ispas et al. [2009]
					60 nm (spherical)	0–1,000 mg/l	LD ₁₀ = 189 mg/l, LD ₅₀ = 361 mg/l, intestinal defects with underdeveloped intestinal epithelial cells (50 mg/l), skeletal muscle defects with the separation of trunk skeletal muscle fibers (LD ₅₀), jaw patterning defects (LD ₅₀)	
					100 nm (spherical)	0–1,000 mg/l	LD ₁₀ = 172 mg/l, LD ₅₀ = 221 mg/l, intestinal defects with underdeveloped intestinal epithelial cells (LD ₁₀), skeletal muscle defects with the separation of trunk skeletal muscle fibers (LD ₅₀), jaw patterning defects (LD ₅₀)	
					60 nm (dendritic/aggregated)	0–1,000 mg/l	LD ₁₀ = 21 mg/l, LD ₅₀ = 115 mg/l, intestinal defects with underdeveloped intestinal epithelial cells (LD ₁₀), skeletal muscle defects with the separation of trunk skeletal muscle fibers (LD ₅₀), jaw patterning defects (LD ₅₀)	
Zebrafish	4–5 hpf	96 hpf	zebrafish culture medium	chitosan	200 nm	5, 10, 20, 30, 40 mg/l	decreased hatching rate (20 and 40 mg/l), increased mortality (30 and 40 mg/l), morphological malformations at 5 mg/l (bent spine, pericardial edema and opaque yolk), increase in cell death (20 and 30 mg/l), high expression of reactive oxygen species (already at 5 mg/l), increase in heat shock protein 70 (significant at 40 mg/l)	Hu et al. [2011]
					340 nm	5, 10, 20, 30, 40 mg/l	decrease in hatching rate (30 and 40 mg/l), increased mortality (40 mg/l), no morphological malformations	
					30 nm	10 mg/l	decrease in hatching rate, increased mortality, elevated expression of heat shock protein 70	
Zebrafish	2 days after fertilization	1–5 days after injection	microinjection into the left flank (behind the yolk sac extension), NP in PBS	silica	200 nm	35–40 nl (10 mg/ml)	no significant increase in mortality or apoptosis, increase in morphological malformations (4.68% of embryos, no statistical significance to control) such as pericardial edema, malformed Meckel's, anterior and posterior brachial cartilage, ethmoid plate, auditory capsule	Sharif et al. [2012]
Zebrafish	4 hpf	96 hpf	suspension in water and then culture media	silica	62 nm	25, 50, 100, 200 µg/ml	increasing mortality and inhibition of hatching rate at 100 and 200 µg/ml, pericardial edema, yolk sac edema, tail and head malformation, dose-dependent increase in cellular death	Duan et al. [2013]

Table 1 (continued)

Species	Embryonic stage	Duration of exposure	Type of exposure	NP	NP size	Concentration	Effect on embryos	Reference
Japanese medaka	blastula (stage 10) to larvae	48 hpf – 60 days after hatching	suspension in water	Ag	20–37 nm	≤500 µg/l 400–1,000 µg/l	no dead embryos developmental retardations, increase in embryonic mortality, heart rate, inhibition of pigmentation, decrease in leucophore expression, decrease in optic tectum width, skeletal flexure and truncation, hemorrhage, eye and finfold abnormalities, edema 100% lethality	Wu et al. [2010]
Japanese medaka	fertilized eggs (8–9 stages)	2 days after hatching	suspension in embryo rearing media solution	silica	50 nm	0, 10, 50, 100 mg/l	increase in mortality and malformations (10 and 50 mg/l), ocular and heart defects, blastula edema with hemorrhage, retarded swimming	Lee et al. [2011]
Fathead minnow	<24 hpf	7 days	fish egg water	TiO ₂	1–110 nm (average 43)	0.01, 0.1, 1, 10, 100, 1,000 µg/ml	no increase in mortality, suppression of neutrophil function, no differences in blood cell number, gene expression changes (IL11, MSFI, NCF2)	Jovanovic et al. [2011a]

N/A = No data available; LD₅₀ = median lethal concentration.

toxic effect was confirmed in zebrafish in the case of using silver NP [Lee et al., 2012a]. Smaller NP could passively diffuse through the chorion and a greater number of larger NP was found to be incorporated in deformed embryos, confirming the size-dependent nanotoxicity [Lee et al., 2012a]. These contradictory results could be explained by differences in the character of NP used.

Indeed, other studies showed that size is not as important for embryonic toxicity as the chemical composition of the nanomaterial used. Comparison of colloidal silver and gold NP revealed that silver NP caused 100% mortality while gold particles caused only 3% mortality under the same experimental conditions (fig. 2) [Bar-Ilan et al., 2009]. Silver NP in sublethal concentrations also generated a variety of developmental defects, including smaller heads and caudal fins, larger yolk sac and larger eyes. On the other hand, gold particles exhibited a very small sublethal toxic effect [Bar-Ilan et al., 2009; Asharani et al., 2011]. Furthermore, graded concentrations of silver particles induced dose-dependent mortality in zebrafish embryos, while gold NP did not exhibit increased mortality at higher concentrations [Bar-Ilan et al., 2009]. A comparison of three metal oxide NP (TiO₂, ZnO and Al₂O₃) also exhibited strong differences in toxicity in zebrafish embryos with dependence on the chemical composition of the NP, where ZnO particles were the most toxic [Zhu et al., 2008].

The influence of NP concentration (particles/cm³) was shown in zebrafish embryos incubated with purified stable silver NP [Lee et al., 2012b]. The number of deformed embryos rose as the NP concentration increased, with considerable dose dependency of the phenotypes. The effect of increasing concentrations on higher mortality was confirmed for silver particles in *Oryzias latipes* [Wu et al., 2010] and in *Pimephales promelas* embryos [Laban et al., 2010]. Zinc oxide [Zhu et al., 2008] and copper NP [Bai et al., 2010a] also exhibited dose-dependent mortality in zebrafish embryos. On the other hand, experiments in zebrafish showed that gold NP accumulated in embryos with increasing concentration, but the effects on embryonic development were not proportionally related to the concentration [Browning et al., 2009].

