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Chapter 2. Developmental toxicity of nanoplastics in the chicken embryo

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Abstract

Nanoplastics and microplastics are an emerging class of pollutants which are becoming a major source of concern due to their potentially harmful effects on the biosphere. Even though the effects of nanoplastics and microplastics are well-studied in aquatic invertebrates and fish, little is known about their effects on warm-blooded animals. The aim of our research is to determine the effects of nanoplastics on the chicken embryo might model. In this chapter, we exposed stage 8 chicken embryos to 25 nm plain polystyrene nanoplastics (PSNPs). Histology, Alcian blue staining and scanning electronic microscopy were used to visualize the effects on the embryonic phenotype 24 h and 4 d post exposure. We found that 25 nm polystyrene nanoplastics caused dose-dependent mortality and malformations of 4 d post exposure chicken embryos. The highest dose (5 mg/mL) caused malformations in 5/5 embryos, and death in 15/20 embryos at 4 d post exposure. The malformations included neural tube defects, microphthalmia and tail bud hypoplasia. We also observed a significant developmental delay in embryos examined at 24 h post exposure. Together, our findings strongly suggest that PSNPs are toxic to the developing chicken embryo. These results call for an in-depth investigation of cellular and molecular mechanisms of toxicity of nanoplastics in the chicken embryo (Chapter 4).

Introduction

In recent decades, many new pollutants such as microplastics and nanoplastics (MPs and NPs) have been identified (Lim, 2021). Plastics are polymers derived from the petroleum industry. They are one of the important basic five materials of human invention which bring the greatest convenience to people's daily life (Ewen, 1997). They are durable, light, water-proof and relatively cheap compared with other materials. Therefore plastic are widely used in different including medicine, the military and high-tech industries (Abbing, 2019; Rosato and Rosato, 2011). The value of global plastic production reached 568.9 billion dollars in 2019 (Roy et al., 2021). Many plastics directly enter the environment after use by consumers (Lim, 2021). In some countries, where they are not collected and disposed of safely, plastics can become degraded in the natural environment into tiny particles called microplastics and nanoplastics (Godfrey, 2019). MPs are defined as plastic particles or fragments ≤5 mm in diameter. Among microplastics there may be even smaller particles called nanoplastics which are ≤100 nm or ≤ 1 µm (Mitrano et al., 2021). MPs and NPs can arise from the physical degradation of larger plastic items such as supermarket carrier bags, plastic bottles, etc. They are also generated during the manufacture of clothing from synthetic fabrics such as nylon or acrylic (Fuschi et al., 2022).

MPs and NPs have been widely studied in aquatic animals such as the zebrafish (Bhagat et al., 2020) and *Daphnia* (Imhof et al., 2017; Liu et al., 2019). In the zebrafish, for example, the major toxic effects of MPs and/or NPs are developmental toxicity, reproductive toxicity, neurotoxicity, immunotoxicity, genotoxicity, metabolome imbalance, behavior variation and oxidative stress (Reviewed in Ref. (Bhagat et al., 2020)). Furthermore, the type, size and surface charge of the MPs and NPs were found to potentially influence their effects (Kögel et al., 2020). The toxicity of MPs and NPs has also been investigated in *Daphnia* spp. Interestingly, MPs and NPs can be found in the offspring of zebrafish (Pitt et al., 2018) and in generations (F_1 and F_2) of *Daphnia manga* (Martins and Guilhermino, 2018). These findings give rise

to the possibility that MPs and NPs could transfer from adult to newborn. Furthermore, one study showed that MPs were present in human placentas including maternal side, fetal side and in the chorioamniotic membranes (Ragusa et al., 2021). Therefore, it is possible that MPs may be transferred to the embryo or fetus.

Knowledge of the effects of MPs and NPs on developing embryos of warm-blooded animals, including humans, is very limited. The chicken embryo is a commonly used model organism to study embryotoxicity. This is because the chicken embryo is a warm-blooded vertebrate which is more closely related to mammals than are the cold-blooded aquatic species (such as zebrafish and *Daphnia*) that are usually used for testing the toxicity of MPs and NPs (Wilson, 1978). For example, the most recent common ancestor of humans and the chicken lived approxiamtely 319 million years ago (Pardo et al., 2020; Rezania et al., 2018; Sánchez-Villagra, 2012; St John et al., 2012); of humans and the zebrafish 429 Mya; and of humans and *Daphnia*, 708 Mya (Kumar et al., 2017). Furthermore, the chicken embryo is highly sensitive to various chemicals and physical agents meaning that it is a good indicator species for toxicity studies (Hill and Hoffman, 1984). Finally, based on the large amount literature about chicken embryo development, it is an ideal model species (Stark and Ross, 2019). Among the literature are classic text books on chicken developmental anatomy (Lillie, 1952; Romanoff, 1960) developmental atlases (Bellairs and Osmond, 2014) and staging tables (Hamburger and Hamilton, 1951).

There are a few studies of the effects of nanomaterials in general on the chick embryo. For example, zinc nanoparticles cause neural crest defects in the chick embryo (Yan et al., 2020; Yan et al., 2021). Further, using various routes and stages of exposure, it has also been shown that 50 nm and 1 µm polystyrene nanoplastics cause neural tube and craniofacial defects in chicken embryos (Nie et al., 2021). The mechanism of neural tube injury was attributed to caveolae mediated endocytosis (Nie et al., 2021).

In this Chapter, we have investigated the toxicity of MPs and NPs to developing chick embryos. We also examined whether the size and concentration of the plastics particles is a determining factor of toxicity they may cause to the chick embryo. Additionally, we have looked for phenotypic effects of the nanoplastics using standard morphological and histological techniques.

Materials and Methods

Preparation and analysis of the nanoplastics

Plain (non-functionalized) polystyrene nanoplastics (PS-NPs) were purchased from Lab 261 (cat. number PST25, PST100 and PST 500, Palo Alto, U.S.), with nominal diameter of 25 nm, 100 nm, 500 nm (1% solid, 1.05 g/cm^3). As supplied by the manufacturer, the PS-NP suspension was in a vehicle of 0.03% Tween®20 in Milli-Q® water. We specifically requested the manufacture to not add azide.

