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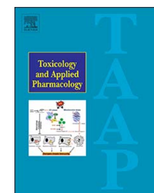

Nanotoxicological and teratogenic effects: A linkage between dendrimer surface charge and zebrafish developmental stages

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ABSTRACT

This article reports novel results about nanotoxicological and teratogenic effects of the PAMAM dendrimers DG4 and DG4.5 in zebrafish (*Danio rerio*). Zebrafish embryos and larvae were used as a rapid, high-throughput, cost-effective whole-animal model. The objective was to provide a more comprehensive and predictive developmental toxicity screening of DG4 and DG4.5 and test the influence of their surface charge. Nanotoxicological and teratogenic effects were assessed at developmental, morphological, cardiac, neurological and hepatic level. The effect of surface charge was determined in both larvae and embryos. DG4 with positive surface charge was more toxic than DG4.5 with negative surface charge. DG4 and DG4.5 induced teratogenic effects in larvae, whereas DG4 also induced lethal effects in both zebrafish embryos and larvae. However, larvae were less sensitive than embryos to the lethal effects of DG4. The platform of assays proposed and data obtained may contribute to the characterization of hazards and differential effects of these nanoparticles.

1. Introduction

Nanomaterials are promising diagnostic and therapeutic tools for *in vivo* applications, such as drug delivery, medical imaging and tissue engineering (Fako and Furgeson, 2009). However, since there is a lack of detailed toxicological information of nanoparticles of medical interest (Asharani et al., 2011), their increased use requires a better understanding. It is necessary to study their potential risk, as well as their *in vivo* toxicity, so as to predict undesirable human health and environmental effects (Geffroy et al., 2012). In addition, due to their complexity and different nature, the effects of different nanomaterials cannot be predicted (Aillon et al., 2009).

The size of materials plays a critical role in how the organism distributes, eliminates and responds to them. This is because a decreased size leads to an exponential increase in surface area relative to volume, which turns the nanomaterial surface more reactive to its surrounding environment (Aillon et al., 2009).

Dendrimers are nanomaterials with a highly branched macromolecular structure, controlled chemical structure and low polydispersity (Tomalia et al., 1985). Depending on the dendrimer type and generation, they have a positive, neutral, or negative charge on the surface. Poly(amidoamine) (PAMAM) dendrimers have an initiator ethylenediamine core and are built up with units attached to it that are repeated radially (Esfand and Tomalia, 2001). Generations go from zero to 10, and include twice the number of functional groups and a larger diameter than the previous generation. Dendrimer G4 (DG4) is a full-generation dendrimer with superficial amine functional groups (cationic), whereas dendrimer G4.5 (DG4.5) is a half-generation dendrimer with superficial carboxylic acid groups (anionic) (Fako and Furgeson, 2009). Dendrimers are being used in numerous biomedical applications, such as gene and antisense therapy, chemotherapy, drug delivery, *in vivo* diagnostics, and also as anti-infective agents (Bosman et al., 1999; Patri et al., 2002; Duncan and Izzo, 2005; Svenson, 2009; Svenson and Tomalia, 2012; Abbasi et al., 2014; Noriega-Luna et al., 2014). However, there is still limited information of the human health risks of DG4 and DG4.5 (Fako and Furgeson, 2009). In addition, a recent investigation with microorganisms, which are at the base of the food web, has reported the potential environmental risk of dendrimers for medical use (Gonzalo et al., 2015).

Zebrafish (*Danio rerio*) is an animal model that offers whole-animal information, impossible to be obtained from *in vitro* studies. Zebrafish developmental biology is well described and its genome is completely sequenced (Kimmel et al., 1995; Rubinstein, 2006). Zebrafish develops most of the main organ systems present in mammals during the first week of life; some organs and tissues have been shown to be similar to those of mammals at anatomic, physiological and molecular levels

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(McGrath and Li, 2008). The heart of the zebrafish embryos resembles that of humans at the third week of gestation, with similar characteristics and functions (Bakkers, 2011; McGrath, 2012; Lee et al., 2013a). The fundamental processes of neurodevelopment in zebrafish are homologous to those in humans (Selderslaghs et al., 2010; Rinkwitz et al., 2011; de Esch et al., 2012). Moreover, the zebrafish blood-brain barrier shares both structural and functional similarities with that of mammals (Fleming et al., 2013).