The differences in embryonic toxicity could also be due to agglomeration of the particles, which can affect the total surface area and change the chemical properties of NP. Because ZnO NP tend to form much larger aggregates in water, the biological effects of these aggregated NP on developing zebrafish embryos and larvae were examined [Zhu et al., 2008]. These aggregates were found to exert dose-dependent toxicity. Furthermore, sonication

of silver NP led to a decrease in aggregate formation, which resulted in a significant increase in mortality compared to exposure to particles that were just stirred [Laban et al., 2010]. No correlation between NP size and any toxic effect was observed in this study.

Some NP in physiological fluids can release ions, which can also influence embryonic development. Comparison of Ag^+ ions and silver NP showed that they produced similar delayed hatching of zebrafish embryos; however, Ag^+ elicited greater dysmorphology and mortality. The results indicated that Ag NP are less potent than Ag^+ with respect to dysmorphology and loss of viability [Powers et al., 2011]. Treatment of mouse blastocysts with both silver NP and Ag^+ induced apoptosis with a stronger effect in the case of Ag^+ application [Li et al., 2010]. Again, the effect of Ag NP on the embryonic developmental potential was weaker than in the case of Ag^+ . Moreover, the dangerous effects of Ag^+ on the post-implantation potential in vivo after embryo transfer were stronger than those of Ag NP [Li et al., 2010]. On the other hand, comparison of nickel NP with soluble nickel salts in zebrafish revealed intestinal defects only in embryos exposed to NP [Ispas et al., 2009]. Additionally, copper NP have a similar effect on zebrafish embryos as Cu^{2+} [Bai et al., 2010a]. Comparative experiments between ZnO NP and dissolved Zn (ZnO NP can release Zn^{2+}) revealed that soluble Zn^{2+} was much less toxic to zebrafish embryos than ZnO NP [Bai et al., 2010b]. Therefore, ions that are released during NP production can cause variable levels of toxicity in embryos, and generation of stable dispersants is critical to avoid increased embryonic lethality by ion-related side effects.

The importance of NP surface modification on in vivo biological responses was shown in zebrafish embryos [Truong et al., 2011], where lead sulfide NP (PbS) were coated with either sodium 3-mercaptopropanesulfonate or sodium 2,3-dimercaptopropanesulfonate (DT) ligands. Both ligands were structurally analogous and had the same carbon backbone with a sulfonate tail group, while differing only in the head group (mono- vs. dithiol). Exposing embryos to these NP revealed differential biological responses, in which mortality was induced in 100% of PbS-3-mercaptopropanesulfonate-treated embryos, while exposure of PbS-DT NP produced less than 5% mortality at the same concentration [Truong et al., 2011]. Citrate-coated or polyvinylpyrrolidone-coated Ag NP only delayed hatching or produced neurobehavioral disruption, while neither affected embryonic survival or morphology at a nominal concentration [Powers et al., 2011]. Citrate-coated NP showed a greater effect, proba-

Fig. 2. Representative micrographs of zebrafish embryos 120 h post-fertilization (hpf) that were exposed to egg water (a) or 250 µM colloidal gold nanoparticles (cAu) and colloidal silver nanoparticles (cAg) of previously synthesized sizes (b) from 4 to 120 hpf [Bar-Ilan et al., 2009]. Blank panels are indicative of 1 survivor out of $n = 12$ with three replicates. Along with a low survival rate between comparable cAu and cAg sizes, the cAg survivors showed a high incidence of sublethal toxic effects. Due to the low survival rate at 250 µM cAg and low incidences of sublethal toxic effects at 25 µM (at 120 hpf), embryos were exposed to an intermediate concentration of 100 µM cAg (c). Scale bar = 0.5 mm. Reprinted with permission from Bar-Ilan et al. [2009]. Copyright 2014 John Wiley and Sons.

bly because the polyvinylpyrrolidone-coated NP agglomeration decreased their surface-to-volume ratio so that less Ag⁺ came into the solution [Powers et al., 2011]. On the other hand, adverse effects of gold NP on muscle development were enhanced by conjugating Au NP with heparan sulfate [Zielinska et al., 2011], but their interaction with taurine did not enhance the phenotype in chicken embryos [Zielinska et al., 2012]. Similarly, use of gold NP with polyvinyl alcohol as a capping agent did not show any toxic effect on zebrafish embryos [Asharani et al., 2011].

Surface charge has also been found to affect biological properties of NP. When silver NP were coated with biocompatible peptides (CALNNK, CALNNS and CALNNE) to prepare positively or negatively charged NP (table 1), the last group was found to be the most toxic for zebrafish embryos [Lee et al., 2013]. So, surface charge was confirmed to be necessary to avoid NP agglomeration.

In summary, there are many contradictory results about size dependence or agglomeration formation effects on embryonic toxicity. This is probably connected to the difficulties with preparing stable, homogenous solutions of NP for in vivo experiments. Therefore, it is necessary to take solution preparation into account, and as many details as possible should be reported in new studies to help reveal the real effects of individual NP parameters on embryonic toxicity.

As in vitro toxic effects of NP can differ from in vivo experiments, it is necessary to analyze the influence of variable NP on early as well as late embryonic development in animal model organisms. In particular, routes of entrance can change possible toxicity of NP. NP can access the body by penetration through the skin, ingestion with food or liquid, inhalation and by transplacental penetration in mammalian embryos. Here, we review individual NP sorted by chemical composition and their observed effect on embryos or larvae of individual model species with focus on developmental defects, which can arise after NP exposure during embryogenesis.

Embryonic Toxicity of NP in Animal Models: Fish Embryos

The zebrafish is a valuable model species for characterizing the toxicity of NP, because of its high sensitivity to even very low levels of environmental pollutants [von Westernhagen, 1988]. Furthermore, embryos are small and translucent, their development is very rapid, and it is easy to obtain them in large numbers [Frayse et al., 2006].

In addition, the embryos of the zebrafish, an anamniote, develop in the aquatic environment where direct exposure to NP can be very high, as particles can penetrate through the chorionic pores into the chorionic space and then into the inner cell mass of the embryo via random Brownian motion [Lee et al., 2007; Lee et al., 2012]. However, some NP were shown to aggregate together and stay trapped inside the chorionic pores [Lee et al., 2012a]. This aggregation clogs the chorionic pores and influences nutrient transportation, which might have adverse effects on embryonic development. Next, NP are distributed into many different organs (e.g. eye, brain and heart) of zebrafish and they stay inside the embryos throughout their development. The accumulation of large numbers of NP inside the embryos can lead to their malformation, increase their mortality and induce behavioral changes (table 1).