In size comparison experiments, different sizes of PS-NP suspension including PST25, PST100 and PST500 were diluted with Ringer's solution (pH 7, 2.5 g/L, cat. number 1.15525, Merck Millipore, Germany), which we autoclaved before use. They were diluted two times for a final concentration of 5 mg/mL. In the concentration comparison experiments, PST25 suspension was then diluted with Ringer's solution. The dilution series in Ringer's was as follows: 2, 10, 100, and 1000 × to yield final PS-NP concentrations of 5 mg/mL, 1 mg/mL, 0.1 mg/mL, and 0.01 mg/mL, respectively.

The sterility of the PS-NPs suspensions was tested by streaking on LB agar plates (File S1). PS-NP size, shape and zeta-potential in Milli-Q® water or Ringer's solution were measured by multi angle dynamic light scattering (MADLS; Malvern Panalytical Ltd., Malvern, UK) and TEM (transmission electron microscopy using a JEOL 1400+). The suspensions were sonicated for 10 min before use using a USC200T ultrasonic cleaning bath (VWR, Amsterdam, the Netherlands).

In vivo embryo toxicity

Fertilized eggs of the White Leghorn chicken (*Gallus gallus*) strain were purchased from a commercial supplier (Drost Loosdrecht B.V., the Netherlands). They were incubated for 29 h at 38 °C on stationary shelves in a humidified, forced-draft incubator (Binder, Germany). Under aseptic conditions, the eggs were windowed and staged as described (Hamburger and Hamilton, 1951). Then, 50 µL Ringers' solution was dripped onto the dorsal side of the embryo to moisten the vitelline membrane. Next, a small hole was made in the vitelline membrane using a sharpened tungsten needle (Brady, 1965; Silver, 1960). It was made beyond the head of the embryo, avoiding the embryo itself. Next, 50 µL of either working solutions of PS-NPs or Ringer's solution as a vehicle-only control, was dripped onto the hole using a Gilson P200 Pipetman®. The egg was then sealed with Scotch® prescription label tape 800 (clear) and returned to the incubator for 24 h, 4 d. At 24 h and 4d post exposure, alive embryos were harvested by Student Vanna Spring Scissors (Cat. 91500-09, Fine Science Tool, Germany) and spoons. Meanwhile, its extra tissues were moved out carefully. Finally, embryos were harvested into cold phosphate buffered saline (PBS).

The somites of the chicken embryo at 24 h post exposure were counted and the embryo staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). For 4 d post exposure chicken embryos, the embryos were staged (Hamburger and Hamilton, 1951) and any phenotypic abnormalities were noted.

Alcian blue wholemounts

This protocol is as previously described by us (de Bakker et al., 2013). Embryos were fixed with 5% trichloroacetic acid at 4 °C degree overnight. They were then transferred into refresh 70% ethanol for 2 h × 2 followed by acid alcohol (20% glacial acetic acid in 70% ethanol for 2 h). Embryos were then stained in 0.03% (W/V) Alcian blue in acid alcohol overnight. Then they were rinsed with acid alcohol for 2 h followed by dehydration through a graded ethanol series from 70% to 100%. Finally, embryos were cleared and stored in methyl salicylate.

Paraffin histology

We performed routine paraffin histology with haematoxylin and eosin staining according to standard protocols (Bancroft and Gamble, 2008). Embryos were fixed in 4% buffered depolymerized paraformaldehyde (pFA) for 24 h at 4 °C. They were then washed 3x with cold PBS and dehydrated in 70% ethanol overnight. Subsequently, the embryos were dehydrated through a graded ethanol series (80%, 90%, 100%), 1 h each. Embryos were cleared with Neo-Clear® (Merck, Darmstadt), 3x 1h, and embedded in paraffin (Paraclean, KP Klinipath/VWR International, Amsterdam) at 60 °C (1x overnight, 1x 1 h). Sections were cut at 7 µm. Because embryos examined at 24 h post-exposure were delicate and difficult to handle, we used a modified protocol 33. After fixing the embryos, they were embedded in a mixture of 2% agarose (Sigma-Aldrich, Zwijndrecht, A-6013) and 2.5% low melting-point agarose (super fine resolution agarose, Electron Microscopy Sciences, Hatfield, PA) at 42 °C. When the mixture had solidified at room temperature (c. 20 min), the agarose blocks containing the embedded embryos were transferred to 70% ethanol for 2 d. They were then dehydrated in graded ethanols, embedded in paraffin and sectioned. The only modification made to the embedding step was that the tissue blocks were in molten paraffin for no more than 3x 1 h.

MicroCT

This protocol for X-ray microtomography (microCT ; Refs.(Metscher, 2009)) was described previously by us (Yi et al., 2021). In brief, embryos were fixed in 4% pFA in PBS (pH 7.4) at 4 °C for 24 h. They were then rinsed 3x with PBS, 4 °C. After dehydration in a graded ethanol series (25%, 50%, 70%) they were stained with phosphotungstic acid (0.3% in 70% ethanol) for 48 h on a rotary shaker. After staining, embryos were stored in 70% ethanol. For scanning, embryos were immobilized in 1% low melting-point agarose in 1 mL pipette tips and sealed with parafilm. For scan parameters, see Table 2-1. The images were analyzed and manipulated using Avizo software (Version: 8.01; Thermo, Fisher Scientific).

Table 2-1. **MicroCT scan parameters**.