Because zebrafish embryos and larvae develop outside their mother and are transparent, they are ideal for nanoparticle and drug analysis (Lin et al., 2013). Zebrafish can absorb compounds in the medium through the skin and gills at embryonic stages, as well as through the digestive system during later larval stages (de Esch et al., 2012; Selderslaghs et al., 2013; He et al., 2013). Some teratogenic substances have been studied in zebrafish embryos and larvae because they exhibit similar phenotype associated with exposure during human development (Rubinstein, 2006; Lee et al., 2013b; Yang et al., 2009). Also, the zebrafish model has been successful in predicting adverse drug effects and demonstrated a good correlation between available data from either human clinical or animal pre-clinical studies (McGrath and Li, 2008; Yang et al., 2009; Fleming, 2007). All these features make the zebrafish model advantageous for toxicological and teratogenic studies.

In this work, we used zebrafish embryos and larvae as a rapid, high-throughput, cost-effective whole-animal model, to provide a more comprehensive and predictive developmental toxicity screening of DG4 and DG4.5, which are nanomaterials with promising medical use. For this purpose, we performed a series of assays to approach different key issues to determine the general toxicity and specific organ damage, as well as the teratogenicity of both dendrimers.

2. Materials and methods

2.1. Dendrimers and reagents

Polyamidoamine (PAMAM) dendrimer G4 ($-NH_2$) (molecular weight = 14,214.17 g/mol, 64 amino end groups) and G4.5 ($-COOH$) (molecular weight = 26,251.86 g/mol, 128 carboxyl end groups), and methanesulfonate were purchased from Sigma-Aldrich, Argentina. Sodium carboxymethylcellulose was from Fluka-BioChemika (Sigma-Aldrich, Argentina) and Entellan® was from Merck KGaA (Darmstadt, Germany). Eosin Yellowish, NaCl, KCl, $CaCl_2$ and $MgSO_4$ were all from BioPack (Buenos Aires, Argentina). Hematoxylin and sucrose were purchased from Anedra (Buenos Aires, Argentina). Agar-agar was from Laboratorios Britania S.A. (Buenos Aires, Argentina). Brine shrimp (*Artemia persimilis*) was from Biosima SRL (Buenos Aires, Argentina). All other reagents used were of analytical grade.

2.2. Animals

Wild-type adult zebrafish were maintained in tanks at 26 ± 1 °C with a 14/10 h of light/dark cycle, and fed with dry fake food (TetraMin PRO®) three times a day and with live brine shrimp once a day. Water was constantly aerated and maintained at pH 7.0–8.0.

The embryos were obtained after spawning and fertilization, as previously described in Igartua et al. (2015), and conserved in E3 medium (NaCl 0.29 g L^{-1}, KCl 0.012 g L^{-1}, $CaCl_2$ 0.036 g L^{-1} and $MgSO_4$ 0.039 g L^{-1} in deionized water, and 50 ppb methylene blue to inhibit fungal growth). Only fertilized eggs in good conditions were selected for further treatments (Rubinstein, 2006). The characteristics of the eggs were determined with a stereomicroscope (Leica Zoom 2000, Wetzlar, Germany).

In this study, embryos refer to zebrafish prior to hatching (0–3 days post-fecundation), whereas larvae refer to post-hatching animals (over 3 days post-fecundation).

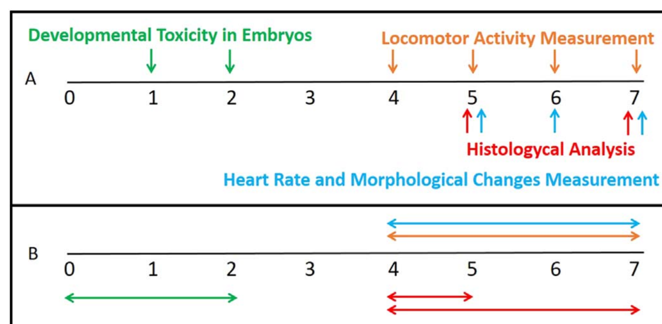

Fig. 1. Diagram of the different treatments in embryos and larvae of zebrafish. Line A represents the days post-fecundation where measurements were performed, and line B shows the time of continuous exposure to the dendrimer (in days post-fecundation).