Exposure of zebrafish embryos to silver NP delayed hatching with a concentration-dependent increase in mortality [Lee et al., 2007; Asharani et al., 2008a, 2011; Lee et al., 2012a]. Developmental changes included fin-fold deformities, spinal cord flexure and truncation, abnormal notochord, and severe eye or cardiac abnormalities [Lee et al., 2007; Yeo and Kang, 2008; Lee et al., 2012a]. Morphological defects also included opaque and nondepleted yolk, small head and jaw, snout and circulatory malformations (hemorrhages and blood clots) [Bar-Ilan et al., 2009]. The developmental toxicity of silver NP has also been investigated in Japanese medaka – *O. latipes* [Wu et al., 2010]. Generally, no distinct species-specific differences in comparison to zebrafish embryos were found (table 1). Furthermore, developmental abnormalities induced by Ag NP are similar to those caused by toxic chemicals such as cadmium [Hallare et al., 2005], indicating possible common targets of malformation during embryogenesis [Lee et al., 2007]; however, a detailed study focusing on the effect of silver on individual cellular developmental processes is necessary.

Similar to silver, gold NP showed diffusion into the chorionic space of zebrafish embryos via their chorionic pore canals [Browning et al., 2009]. The quantity of gold NP accumulated in the embryos increased with the concentration, but the effect on embryonic development was not proportionally related to concentration. Gold NP were shown to be nontoxic in comparison to silver particles [Asharani et al., 2011]. On the other hand, zebrafish embryo screening methods revealed that bare colloidal gold also induced less than 3% mortality [Bar-Ilan et al., 2009] or 24% dead embryos [Browning et al., 2009], de-

pending on experimental conditions, particle preparation techniques and concentration (table 1).

The common NP used in cosmetics, electronics, photonics and ceramics are ZnO particles [Wang, 2004]. They can be produced in different shapes (nanocombs, nanorings, nanosprings, nanobelts and nanowires), which enable their wide variability of usage. Exposure of zebrafish embryos to ZnO NP caused hatching retardation, tail malformations and shortened body length of the larvae at lower concentrations and embryonic mortality at higher concentrations [Zhu et al., 2008; Bai et al., 2010b]. Zinc ions released from the NP probably contributed to their high toxicity, which is in contrast to other metal nontoxic NP such as Al_2O_3 and TiO_2 [Zhu et al., 2008]. Differences in NP shape (strip shape or spherical/elliptical shape) did not produce variability in embryonic phenotype, probably because of particle agglomeration into similarly shaped clusters in the water [Zhu et al., 2008].

Analysis of the potential toxicity of a suspension of copper NP on zebrafish embryos [Bai et al., 2010a] revealed that Cu NP delayed embryo hatching, caused morphological malformations of the larvae or even terminated development at the gastrula stage when a higher concentration was used (table 1). In contrast to ZnO particles [Bai et al., 2010b], no significant difference was found regarding the effect of Cu NP from Cu^{2+} at the corresponding concentrations. Therefore, Cu NP by themselves do not seem to produce additional cellular processes increasing their embryonic toxicity.

Titanium dioxide (TiO_2) NP have recently become common in industrial applications as components of sunscreens and lacquers with 'invisible' UV filters, soaps, shampoos and toothpastes, as an additive in paint with photocatalytic-induced self-cleaning properties and in other building materials [Melquiades et al., 2008]. The exposure of zebrafish or fathead minnow embryos to TiO_2 NP did not produce an increase in mortality or morphological malformations [Zhu et al., 2008; Jovanovic et al., 2011a]. However, the treatment with NP stimulated the immune system and upregulated gene expression of several inflammatory markers (e.g. IL-11, MSF-1 and NCF2) [Jovanovic et al., 2011a] at levels close to estimated environmental concentrations. Also, microinjection of sublethal TiO_2 concentrations into the zebrafish otic vesicle did not produce any significant toxicity in zebrafish embryos and larvae [Jovanovic et al., 2011b]. The hatching rate was similar to control animals and neither embryos nor larvae showed any significant malformations. However, a more detailed analysis using a microar-

ray approach revealed changes in genes related to circadian rhythm, cell kinase activity and intracellular trafficking indicating the toxic potential for behavioral impact [Jovanovic et al., 2011b]. As TiO_2 is classified as a human carcinogen (IARC, 2006), further study of the effect of TiO_2 during long-term exposure of whole embryos is required.

The Cu-doped TiO_2 system is considered as a potential photocatalyst due to its promising performance in degrading various environmental pollutants [Choi and Kang, 2007]. The exposure of zebrafish embryos to Cu-loaded TiO_2 NP revealed abnormal embryonic morphology, including edema and absence of tail and head (table 1) [Yeo and Kang, 2009]. It was proposed that Cu_xTiO_y could enter into cells and affect their ion regulation, causing lethal injury to embryos. Developmental abnormalities could also be caused by Cu blocking ion transportation as copper can act as sodium analogues and competitors in gills [Grosell and Wood, 2002].

Nickel NP have recently been adopted in the manufactures of jewelry, medical implants, stainless steel, Ni-Cd batteries, and catalytic and electronic applications. Exposing zebrafish embryos to nickel NP produced intestinal defects, suggesting that the gut is a major route of exposure during embryogenesis [Ispas et al., 2009]. Furthermore, nickel NP exposure resulted in skeletal muscle abnormalities and defects of the head and jaw cartilage. The phenotype increased with higher NP concentration. The influence of nickel NPs was less than or equal to that of soluble nickel, while particle agglomerations were more toxic. It was proposed that defects from Ni NP arise by different biological mechanisms or entry routes than from soluble nickel [Ispas et al., 2009] because soluble Ni does not produce any obvious digestive or skeletal muscle defects except at very high concentrations.

Platinum NP are used in medicine for cancer therapy and in catalytic techniques using oxygen, hydrogen and other gases. Exposing zebrafish embryos to platinum particles led to delays in hatching as well as a concentration-dependent drop in heart rate and axis curvatures [Asharani et al., 2011]. Accumulation of platinum in embryos was lower in comparison to silver and gold NP, probably due to their smaller size.

Chitosan [poly(1,4- β -D-glucopyranosamine)] has recently been used as a drug or gene delivery vehicle [Dyer et al., 2002; Huang et al., 2004]. However, the exposure of zebrafish embryos to biodegradable chitosan NP caused a decrease in hatching rate and increased mortality (table 1) that was concentration dependent [Hu et al., 2011]. The rate of malformations increased with increasing con-

centrations. Furthermore, embryos exposed to chitosan NP exhibited an increased rate of cell death and enhanced expression of intracellular reactive oxygen species, as well as overexpression of heat shock protein 70, indicating that chitosan NP can induce a higher level of cellular oxidative stress [Hu et al., 2011].