Micro-CT Xradia 520 Verda 3D X-ray microscope (Zeiss)

Embryo code	Treatment (PSNPs mg/mL)	pixel size (μm)	KV/W	exp. time (sec.)	Intensity
1	5 mg/mL	43.468	40/3	1.3	5000-6500
2	Ringer's	62.048	40/3	6	5000-6600
3	5 mg/mL	16.040	40/3	9	5000-6300
4	Ringer's	26.594	50/3	3	5000-8000
5	Ringer's	26.586	50/3	2.5	5500-7000
6	5 mg/mL	17.543	50/4	4	5000-7500

Scanning electron microscopy

The embryos were fixed in 2% glutaraldehyde (GA) and 2% PFA in sodium cacodylate buffer at room temperature for 2 h, followed by 4 °C for 22 h. After fixation, embryos were rinsed 3 × in buffered PBS, and dehydrated through a graded acetone series (25%, 50%, 75% and 100%). Then they were critical-point dried with a BAL-TEC critical point drier 030 (BalTec, Switzerland). Finally, the embryos were sputter-coated with palladium and platinum using a Q 150T S Plus Sputter Coater (Quorum, United Kingdom). The specimens were imaged using a Joel JSM-7600F field emission scanning electron microscope.

Results

Characterization of polystyrene nanoplastic particles

The plain polystyrene nanoplastics (PST25; PS-NPs) were 29.16 ± 0.23 nm in diameter and their zeta potential was 14.65 ± 4.16 mV measured in Ringer's solution. They were spherical in sterile Milli-Q water as confirmed by TEM (Fig. 2-1a). Their size distribution dispersed in Ringer's solution was monitored by MADLS (Fig. 2-1b). The

sterility of the particles, as supplied by the manufacturer was confirmed by a lack of microbial growth after streaking the particles on LB agar (File S1).

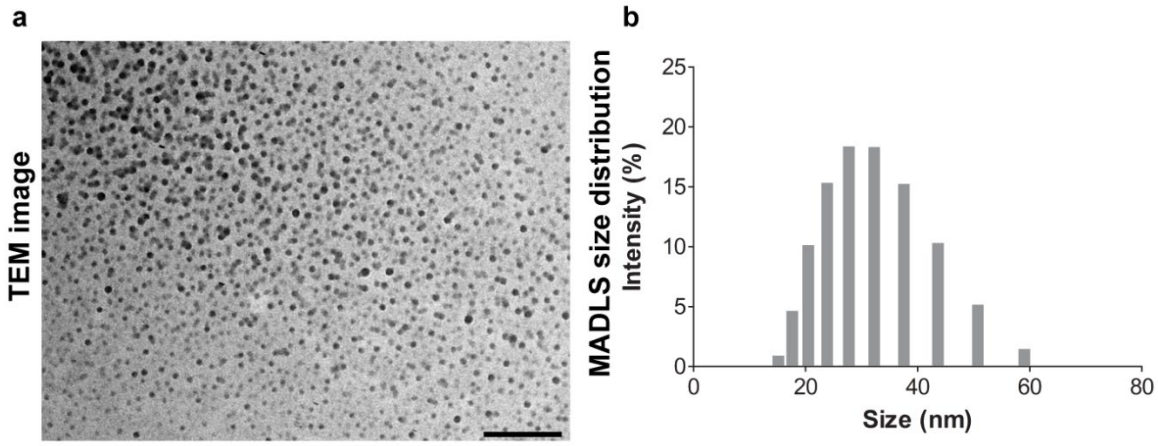

Fig. 2-1. **Characterization of 25 nm plain polystyrene nanoplastics**. (**a**), transmission electron microscopy (TEM) image. (**b**), the size distribution of 5 mg/mL 25 nm plain polystyrene nanoplastics in Ringer's solution measured by multi angle dynamic light scattering (MADLS).

PS-NPs may cause size-dependent mortality and malformations

A preliminary series of experiments was performed with 25, 100 and 500 nm plain PS-NPs. Exposure was at stage 8 and harvesting was at 4 d post-exposure (stages according to Hamburger-Hamilton (Hamburger and Hamilton, 1951)). Our statistical analyses of these data are given in File S2. We found that, using a standard exposure concertation of 5 mg/mL, the 25 nm particles produced the highest percentage of malformed embryos, and the highest mortality (File S2). Analysis suggested that the relative number of particles might provide a more reliable model than the particle size itself. There seemed to be a clear, increasing probability of malformations and mortality if the number of particles increased (File S2). But these outcomes could be a combined effect of particle size and/or the number of particles. In order to reduce the number of variables, we used 25 nm PS-NPs for all subsequent experiments. In all cases, we used plain PS-NPs except in the experiments where fluorescent particles were used to track the location of particles.

Dose-dependent mortality of 25 nm PS-NPs

The dose-response parameters of 25 nm PS-NPs were tested in chicken embryos exposed at stage 8, and examined at 4 d post-exposure. Six chicken embryos were treated for each concentration step and each experiment was repeated three times. Thus, *n* = 18 for each concentration (File S3). The mortality in the control group (Ringer's only) was 27.78 ± 5.55% (Fig. 2-2c). The fact that nearly 30% of embryos in the control group died could be due to the fact that such young embryos (stage 8) are very sensitive to manipulation. The highest concertation group (5 mg/mL) had significantly higher mortality (75 ± 4.81%; $P<0.001$) compared to the control group (Fig. 2-2c; File S3).

Dose-dependent teratogenicity of 25 nm PS-NPs

In controls (Ringer's only), the baseline teratogenicity (incidence of malformations) was 8.33 ± 8.33% (Fig. 2-2d). This baseline level of malformations could reflect the sensitively of young embryos to manipulation *in ovo* or could be due to an ageing flock (Boerjan, 2002; Pawłowska and Sosnówka-Czajka, 2019). All PS-NPs treated embryos showed excess of malformations compared to the control group. For the highest two doses in the series (1 mg/mL and 5 mg/mL) all embryos showed malformations (Fig. 2-2d; File S3).