2.3. Ethics statement

All animal procedures were performed in strict accordance with the International Guidelines for animal care and maintenance. The protocols were approved by the Institutional Committee for the Care and Use of Laboratory Animals and the Ethic Committee of the National University of Quilmes (CE-UNQ 2/2014, CICUAL-UNQ 013-15 and CICUAL-UNQ 014-15) (Buenos Aires, Argentina).

2.4. Exposure to DG4 and DG4.5

Stock commercial solutions of DG4 and DG4.5 were dried under nitrogen flow to remove methanol, and then resuspended in E3 medium. No further characterization of dendrimers was conducted, since they were commercial stock solutions.

Embryos and larvae were maintained at 28 ± 1 °C in a 96-well microplate in E3 medium, with a 14/10 h light/dark cycle. Embryos and larvae were incubated at 1-hour post-fecundation (hpf) or 4 day post-fecundation (dpf), respectively, adding the dissolved dendrimer in E3 medium into each well. In all cases, the treatment solution was not removed during the study (Fig. 1). In order to determine the concentration range to be tested, embryos and larvae were incubated with DG4 and DG4.5 0.031–20 μM. Finally, two-fold serial dilutions between 0.031 and 0.5 μM for DG4 and between 1.25 and 20 μM for DG4.5 were tested. Selected differential concentrations were related to the range of effect of each dendrimer.

For the developmental toxicity study, 16 embryos for each concentration of dendrimer were incubated (one per well), whereas for the other studies, 24 larvae were incubated (three larvae per well). The same larvae used for locomotor activity studies were analyzed by histological sectioning; cardiotoxicity and morphological changes were assessed in another group of larvae, which were incubated in the same conditions. Each assay was performed three times.

2.5. Developmental toxicity in embryos

This study was developed to determine the toxic effect of water-soluble substances as an alternative acute toxicity assay in adult fishes (Lammer et al., 2009). Embryos of 1 hpf (2–8 cells) were transferred into each well of a 96-well microplate and incubated for 48 h, before hatching.

The embryos were observed at 24 and 48 hour post-incubation (hpi) (1 and 2 dpf, respectively) and morphological endpoint parameters of lethality and sub-lethal damage (teratogenesis) were determined and recorded as negative. Lethal parameters were coagulation (24 and 48 hpi), lack of tail detachment (24 and 48 hpi), malformation of the somites (24 and 48 hpi), and absence of heartbeat (48 hpi). Teratogenic parameters were abnormal eye development (24 and 48 hpi), lack of spontaneous movement (24 and 48 hpi), abnormal heart rate (48 hpi),

Table 1
Endpoint parameters of developmental toxicity. Zebrafish embryos were incubated at 1 hpf with the dendrimer solution.

	Endpoint	24 hpi	48 hpi
Lethality	Coagulation	✓	✓
	Lack of tail detachment	✓	✓
	Malformation of somites	✓	✓
	Absence of heartbeat		✓
Teratogenicity	Abnormal eyes development	✓	✓
	Lack of spontaneous movement	✓	✓
	Abnormal heart rate		✓
	Lack of pigmentation		✓
	Edema		✓

lack of pigmentation (48 hpi), and edema (48 hpi) (Table 1) (Kimmel et al., 1995; Nagel, 2002; Braunbeck et al., 2005; OECD, 2006; ISO, 2007; Selderslaghs et al., 2009; Embry et al., 2010; Weigt et al., 2011). The concentration that caused 50% of lethality (LC_{50}) and the concentration that caused 50% of malformation (EC_{50}) were determined by Probit Analysis of survival, StatPlus 2007 Professional. In order to characterize the teratogenic potential of DG4 and DG4.5, the teratogenicity index (TI), defined as the ratio of LC_{50}/EC_{50}, was calculated. If the TI is greater than one, the substance is considered to be teratogenic, and if the TI is below or equal to one, the substance produces mainly embryo-lethal effects (Weigt et al., 2011).