Mesoporous silica NP can also be used as a compound delivery system for therapeutic drugs [Ravi Kumar et al., 2004]. The application of silica particles to zebrafish embryos did not affect survival or morphology of the embryos [Fent et al., 2010; Sharif et al., 2012]. Furthermore, cell death or immune responses measured by leukocyte infiltration were not induced [Sharif et al., 2012]. Developmental toxicity of fluorescent silica NP was also evaluated in *O. latipes* embryos [Lee et al., 2011]. NP showed a minor effect on the hatchability of the embryos, with slight increases in mortality and morphological defects with increasing particle concentration (table 1). Use of the highest concentration led to aggregation formation resulting in reduced toxicity to embryos. Fluorescence caused by the particles was mostly detected in the gut tissues, indicating that the main route of NP entrance into the larval body is by ingestion and not by skin penetration [Lee et al., 2011].

Carbon nanotubes are single or multiple concentric graphene sheets used in electronics, computers and architecture [Ajayan and Zhou, 2001]. Single-wall carbon nanotubes (SWCNT) have a small diameter of around 1–2 nm, while multiple-wall carbon nanotubes (MWCNT) are composed of several layers of carbon cylinders with a total diameter of around 10–30 µm. Carbon nanotubes represent a promising vector for drug delivery [Bianco et al., 2005; Lacerda et al., 2006] because of their ability of intrinsic transport and long blood circulation time; however, their usage is now of great interest with an effort to understand processes of cellular NP-caused damage [Shvedova et al., 2012]. Zebrafish embryos exposed to MWCNTs exhibited delayed hatching, abnormal spinal cord and a slimy mucous-like coating around embryos with a concentration-dependent phenotype [Asharani et al., 2008b]. As properties of carbon nanotubes vary highly depending on the manufacturing process [Baughman et al., 2002], the effects of different durations of MWCNT sonication in a nitric acid solution on zebrafish embryos were analyzed [Cheng and Cheng, 2012]. The length of sonication time influences the final size of nanotubes and the length of carbon nanotubes greatly affected their toxicity in embryos. A longer sonication time was shown to produce severe developmental toxicity, while a shorter sonication time did not generate

obvious changes in embryonic development [Cheng and Cheng, 2012]. The most affected embryos displayed developmental arrest until death, epiboly was not initiated and embryos were maintained at the sphere stage. The phenotype of older embryos was concentration dependent (table 1). Interestingly, just a small change in the length of carbon nanotubes induced a large difference in the bioproperties of the produced nanomaterial.

Exposure of zebrafish embryos to SWCNT led to a hatching delay only when using high concentrations and double-walled nanotubes at even higher concentrations [Cheng et al., 2007] and no increase in embryo mortality was observed. As SWCNT have a tendency to agglomerate, large clusters of NP did not pass through the chorion pores but induced changes in the chorion membrane and could contribute to a hatching delay [Cheng et al., 2007]. Toxicity of high concentrations was probably also caused by the presence of Co and Ni catalysts, which are used for NP synthesis. Microinjection of single-well nanotubes conjugated with an anti-angiogenic binding site (cyclic arginine-glycine-aspartic acid) and an anti-angiogenic drug (thalidomide) inhibited wound-healing-associated angiogenesis [Cheng et al., 2011] without an increase in embryonic mortality, which showed their possible use as vector for drug delivery. Furthermore, NP combined with a drug and a fluorescent probe enable better-targeted cancer therapy.

Fluorescent nanocrystals or QD have recently been used in biomedical imaging, diagnostics and tumor targeting [Michalet et al., 2005]. QD are coated to reduce leaching of metal into the solutions or to facilitate their entry into biological targets [Akerman et al., 2002; Derfus et al., 2004]. However, coating or size of QD can influence the embryonic toxicity, as was shown in case of CdSe/ZnS QD coated with PEG or polylysine in zebrafish embryos [King-Heiden et al., 2009]. QD induced higher mortality than the equivalent amount of Cd²⁺, probably due to the specific organic coating, QD scale or degree of their agglomeration in solution. Similarly, cadmium selenium (CdSe) QD coated with mercaptopropionic acid exhibited toxicological effects reflected in a concentration-dependent decrease in the hatching rate of zebrafish and morphological malformations (table 1) [Zhang et al., 2012], while use of mercaptopropionic acid alone did not show any obvious toxic effect on zebrafish. Increased cellular apoptosis was concentration dependent for used QD and it was proposed to be a more sensitive diagnostic marker of early nonlethal changes during experimental conditions.

Table 2. Effect of NP on amphibian embryos

Species	Embryonic stage	Duration of exposure	Type of exposure	Nano-particle	Size of nanoparticles	Concentration	Effect on embryos	Reference
<i>Pelophylax perezi</i>	Gosner stage 24–25	96 h	suspension in water	TiSiO ₄	<50 nm	8.2, 10.2, 12.8, 16, 20 mg/l	increased catalase activity, increased LDH activity, mortality <11%, lactate and alanine contents significantly reduced	Salvaterra et al. [2013]
<i>Xenopus laevis</i>	2-cell stage, 8-cell or blastula	late tadpole stages	injection into individual cells	ZnS/CdSe QD	10–15 nm	2×10 ⁹ QD/cell 5×10 ⁹ QD/cell	phenotype same as control abnormal phenotype	Dubertret et al. [2002]

Embryonic Toxicity of NP in Animal Models: Amphibian Embryos

Amphibians are widely used for a variety of developmental and toxicological studies [Dawson and Bantle, 1987; Fort et al., 2000; Leconte and Mouche, 2013; Yu et al., 2013]. However, up to now, there have been only a few studies evaluating the effect of NP on tadpole development (table 2) or establishing new imaging methods [Umanzor-Alvarez et al., 2011; Charalambous et al., 2012; Salvaterra et al., 2013]. Moreover, it is necessary to mention that *Xenopus* embryos exhibit a high degree of auto-fluorescence [Beumer et al., 1995] and, therefore, an evaluation of QD fluorescence is more difficult in comparison to other species.