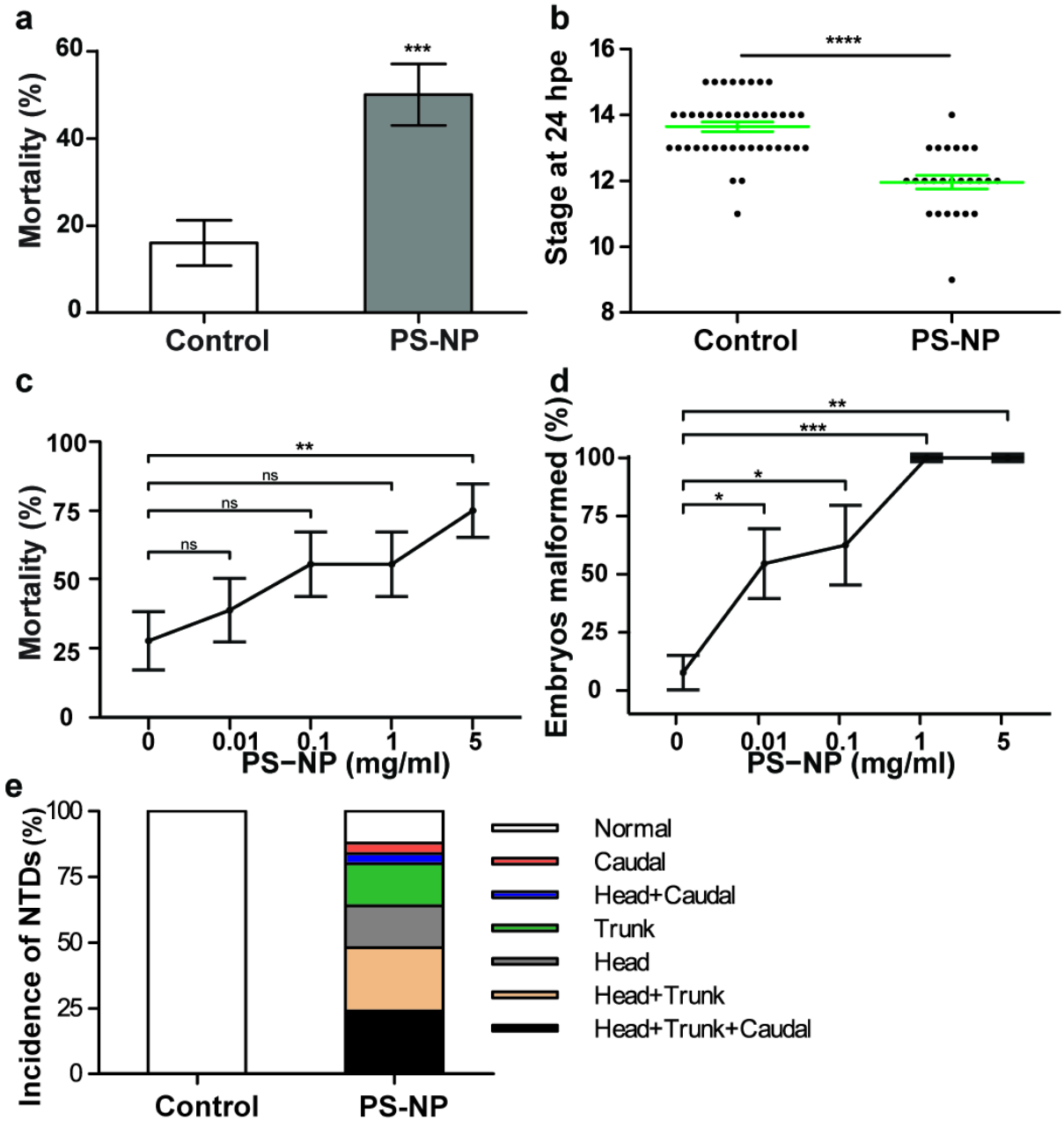

Fig. 2-2. **25 nm PS-NP exposure leads to increased mortality, developmental delay and malformations in chicken embryos.** Embryos were exposed at stage 8. **a**, **b**, 5 mg/mL PS-NP, analysed at 24 h post-exposure. **a**, mortality rate. *n* = 50 (for both control and PS-NPs-treated group) from 5 independent experiments; data are mean ± s.e.m. Chi square test, P = 0.0007. **b**, developmental delay (as expressed in stage; stages according to Ref. (Hamburger and Hamilton, 1951)). *n* = 42 for control and *n* = 25 for PS-NP-treated from 5 independent experiments; data are mean ± s.e.m. Chi square test, P < 0.0001. This delay caused by PS-NPs has also been noted by Nie and colleagues (Nie et al., 2021). **c**, **d**, dose-response series, analysed at 4 d post-exposure. **c**, mortality. *n* = 18 for 0, 0.01, 0.1 and 1 mg/mL group; *n* = 20 for 5 mg/mL group from 3 independent experiments; data are mean ± s.e.m. Chi square test, P = 0.7237 (0 mg/mL vs 0.01 mg/mL), P = 0.1763 (0 mg/mL vs 0.1 mg/mL), P = 0.1763 (0 mg/mL vs 1 mg/mL), P = 0.0097 (0 mg/mL vs 5 mg/mL PS-NPs-treated). **d**, malformation rate (all malformations). *n* = 13 (0 mg/mL), *n* = 11 (0.01 mg/mL), *n* = 8 (0.1 mg/mL), *n* = 8 (1 mg/mL), and *n* = 5 (5 mg/mL) from 3 independent experiments; data are mean ± s.e.m. Chi square test, P = 0.0387 (0 mg/mL vs 0.01 mg/mL), P = 0.0276 (0 mg/mL vs 0.1

mg/mL), P = 0.0002 (0 mg/mL vs 1 mg/mL), and P = 0.0016 (0 mg/mL vs 5 mg/mL). **e**, axial level affected by neural tube defects (per embryo), 5 mg/mL PS-NP concentration 24 h post-exposure. *n* = 42 for control and *n* = 25 for PS-NP-treated from 5 independent experiments. *Key*: NTDs, neural tube defects; hpe, hours post-exposure; PS-NP, polystyrene nanoparticles; significance of difference between control and experimental groups indicated by asterisks as follows: ****, P <0.0001; ***, P <0.001; **P <0.01; *P <0.05; ns for P >0.05.