The test was performed in triplicate with embryos of different progenitors, and was accepted as valid when the viability was > 90% for the control group.

2.6. Toxicity in larvae

2.6.1. Measurement of locomotor activity

Activity events were recorded for 15 min at 4, 24, 48 and 72 hpi at room temperature with an automated system (WMicrotracker, Designplus SRL, Buenos Aires, Argentina) (Simonetta and Golombek, 2007; Bichara et al., 2014). The system is based on an infrared microbeam arrangement that detects larvae movement as photo-beam interruptions in real time. Locomotor activity was determined as the number of interruptions for 15 min and data were relativized to the control (Prieto et al., 2012; Igartua et al., 2015; Feas et al., 2017).

Also, the 50% of the locomotor activity (LAC_{50}) was determined as the concentration that reduces by half the swimming activity respect to the control.

2.6.2. Measurement of the heart rate and morphological changes

The heart rate and morphological changes were assessed at 24, 48 and 72 hpi. Control and treated zebrafish larvae were individually transferred into a well of a 96-well microplate and immobilized with sodium carboxymethylcellulose. A total of five larvae per condition were photographed and a video was recorded with a Microsoft LifeCam Studio camera coupled to a trinocular microscope Nikon SMZ800 (Nikon Corporation, Tokyo, Japan) (Supplementary Video 1).

The heart rate was measured by counting the number of beats over 15 s and reported as beats per minute (bpm) (Prieto et al., 2012; Lee et al., 2013a; Igartua et al., 2015). Larval eye area, rostrocaudal length, spinal cord length, uninflated swim bladder, arched body, tissue ulceration and pericardial edema were analyzed with ImageJ Software (US NIH, Bethesda, Maryland, USA) (Igartua et al., 2015; Feas et al., 2017; Padilla et al., 2011).

2.6.3. Histological analysis

2.6.3.1. Preparation of histological sections. Treated and control animals were anesthetized with a tricaine methanesulfonate 0.3 $g\,L^{-1}$ solution. Then, larvae were fixed by immersion in 4% w/v paraformaldehyde in Phosphate Buffer 0.1 M pH 7.4 (PB) at 4 °C overnight. Paraformaldehyde was removed with five washes for 5 min in phosphate-buffered saline 10 mM pH 7.4 (PBS). The samples were embedded and oriented in a mixture of agar 1.5% and sucrose 10% in PB previously heated, and then placed into plastic molds until the mixture was solidified. The blocks were cryoprotected in a 30% w/v sucrose solution in PB overnight at room temperature with constant agitation. Finally, the blocks were frozen in a Leica CM 1850 cryostat (Leica Microsystems, Nussloch, Germany) and then cut at − 22 °C to obtain parasagittal serial sections of 10 μm thickness, which were collected on positively charged slides (BioTraza®, Instrumental Pasteur SRL, Buenos Aires, Argentina). Histological sections of 10 larvae per treatment and control were performed.

2.6.3.2. Hematoxylin-eosin staining. Histological sections were obtained as described above and stained with hematoxylin-eosin to observe possible morphological changes in the brain, liver and spinal cord. Briefly, the sections were washed three times in PBS to remove the agar of the slide. Then, the slides were immersed in Harris's hematoxylin for 1.5 min, washed for 30 min with running water and further immersed in eosin for 1 min and a rapid passage in distilled water. Each sample was dehydrated in increasing ethanol concentrations for 2 min and underwent three passages in xylene for 3 min. The slides were mounted with Entellan®.

Finally, images of the sections of the treated larvae and controls were taken using an inverted fluorescence microscope Nikon Eclipse TE2000-U (Nikon Corporation, Tokyo, Japan), coupled to a digital camera Nikon Digital Sight DS-5M-U2 (Nikon Corporation). The brightness and contrast were fit with Adobe® Photoshop® CS2 version 9.0 (Adobe Systems, San Jose, CA, USA).