The toxicity of titanium silicate NP (TiSiO₄) was studied in tadpoles of *Pelophylax perezi* (Seoane) [Salvaterra et al., 2013]. The mortality of the embryos was less than 11% at all tested concentrations and no morphological changes were described. On the other hand, biochemical and metabolic changes were observed when analyzing levels of lactate dehydrogenase and catalase activities, indicating increased oxidative stress following NP application (table 2).

A comparison of two methods of delivering QD into embryos was performed in *Xenopus* where near-infrared laser injection was compared to standard manual injection using micropipettes [Umanzor-Alvarez et al., 2011]. Most of the embryos survived the laser treatment without obvious developmental defects from the QD delivered to the target tissue; however, pulses of higher intensities caused permanent damage to the targeted cells. Both application methods showed comparable results; however, using laser injection, it was possible to employ much smaller cell types [Umanzor-Alvarez et al., 2011].

Furthermore, a new method of monitoring QD conjugates was introduced using NP in *Xenopus* embryos

[Charalambous et al., 2009, 2011, 2012; Ioannou et al., 2012]. QD were conjugated with protein or RNA by an intein-mediated ligation system. After application into embryos, it was possible to evaluate the localization of infrared-emitting QD to confirm in vivo targeting of a selected protein.

ZnS/CdSe QD coated with phospholipids were successfully used for cell tracking in *Xenopus* embryos [Dubertret et al., 2002]. Individual cells were injected by particles, but abnormalities were observed only after the application of higher concentrations of QD. It was proposed that osmotic changes contributed to altered development; however, a more detailed study of the cellular mechanisms is necessary.

Embryonic Toxicity of NP in Animal Models: Avian Embryos

Chicken embryos represent a conventional and easily accessible model that has been used in developmental biology and biomedical research for a long time [Dieterlen-Lievre, 1997; Coleman, 2008; Rashidi and Sottile, 2009]. Embryos grow quickly and independently from the mother and individual developmental stages are morphologically well defined, which makes the comparison between experiments easier. It is also suitable for developmental toxicity testing because of the possibility of large-scale analysis with easy in ovo manipulations during different developmental stages. Surprisingly, similar to amphibian models, there have been only a few studies analyzing the embryonic toxicity of NP in this experimental model, but their number has been increasing in the last few years (table 3).

In contrast to zebrafish, the application of colloidal silver NP to chicken eggs showed no influence of the NP on embryo development, but it is necessary to mention that

Table 3. Effect of NP on bird embryos

Species	Embryonic stage	Duration of exposure	Type of exposure	NP	NP size	Concentration	Effect on embryos	Reference
Chicken	application 3 times on days 5,11 and 17 of incubation	collection on day 18 of incubation	injection into egg	colloidal Ag	N/A	10 ppm (0.2 ml)	number and size of lymph follicles were decreased in the bursa of Fabricius	Grodzki and Sawosz [2006]
Chicken	prior to incubation of eggs	20 days	injection into egg	hydrocolloidal Ag	N/A	50 ppm (0.3 ml)	deposition in femur, influence on bone mineralization, increasing mineral content	Sikorska et al. [2010]
Chicken	day 4	4–20 days of incubation	injection into egg (albumen)	hydrocolloid Ag/Pd	from a few to 100 nm	50 ppm (0.3 ml)	normal embryonic survival, development without any abnormalities	Studnicka et al. [2009]
Chicken	day 4	48 h, 18 days	injection into egg (albumen)	colloidal Ag – Ag/Cu – Ag/Pd	2–6 nm	50 ppm (0.3 ml)	no negative effect on embryo survival, growth, development and morphology, did not affect activity of multiple enzymes (asparagine transferase, alanine transferase and alkaline phosphatase), concentrations of glucose, triacylglyceride and cholesterol in blood serum or genotoxicity measured as a concentration of 8-oxo-2deoxyguanosine in the liver DNA	Sawosz et al. [2009]
Chicken	day 1	21 days	injection into egg (air sack)	hydrocolloids Ag	2–35 nm	50 mg/kg of egg	Ag but no Au affected metabolic rate of layer embryos but not broiler embryos	Pineda et al. [2012]
Chicken	day 3	20 days	injection into egg (air sack)	Au, Au + taurine, taurine	N/A	gold: N/A, taurine: 4.32 mg/ml	gold did not change PCNA expression, while taurine decreased this marker	Zielinska et al. [2010]
Chicken	fertilized eggs (day 1)	20 days	injection into egg (air sack)	Au, Au + heparan sulfate, heparan sulfate	5–70 nm	gold: 50 mg/l, heparan sulfate: 0.032 mg/l	no effect on mortality, organ weight and homeostasis of the embryos, nano-Au + heparan sulfate complex contributed to the increased number of myocytes and nuclei in chicken embryo muscles	Zielinska et al. [2011]
Chicken	day 3	from 3 to 20 days of incubation	injection into egg (air sack)	Au, Au + taurine, taurine	10–30 nm	gold: 50 mg/l, taurine: 4.32 mg/ml	gold NP affected the number of muscle cells during development, taurine exhibited the strongest effect	Zielinska et al. [2012]
Chicken	day 4	from 4 to 20 days of incubation	injection into egg (albumen)	hydrocolloid Pt	2–19 nm	1, 5, 10, 15 or 20 µg/ml	did not affect the growth and development of the embryos, no changes in the number of cells in the brain cortex, weight of the brain, heart, spleen or bursa of Fabricius, induced apoptosis measured by caspase-3-positive cells and decreased the number of proliferating cells measured by PCNA-positive cells in the brain tissue	Prasek et al. [2013]
Chicken	day 3	days 11 and 14 of incubation	micro-injection	SWCNT	0.7–1.1 nm	25 µg	inhibition of angiogenesis of the chorioallantoic membrane and in the chicken embryo, harmful effect on embryonic development, deregulation of important genes	Roman et al. [2013]
Chicken	fertilized eggs	72 h	injection into egg	maltoedextrin-coated cadmium sulfide semiconductor NP	3 nm	3.28, 6.56, 8.20, 9.84, 11.48, 13.12, 14.76 and 16.4 nm	significant abortifacient activity, observed morphological alterations at concentrations >9.84 nm, dose-dependent increase in anomalies that, starting at 9.84 nm, centered on the heart, central nervous system, placodes, neural tube and somites, no toxic effect observed with concentrations <3.28 nm	Rodriguez-Fragoso et al. [2012]

N/A = No data available; PCNA = proliferating cell nuclear antigen.

the number and size of lymph follicles in the bursa of Fabricius decreased [Grodzik and Sawosz, 2006]. Furthermore, the experiment started on day 5 of incubation (table 3), which is already quite late in development and, therefore, early changes in development could not be observed in this experimental design.