Neural tube defects, developmental delay and mortality at 24 h post-exposure

We performed a series of experiments in which we recorded the mortality and selected malformations in embryos exposed to 25 nm PS-NPs at stage 8, then examined at 24 h post-exposure. For each experiment there 10 treated (5 mg/mL 25 nm PS-NPs) and 10 controls (Ringer's only). The experiment was repeated three times (*n* = 30, both for the control and treated groups). Our results are shown in Fig. 2-2a-b. The mortality in the treated group was 60 ± 8.16% which was significantly higher (*P* < 0.01) than in the control group (Fig. 2-2a). A significant developmental delay was seen in the surviving, treated embryos (Fig. 2-2b). The delay was based on somite count of the embryos. When we converted the somite count into Hamburger Hamilton stage, the average stage reached was 11.50 ± 1.24 for treated embryos, and 13.67 ± 1.09 for control embryos (Fig. 2-2b; Table S1). In the same experimental series, neural tube defects were never seen in the control embryos (*n* = 24/30; Fig. 2-2e; Movies S1). By contrast, all surviving embryos treated with PS-NPs (*n* = 12/30) showed neural tube defects (failure of the neural tube to close dorsally; Fig. 2-3; Movies S2; Movies S3). The defects were either in the head, trunk or tail, or in a combination of these regions (Fig. 2-2e; Fig. 2-3; Table S1). At the stages examined in this Chapter, the heart has not completed septation; we therefore reserve a detailed analysis of heart malformations for Chapter 3, where we examine older embryos.

Fig. 2-3. **Neural tube defects caused by exposure of chick embryos to 25 nm PS-NPs. a-j**, micro-CT images of control (**a**-**e**) and 25 nm PS-NPs treated embryos (**f**-**j**). *n* = 2 for both groups. **a**, stage 14, volume rendering of wholemount control chick embryo. (**b**-**e**), virtual transverse sections. f, stage 12, volume rendering of wholemount PS-NPs treated embryos. (**g**-**j**), virtual transverse sections. White arrowheads in f-j indicate neural tube abnormalities. (**k**-**u**), wholemount and histological sections of control (**k**-**n**) and 25 nm PS-NPs treated embryos (**o**-**u**). **k**, stage 13, control embryo. **l**-**n**, transverse sections, haematoxylin and eosin stain. o, stage 12, PS-NP-treated embryo. (**p**-**u**), transverse sections, haematoxylin and eosin stain; black arrowheads, neural tube abnormalities; black arrows, clumps of detached. Scale bars, 500 µm in (**a**, **f**, **k**, **o**), 200 µm in (**b**-**e**, **g**-**j**, **l**-**n**, **p**-**u**). *Key*: In figures a, f, k and o, the dashed horizontal lines indicate the plane of the associated transverse sections. PS-NP, polystyrene nanoparticles. Note that the embryos in f and o both have neural tube defects.

Phenotypic analyses of malformations caused by PS-NPs exposure at 4 d post exposure

Phenotypic analyses of embryos at 4 d post-exposure are shown in Fig. 2-4-Fig. 2-7. In all cases, the embryos were exposed to either 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL or 5

mg/mL of 25 nm PS-NPs at stage 8. A consistent feature of our treated embryos was that, no matter how malformed the embryos were, they all had apparently normal limbs (wings and legs). Half of the embryos (*n* = 16/32, Table S2) showed a failure of the normal processes of flexure (dorsoventral bending of the primary axis), and/or torsion (rotation along the primary axis). These failures lead to the embryo being abnormally flat, or straight, or both (Fig. 2-4c-e and f-g). Microphthalmia was present in 8/32 embryos in the form of gross hypoplasia or dysplasia of the optic cup and lens (Fig. 2-4c and d, Fig. 2-5d-f, i and j, Table S2). The microphthalmia was unilateral in three embryos and bilateral in five (Table S2). In some embryos, the caudal neural tube showed gross dysplasia, presenting as an asymmetric mass of disorganized tissue (Fig. 2-6d-i). In other embryos, the caudal neural tube showed gross dysplasia, presenting as an asymmetric mass of disorganized tissue (Fig. 2-7d-f). The tailbud was hypoplastic or absent in 5/32 embryos at 4 dpe (Table S2).