When hydrocolloids of silver NP were applied to fertilized eggs before incubation, silver was deposited into femurs without affecting the structure or mechanical properties of the bone [Sikorska et al., 2010]. However, an influence on bone mineralization was observed, with a tendency towards increasing mineral content. As the skeleton can absorb silver NP without affecting bone structure and biomechanical properties, these particles can be used to carry drugs into the developing bones [Sikorska et al., 2010].

Similarly, in ovo injection of silver/copper or silver/palladium NP into chicken embryos did not influence embryonic development [Sawosz et al., 2009; Studnicka et al., 2009] or produce any negative effects on embryonic survival, growth, development or morphology of chicken embryos. Interestingly, a comparison between different breeds revealed that silver NP affected the metabolic rate of layer but not broiler embryos but had no effect on the growth rate of embryos [Pineda et al., 2012].

In the light of previous experiments, it seems that silver does not influence chicken development as negatively as in zebrafish. However, it is necessary to take into account that only a small increase in Ag in the embryonic body was observed [Sikorska et al., 2010] and injection into the air sac of egg or albumen was used as the main application. Thick albumen could lead to lower distribution and penetration of silver NP in contrast to the water environment of zebrafish. Furthermore, the concentrations used were much smaller with no testing of concentration gradients.

Likewise, gold NP showed no adverse effect on mortality, organ weight or homeostasis of chicken embryos, but did influence muscle development where an increased number of myocytes was observed following the conjugation of gold NP with heparan sulfate [Zielinska et al., 2010, 2011, 2012]. As gold particles are more biocompatible and stable than silver particles [Browning et al., 2009], they have been recommended for use as probes for in vivo experiments, cellular imaging or as vehicles for drug delivery. However, the concentration of particles has to be taken into account to avoid unintentional side effects.

Similarly, platinum NP also did not affect the growth and development of chicken embryos [Prasek et al., 2013]. Nevertheless, NP induced apoptosis (table 3) and de-

creased the number of proliferating cells in the brain tissue [Prasek et al., 2013]. On the other hand, concentrations of platinum NP were lower in chicken embryos in comparison to zebrafish [Asharani et al., 2011]. As they observed a concentration-dependent increase in apoptotic cells [Prasek et al., 2013], it is highly probable that morphological abnormalities would occur after the application of higher concentrations, similar to zebrafish.

The application of SWCNT inhibited the angiogenesis of the chorioallantoic membrane in chicken embryos and embryos died before day 12 of incubation. Embryos were smaller, although without significant morphological anomalies. Gene expression of cell proliferation regulators as well as genes of apoptosis, survival and angiogenesis were downregulated [Roman et al., 2013].

Cadmium, which is a component of QD, is known to be toxic to embryos [Fein et al., 1997]. Protection of the core by capping can decrease the toxicity of the NP. Exposure of chicken embryos to cadmium sulfide semiconductor NP coated with maltodextrin resulted in dose-dependently increased developmental anomalies (table 3) [Rodriguez-Fragoso et al., 2012]. However, no toxic effect was observed with low concentrations, which enables them to be used in bioimaging applications at this low concentration [Rodriguez-Fragoso et al., 2012].

Embryonic Toxicity of NP in Animal Models: Mammalian Embryos

The mouse is a common mammalian model for embryonic toxicity studies [Bailey et al., 2009; Wise et al., 2009; Khan et al., 2013; Wolff et al., 2013]. Because NP can cross the placental barrier in mammals and can accumulate in embryonic as well as surrounding extra-embryonic tissues, studying their effect on embryos is critical [Chu et al., 2010]. The effects of NP on mammalian embryos and their transplacental crossing have recently become the center of attention (table 4). As mammalian species exhibit large variability in placental morphology and gestational length, the comparison of embryonic toxicity between species is essential.

In mouse blastocysts, silver NP can induce apoptosis in the trophectoderm and the inner cell mass, and significantly inhibit cell proliferation [Li et al., 2010]. To discover the impact of Ag NP on later mammalian development in vivo, untreated control and silver-pretreated mouse blastocysts were transferred to females and the embryos were examined 13 days later [Li et al., 2010]. The implantation ratio of NP-treated blastocysts was signifi-

Table 4. Effect of NP on mammalian embryos

Species	Embryonic stage	Duration of exposure	Type of exposure	NP	NP size	Concentration	Effect on embryos	Reference
Mouse	oocytes	24 h	treatment during in vitro maturation	CdSe QD	3.5 nm	0–500 nM	increased resorption of post-implantation embryos and decreased placental and fetal weight, decreased cell number, induced apoptosis and inhibited post-implantation development, possibly due to a teratogenic effect	Hsieh et al. [2009]
Mouse	blastocyst	24 h	suspension in medium	CdSe-core QD	3.5 nm	250, 500 nM	induced apoptosis in mouse blastocysts, inhibited cell proliferation, retarded early post-implantation blastocyst development and increased early-stage blastocyst death in vitro and in vivo, cytotoxicity was significantly reduced by the addition of a ZnS coating	Chan and Shiao [2008]
Mouse	blastocyst	24 h	suspension in medium	Ag	13 nm	25, 50 μ M	implantation ratio of NP-treated blastocysts was significantly lower, weight of the placenta and fetal weight were reduced in the treated group	Li et al. [2010]
Mouse	pregnant mouse	injection for 2 consecutive gestational days (E16, E17), collection at E18	intravenously	Si	70, 300 and 1,000 nm	0.8 mg/mouse	particles were found in placental trophoblast cells, fetal liver and brain already 24 h after injection, they induced smaller uteri and fetuses, silica NP induced morphological or functional changes in the offspring only when small particles (<100 nm) were applied while after larger particle treatment (300 and 1,000 nm) embryos were normal, alterations in the placenta morphology, with cell damage and induction of apoptosis	Yamashita et al. [2011]
				TiO ₂	35 nm	0.8 mg/mouse	particles were found in placental trophoblast cells, fetal liver and brain already 24 h after injection, structural changes including smaller uteri, smaller fetuses	
Mouse	pregnant mouse	up to 4 postnatal days, 6 weeks old	intravenously	TiO ₂	25–70 nm	1 mg/ml	functional and pathologic disorders, reduced daily sperm production, numerous caspase-3-positive cells in olfactory bulb	Takeda et al. [2009]
Mouse	pregnant mouse	8–18 days of gestation, 1 h/day	inhalation	TiO ₂	21 nm	42 mg/m ³	moderate neurobehavioral alterations, lung inflammation in mothers	Hougaard et al. [2010]
Mouse	gestational days 6, 9, 12 and 15	up to E16 and postnatal days 2, 7, 14 and 21	subcutaneous injection	TiO ₂	2,570 nm	1 μ g/ μ l	changes in the expression of genes associated with apoptosis in newborns, oxidative stress markers were changed in the brains of 2- and 3-week-old mice, expression of genes associated with neurotransmitters and psychiatric diseases was altered	Shimizu et al. [2009]
Mouse	gestational day 10	gestational day 19	oral gavage	TiO ₂	51–65 nm	10, 100 or 1,000 mg/kg	no obvious signs of maternal toxicity, increase in fetal deformities (exencephaly, open eyelids, leg defects, tail defects) and mortality	Philbrook et al. [2011]
				Ag	24–47 nm		no obvious signs of maternal toxicity, increase in fetal mortality	

Table 4 (continued)

Species	Embryonic stage	Duration of exposure	Type of exposure	NP	NP size	Concentration	Effect on embryos	Reference
Mouse	morula	24 h	suspension in medium	chitosan	up to 100 nm	10–200 µg/ml	blastocyst complications that had either no blastocoel cavity or a small cavity, treated embryos showed lower expression of trophectoderm-associated genes as well as pluripotent marker genes, reduction in total cell numbers, enhanced apoptosis at the blastocyst stage, the implantation rate of embryos was reduced, implanted embryos were subsequently resorbed	Park et al. [2013]
Mouse	days 5.5 or 14.5 of gestation	10 min, 1 and 4 h after injection	injection	SWCNT	N/A	0.1–30 µg/mouse	no adverse effects on embryos up to the dose of 10 µg, occasional teratogenic effects associated with placental damage was observed at the dose of 30 µg	Campagnolo et al. [2013]
Mouse	pregnant mice	immediately after birth/for QD distribution study 22–24 h after application/for study of transplacental transport 5.5, 21 and 50 h after injection.	injection into tail vein	CdTe/CdS QD	N/A	20, 50, 86 or 125 µg	QD may be transferred from female mice to fetuses across the placental barrier, smaller QD are more easily transferred than larger QD and the number of QD transferred increases with increasing dosage	Chu et al. [2010]
Rat	from gestation day 6–19	gestation day 20	orally	MWCNT	10–15 nm in diameter, 20 µm in length	0, 40, 200 and 1,000 mg/kg/day	no treatment-related differences in gestational index, embryo-fetal mortality, or fetal and placental weights were observed	Lim et al. [2011a]
Rat	from gestation day 6–19	gestation day 20	orally	MWCNT	10–15 nm in diameter, 20 µm in length	0, 40, 200 and 1,000 mg/kg/day	morphological examinations of the fetuses demonstrated no significant difference in incidences of abnormalities	Lim et al. [2011b]
Rat	pregnant rats	from gestation day 1–19, 5 h/day	inhalation	NP-rich diesel exhaust	20–30 nm	148.86 g/m ³	the relative weights of the seminal vesicle and prostate to body weight were decreased; serum concentrations of testosterone, progesterone, corticosterone, follicle-stimulating hormone and testicular concentrations of steroidogenic acute regulatory protein and 17β-hydroxysteroid dehydrogenase mRNA were decreased after exposure	Li et al. [2009]
Rat	pregnant rats	from gestation day 1–19, 5 h/day	inhalation	NP-rich diesel exhaust	22–27 nm	148.86 g/m ³	suppressed function of corpora lutea, stimulation of adrenal cortex function, greater body weight and crown-rump length of fetuses, lower concentration of maternal progesterone, luteinizing hormone and corticosterone significantly higher	Li et al. [2013]
N/A = No data available.								

cantly lower in comparison to the untreated controls and the implanted embryos mostly failed to develop (table 4). Furthermore, the potential for subsequent development was reduced. A comparable effect was observed in mice after cadmium exposure during the pre-implantation period [De et al., 1993] indicating a common embryotoxic effect of silver NP resembling other chemicals.

The intravenous injection of titanium dioxide into pregnant mice [Yamashita et al., 2011] induced structural changes, including a smaller size of uteri as well as fetuses (table 4). The toxicity was dependent on the NP concentration and could be prevented by coating the surface with carboxy or amino groups. Damage of the genital system, including defects in daily sperm production or defects of cranial nerves, was observed in the pups after subcutaneous injection of TiO₂ NP into pregnant mice [Takeda et al., 2009]. More detailed microarray analysis revealed changes in the expression of genes associated with apoptosis, brain development and function of the central nervous system in embryos [Shimizu et al., 2009]. Not only injection but also maternal inhalation of TiO₂ induced moderate neurobehavioral alterations in the offspring [Hougaard et al., 2010] and maternal oral dose resulted in increases in fetal deformities and their mortality [Philbrook et al., 2011].

Similar to zebrafish, the exposure of mouse morulae to chitosan NP caused several pre- and post-implantation defects in embryos [Park et al., 2013]. Blastocysts exhibited an abnormal blastocoel cavity and reductions in total cell numbers with enhanced apoptosis. Furthermore, expression of the *Wnt3a*, *B3gnt5* and *Eomes* genes (markers of developmental progression in blastocysts) was down-regulated in agreement with the severity of the morphological phenotype [Park et al., 2013]. As chitosan NP exhibit much higher cytotoxicity than chitosan [Qi et al., 2005], embryonic toxicity of these NP should be taken into account before using chitosan for drug delivery.

The analysis of silica NP distribution in maternal organs and fetuses after intravenous injection into pregnant mice [Yamashita et al., 2011] revealed a similar distribution to that seen with titanium dioxide particles. However, silica NP induced morphological or functional changes in the offspring only when small particles were applied (table 4).

When carbon nanotubes were orally given to pregnant rats, minimal maternal toxicity without an increase in embryonic mortality or fetal weight was observed [Lim et al., 2011]. Similarly, SWCNT fused with PEG chains caused occasional teratogenic effects only when using high concentrations in the mouse [Campagnolo et al.,

2013]. As embryonic malformations arise when using concentrations beyond a threshold dose, then only low concentrations of nanotubes should be used for potential therapeutic treatments.