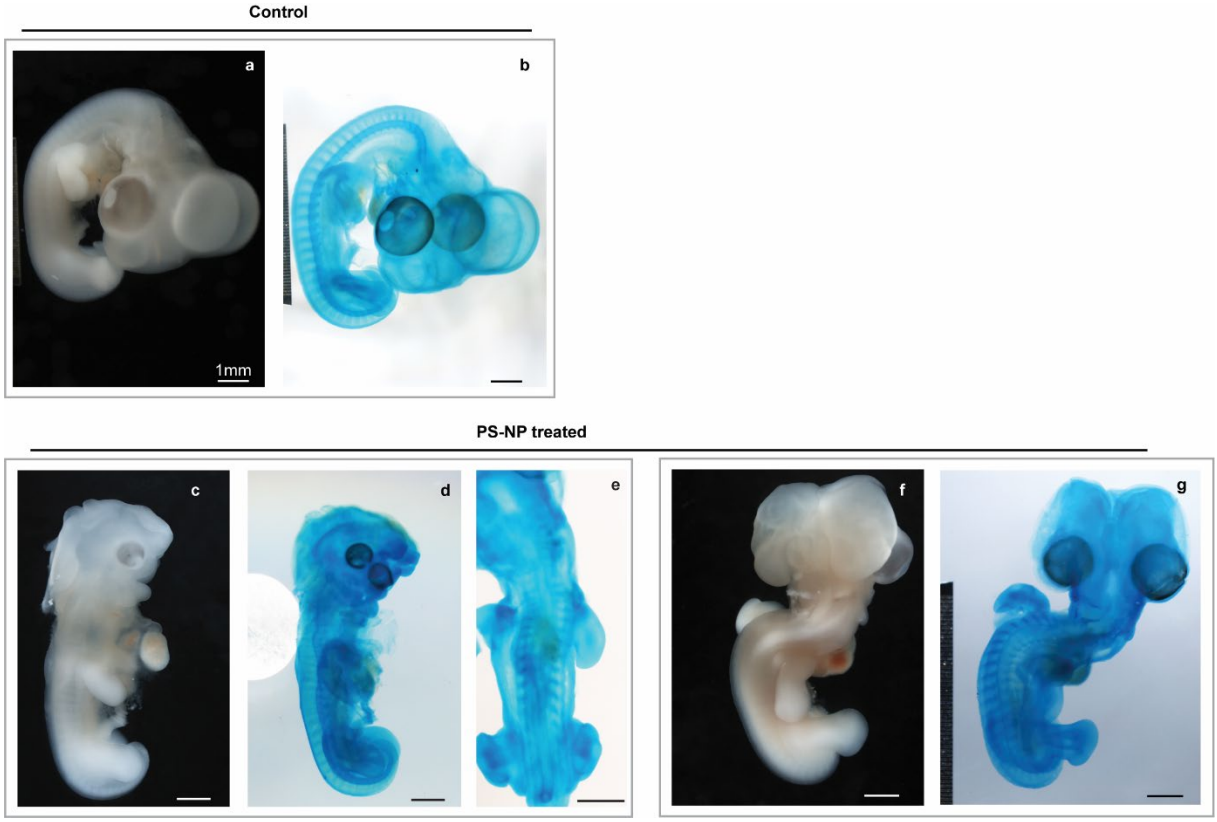

Fig. 2-4. **Phenotypic analysis of malformations 4 d after exposure to PS-NPs at stage 8. a**, **b**, the chicken embryo from the control group. **c**, **d**, **e**, PS-NPs treated chicken embryo, with microphthalmia, anencephaly and abnormal cervical flexure. **f**, **g**, PS-NPs treated chicken embryo, with exencephaly and failure of cervical flexure.

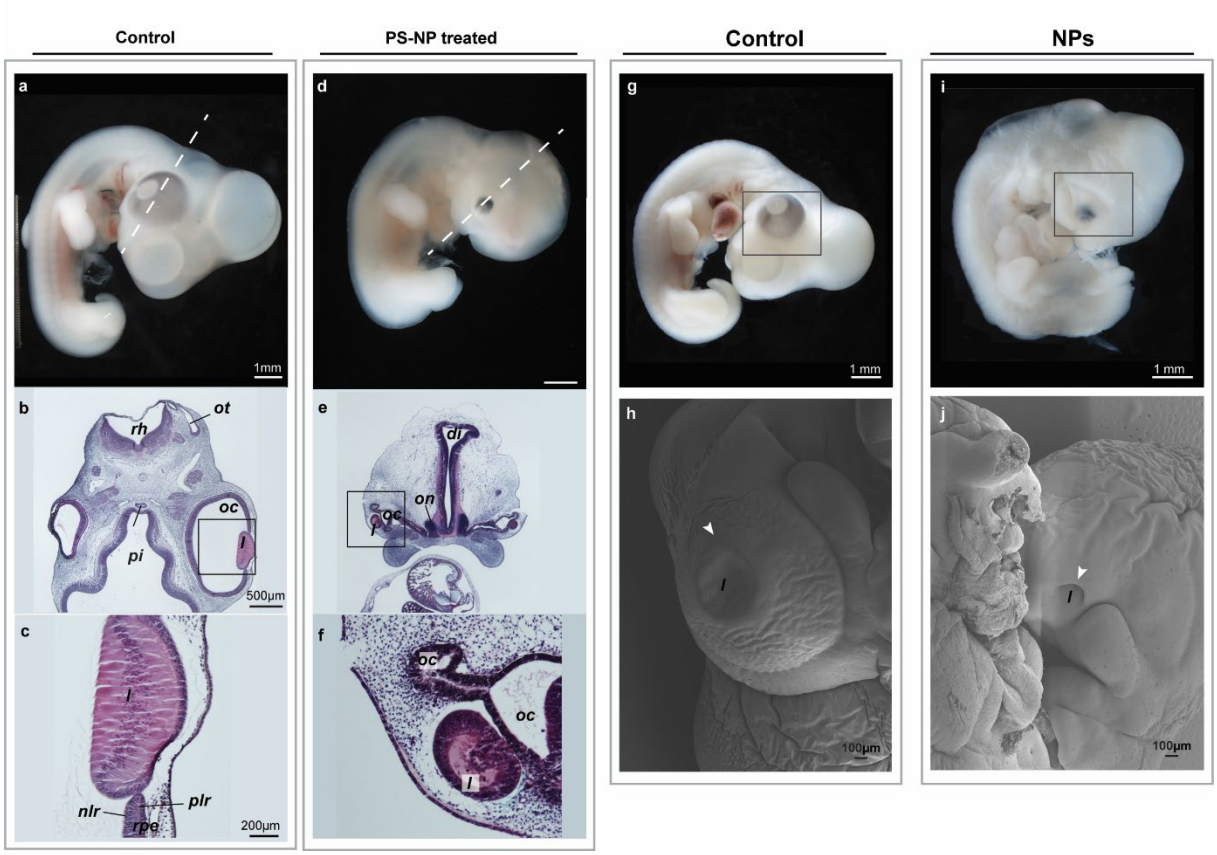

Fig. 2-5. **Phenotypic analysis of eye defects 4 d after exposure to PS-NPs at stage 8. a**, the wholemount chicken embryo from the control group, stage 25. **d**, the chicken embryo from PS-NPs treated group with microphthalmia anencephaly and caudal neural tube defect. **b**, **c**, **e** and **f** histological examinations od HE staining. **g**, the chick embryo from the control group, stage 25. **i**, the chick embryo from the PS-NPs group with microphthalmia, stage 24. **h** and **j**, the scanning of the eye from control and PS-NPs treated embryos; white arrowheads, the normal (**h**) and abnormal eye (**j**). **j**, the chick embryo with small lens. *Key*: di, diencephalon; l, lens; nc, notochord; nlr, neural layer of retina; on, otocyst; pi, pituitary; rpe, retinal pigment epithelium; rh, rhombencephalon; te, telencephalon.

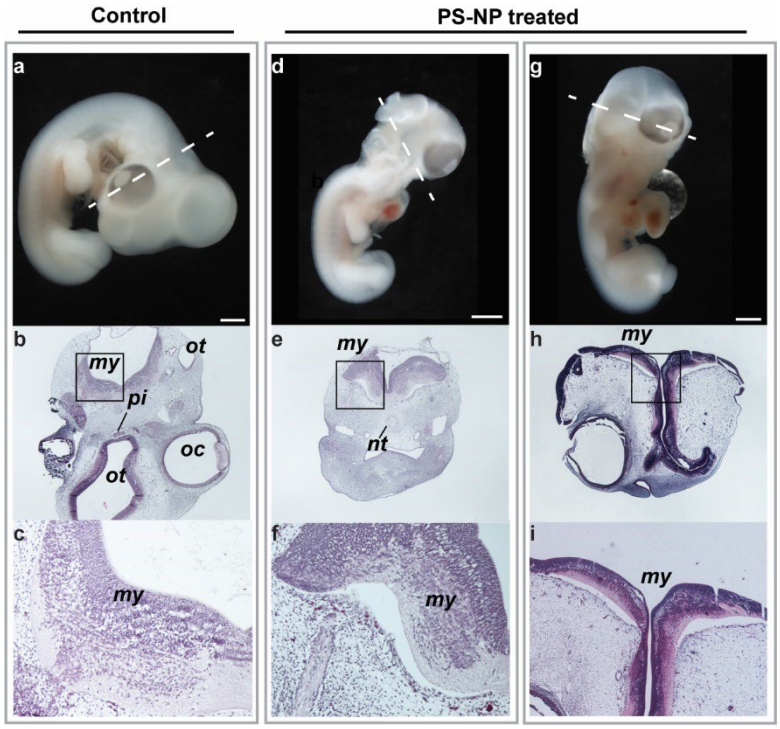

Fig. 2-6. **Phenotypic analysis of neural tube defects at 4 d after exposure to PS-NPs at stage 8. a**, the chicken embryo from the control group, stage 25. **d** and **g**, the chicken embryo from the PS-NPs treated group with anencephaly and abnormal cervical flexure. my, myelencephalon; nc, notochord; nt, neural tube; on, otocyst; pi, pituitary.

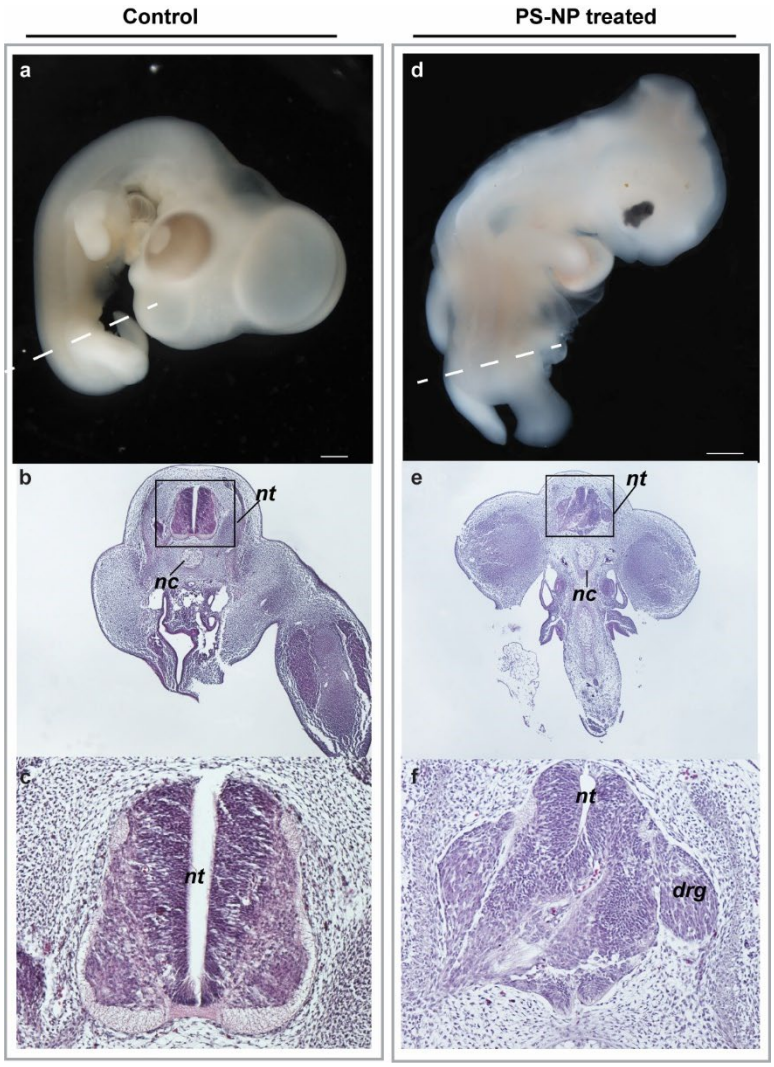

Fig. 2-7. **Phenotypic analysis of neural tube defects 4 d after exposure to PS-NPs at stage 8. m**, the chick embryo from the control group. **n**, the chick embryo from PS-NPs treated group with caudal neural tube defects, microphthalmia, anencephaly and abnormal cervical flexure. *Key*: drg, dorsal root ganglia; nc, notochord; nt, neural tube.

Discussion

We exposed chicken embryos, of Hamburger-Hamilton stage 8, to 25 nm PS-NPs; we then returned the eggs to the incubator and analyzed them 24 h or 4 d post exposure. In a first series, we examined the effect of PS-NP size. This examination showed the highest mortality and malformation rate in the chicken embryos exposed to the smallest (25 nm) PS-NPs. In a recent study , Nie et al found that 60 nm polystyrene nanoparticles cause more cases of serious malformation in chicken embryos at 24 hpe than did 900 nm nanoparticles (Nie et al., 2021). Furthermore,

smaller polystyrene nanoplastics (0.05 μm) produce higher mortality in the copeopod *Tigriopus japonicus* than larger-sized nanoplastics (0.5 μm) (Lee et al., 2013).