Treatment of mouse oocytes with CdSe QD during in vitro maturation led to increased resorption of post-implantation embryos and decreased placental and fetal weights [Hsieh et al., 2009]. However, ZnS-coated CdSe QD did not affect development; therefore, surface modification of the core helped decrease the embryonic toxicity of the NP. A similar cytotoxic effect of CdSe core QD was observed at later developmental stages of mouse blastocysts in a dose-dependent manner [Chan and Shiao, 2008]. QD composed of a CdTe core encased in mercaptopropionic acid [Chu et al., 2010] can be transported across the placental barrier depending on their size, with smaller QD demonstrating higher Cd levels and greater toxicity. When the particles were stabilized with a SiO₂ or PEG coating, their transport and toxic effect decreased [Chu et al., 2010].

NP-rich diesel exhaust represents a serious environmental problem leading to bronchial asthma [Miyabara et al., 1998] or lung cancer [Ichinose et al., 1997]. Exposure of pregnant rats to NP from diesel exhaust led to a decrease in the weight of seminal vesicles and prostate in male offspring [Li et al., 2009]. Moreover, the level of testosterone, progesterone and FSH was significantly decreased in the serum of offspring. Taken together, these data show that prenatal exposure suppresses testicular function in immature offspring. Maternal progesterone production was also affected [Li et al., 2013], confirming that hormonal regulation of pregnant animals was disrupted, which increases the potential risk of abortions (table 4).

Toxicity of NP in Human Embryos

Animal models are very useful for primary investigations of NP toxicity; however, they can fail to predict teratogenicity, as in the case of thalidomide [Marx-Stoelting et al., 2009]. Therefore, further studies confirming their possible safety for human embryos are necessary. Recently, several models have been established to study human-specific teratogenicity of NP. Human embryonic stem cells were utilized to derive a 3-dimensional in vitro model that allows the testing of potential developmental neurotoxicants [Hoelting et al., 2013]. This model enables processes to be studied at the molecular as well as cellular level in detail.

Furthermore, an ex vivo human placental perfusion model was used to study the potential of NP for transpla-

cental transfer. Gold NP (10–30 nm) were not transferred from perfused human placenta [Myllynen et al., 2008] into the fetal circulation within the examined time period (6 h). On the other hand, fluorescent polystyrene particles (240 nm) were able to cross the placental barrier [Wick et al., 2010]. Therefore, more detailed studies are necessary for different classes of particles in order to evaluate their potential safety risk for human embryos.

Toxicity of NP: Potential Mechanisms behind Their Cellular Effects during Embryonic Development

Because of their small size, NP can penetrate into the circulatory system and directly propagate into cells thus causing different interactions with cellular organelles. While the toxicity of NP and their effect on embryonic mortality and developmental defects have recently been in focus, the exact toxic mechanism is still unclear, and only a few studies have centered their attention on the cellular response in embryos [Sawosz et al., 2009; Shimizu et al., 2009; Xia et al., 2011; Park et al., 2013].

The toxicity of NP can be explained by their different biological activities, including oxidative stress that damages lipids, carbohydrates, proteins and DNA. Moreover, their penetration into mitochondria, activation of the immune response and changes in receptor or ion channel functions after NP incorporation must be considered. Also, interactions of NP with enzymes, potential apoptotic or necrotic factors and also induction of inflammatory processes must be taken into account [Kohen and Nyska, 2002; Yeo and Kang, 2009; Park et al., 2011, 2013; Zhao et al., 2013]. Furthermore, NP show virus-like properties, which can cause DNA damage and delays in cell proliferation [Asharani et al., 2008a]. Furthermore, they can reduce mitochondrial function [Chen and Schluesener, 2008] and generate cellular morphological abnormalities [Hussain et al., 2005]. The mechanism of NP toxicity can also vary among different types of NP, depending on their chemical composition [Buzea et al., 2007].

Conclusion

Nanomaterials are ubiquitous in the environment as they are widely used, e.g. in the production of many medical products such as surgical instruments or prosthetic devices, deodorants, cleaning reagents, building materials and clothing. However, confirmation of their safety is still limited. As tiny particles, they can easily accumulate with-

in bodies, where many of their interactions remain unknown. The study of their embryonic toxicity and knowledge of their impact on embryonic development has also become critical because of their potential transfer through the placental barrier. NP may also cross the blood-brain barrier in the fetus and evoke neurobehavioral changes. In general, the penetration of particular NP into different organs, their circulation, along with life span and final effects should be understood in order to further optimize their application with no or minimal side effects. Moreover, only small variations in the structure, size and chemical composition of NP can have a significant effect on bioproperties of produced material as well as on the level and mechanisms of cellular or organismal response. Further studies are also necessary to establish efficient protection strategies against the cytotoxic effects of these NP, which can include coating or other modifications of their surfaces with the aim of creating safer nanomaterials for the future.

Up to now, only some NP have been tested in a variety of model organisms and, therefore, the possibility for comparisons are limited and it will be necessary to fill the gaps in the present knowledge to separate species-specific responses from general toxic effects. However, some species-specific differences in the toxicity of the same NP have already been observed. In particular, chicken embryos seem to be more resistant to NP treatment. This phenomenon can be affected by the type of the application, where injection into the air sac or albumen was preferentially used in chicken embryos while zebrafish embryos were floating directly surrounded by egg water with dispersed NP. NP could agglomerate in albumen or bind into albumen protein and penetration into the embryo could be much lower. While embryonic uptake has been studied in zebrafish, it would be necessary to study the diffusion into individual embryonic organs and their accumulation in chicken embryos as final concentration inside the embryo can highly differ between species. Assays to determine the metal content could reveal the intake of NP with different chemical compositions similar as applied in zebrafish where variable accumulation of silver, gold and platinum was found [Asharani et al., 2011]. It is also necessary to take in account that not only chemical composition and physical parameters affect the final toxicity, but also the application and routes inside organisms differ among diverse species and significantly influence the possible toxicity of NP.

Many questions still remain open regarding the safety of NP and it will be essential to solve them quickly as there is a growing speed of NP applications in many branches of medicine and industry.

Acknowledgments

This study was supported by the Grant Agency of the Czech Republic (P503/11/2315 and P503/12/G147) and institutional support (RVO:67985904). We thank Dr. Filip Mika (Institute of Scientific Instruments of the ASCR, Brno, Czech Republic) for scanning electron microscope images.

Disclosure Statement

The authors declare that they have no competing interests.

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