In some species, the situation appears to be reversed. This, when embryonic and larval stages of the sea urchin *Sphaerechinus granularis* were exposed to different sizes of PS-NP (10 nm, 80 nm and 230 nm); the larger size particles caused higher incidence of malformations than did the smaller (Trifuoggi et al., 2019). Therefore, it is possible that the relationship between nanoplastics size and toxicity is species-specific (Kögel et al., 2020).

There are other reasons why the relationship between particle size and toxicity is not straightforward (Wang et al., 2023). Thus, particles of different sizes will have different surface areas at the same mass. Therefore, the size-dependent toxicity may be caused by the combination of these characterizations of particles. In this Chapter, after we had completed the preliminary experiments, we then maintained a fixed concentration (5 mg/mL) in all subsequent experiments. This meant that we could not disentangle the effects of particle size and particle number. Therefore, the higher mortality and malformation rates at the smaller particle size (25 nm) might be due, in part, to the correspondingly higher number of particles, and their higher total surface area.

We also found that 25 nm plain polystyrene nanoplastics cause dose-response toxic effects on mortality and malformation rate of 4 dpe chicken embryos. In one study, the toxicity of three different types of 50 nm PS-NP nanoplastic particle were tested on oysters (Tallec et al., 2018). The particles tested were plain, carboxyl-modified or amine modified. Each type of particle had a different dose-response effect on fertilization yield of the oyster gametes (Tallec et al., 2018). In a study on chicken embryos, there was a dose-dependent increase in the frequency of exomphalos or omphalocele (exposure of abdominal viscera through the ventral body wall), and in the mortality (Nie et al., 2021). In summary, there is evidence of a relationship

between nanoplastic dose, and the incidence of mortality and malformations caused in exposed embryos.

In this chapter, we observed that exposure of chicken embryos to PS-NPs caused a developmental delay. Developmental delay is a typical consequence of exposure of embryos to teratogens (Harnett et al., 2021; Teixidó et al., 2013). Development delay has been reported after the exposure of chicken embryos to NPs (Nie et al., 2021) and the exposure of zebrafish embryos/larvae to 35 nm gold nanorods (Mesquita et al., 2017). Overall, our results are aligned with previous findings which confirm that nanoplastics could be considered as a teratogen in the early stages of embryos. In additional to developmental delay, Nie et al have shown that the exposure of chicken embryos to various sized PS-NPs can cause major malformations of the neural tube (Nie et al., 2021). The mechanism proposed by Nie et al was that PS-NPs are taken up by neural tube cells by endocytosis; this in turn led to led to cell damage and cell death through the increased production of reactive oxygen species (Nie et al., 2021).

In this chapter, we found neural tube defects in the head, trunk, tail, or a combination of these sites. This variation in location of the neural tube defects could be because of differences in the distribution of PS-NPs between individual treated embryos; or could be a result of multiple neural tube closure sites in one embryo, as has been postulated for human malformations (Seller, 1995). Neural tube defects result from a failure of the neural folds to meet and close (Copp and Greene, 2013; Copp et al., 2013). In one previous study, mouse embryos exposed to 6.5 nm titanium dioxide (TiO_2) particles showed neural tube defects (Hong et al., 2017).

Results similar to those in this chapter were also seen in a previous study (Nie et al., 2021) in which chicken embryos at early stages were exposed to 60 and 900 nm PS-NPs *in vitro*. Specifically, defects were noted in the cranial and truncal neural tube at 36 hpe (Nie et al., 2021). One difference with that study is that we observed in this chapter, neural tube defects in caudal regions, which were not reported in the chicken embryo before (Wang et al., 2023). There is a junction between regions of

primary and secondary neurulation (Dady et al., 2014). The secondary neurulation starts around stage 8 which suggest that PS-NPs may affect the process (Dady et al., 2014). This could explain the neural tube defects in the caudal region seen in this chapter. Furthermore the caudal region of the embryo develops later than the truncal and cranial regions and so that study (Nie et al., 2021) may have exposed the embryos too early.

Another new observation of this chapter is the presence of microphthalmia in the PS-NPs treated group at 4 dpe, something not reported in a previous study of chick embryo exposure to PS-NPs (Nie et al., 2021). Microphthalmia is a birth defect in which the newborn have abnormally small eyes. The occurrence of microphthalmia was differ between individuals. Specifically, either one or both eyes was abnormally small (Verma and FitzPatrick, 2007). In humans and mice, microphthalmia can have a genetic or environmental causes (Verma and FitzPatrick, 2007). We cannot be sure that nanoplastics directly impair affect eye development. However, it has been demonstrated that nanogold functionalized with a cationic ligand, N,N,N-trimethylammoniumethanethiol, can disrupt eye development in the zebrafish, leading to microphthalmia (Kim et al., 2013). In addition, copper nanoparticles have been found to reduce the size of eyes by decreasing the cell number of ganglion cell layer of the retina in zebrafish embryos (Zhao et al., 2020). Microphthalmia has also been reported in zebrafish embryos exposed to PS-NPs and polybrominated diphenyl ethers (Wang et al., 2022).

Skeletal abnormalities were observed among the PS-NPs-treated embryos analyzed at 4 dpe. These abnormalities included an abnormally curved vertebral column, and an absent or short tail-bud. Similar results have been found in other studies in which different species were exposed to various types of nanoparticles at early developmental stages. For instance, zebrafish embryos exposed at 6 hours post fertilization to polyethylene terephthalate nanoparticles, showed abnormal curvature of the vertebral column and tail (Bashirova et al., 2023). In mouse embryos, exposure

to 6.5 nm titanium dioxide (TiO_2) particles caused skeletal abnormalities associated with abnormal cartilage development (Hong et al., 2017).

In conclusion, we have confirmed the results of previous studies in vertebrate model species, showing that nanoparticles can cause defects of the neural tube, vertebral column and, in zebrafish, in the tail. In addition, we have shown for the first time that PS-NPS can cause microphthalmia and tail abnormalities in the chicken embryo. In Chapter 3, we will look at defects in later-developing organ systems, particularly the heart and facial region; and in Chapter 4 will explore the cellular mechanisms underlying the malformations caused by PS-NPs.

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