2.7. Statistical analysis

Data are presented as mean ± standard deviation (SD) or the standard error of the mean (SEM), and analyzed by one-way analysis of variance (ANOVA) and multiple comparisons test of Dunnett. Each sample was compared to the control. For non-normally distributed data or when homoscedasticity was not supported, Kruskal-Wallis followed by the test of Dunn were performed. In addition, homoscedasticity was determined with the test of Bartlett. GraphPad Prism version 6.0 was used to conduct all statistical analyses. Only values with $p < 0.05$ were accepted as significant.

3. Results and discussion

3.1. Developmental toxicity in embryos

By monitoring endpoint parameters of developmental toxicity in zebrafish embryos at 24 and 48 hpi, it was possible to determine the LC_{50} and EC_{50}. Embryos were exposed within 1-hpf (2–8-cell).

The LC_{50} and EC_{50} were 0.34 μM at 24 hpi in the DG4 treatment. The LC_{50} and EC_{50} were 0.21 μM at 48 hpi. Since there was a decrease in the LC_{50} between 24 and 48 hpi, DG4 seems to be more toxic in advanced stages of the zebrafish development. Similar values of LC_{50} were obtained by Oliveira et al. (2014) at 48 hpi. The TI of DG4 was one at 24 and 48 hpi; therefore, the predominant effect of this dendrimer was embryo lethality.

The LC_{50} and EC_{50} for DG4.5 could not be determined in the concentration range analyzed at 24 and 48 hpi. The estimated concentrations were obtained by Probit Analysis (StatPlus 2007). The theoretical TI of DG4.5 was 0.8 at 24 hpi and 1.1 at 48 hpi. This indicates a trend to induce mainly embryo-lethal effects in early stages and a slight teratogenic effect in later developmental stages.

When the concentrations that induced adverse effects were compared between dendrimers, it was observed that DG4 had a LC_{50} 100-fold higher than DG4.5 at 24 and 48 hpi (Table 2). This indicates that DG4 was 100-fold more toxic than DG4.5. Because DG4 is a cationic

Table 2
LC_{50} and EC_{50} values of treated embryos (exposed since 1 hpf) were determined at 24 and 48 hpi.

	24 hpi			48 hpi		
Dendrimer	LC_{50} (μM)	EC_{50} (μM)	TI	LC_{50} (μM)	EC_{50} (μM)	TI
DG4	0.34 ± 0.02	0.34 ± 0.02	1	0.21 ± 0.01	0.21 ± 0.01	1
DG4.5	30.02 ± 2.12	36.45 ± 2.83	0.8	30.32 ± 2.11	27.47 ± 2.09	1.1

dendrimer, it might destabilize the cell membrane and cause cellular lysis of blastomeres, and finally induce embryo death. Dendrimers could also interfere with critical signaling cascades during development. In agreement with that observed in this study, previous studies with zebrafish embryos have reported that DG4 causes a fast, direct, systemic and irreversible toxicity (Duncan and Izzo, 2005; Heiden et al., 2007). Oliveira et al. (2014) observed that DG4 acts as an inducer of the innate immune response in zebrafish embryos, but not of the oxidative stress response that is usually observed in *in vitro* studies (Oliveira et al., 2014).

An important consideration of zebrafish embryos is the presence of the chorion, an acellular envelope made of three intercrossing layers that surround the embryo during development. Although the chorion possesses pores or channels of 0.5–0.7 μm in diameter, it allows small materials, such as DG4 and DG4.5 (4.5–5 nm), to pass through the embryo *via* passive diffusion (Fako and Furgeson, 2009). Heiden et al. (2007) and Bodewein et al. (2016) observed a differential toxicity between embryos with chorion and dechorionated embryos after 48 hpi with DG4, when the incubation was at 24 hpf (Heiden et al., 2007; Bodewein et al., 2016). However, the results of both articles are contradictory, although they were carried out under comparable experimental conditions. Heiden et al. (2007) also observed that there was no significant difference in the LC_{50} at 120 hpf (hatched eggs) between embryos with chorion and those without chorion when they were incubated at 24 hpf. The authors concluded that the effect was transitory, and the damages were not permanent, a phenomenon not observed with anionic dendrimers (Heiden et al., 2007). Nevertheless, DG4 could have interacted with the proteins that form the chorion. This interaction may have accumulated the nanoparticle in the proximity of the embryo and this could have induced a decrease in the concentration necessary to produce a toxic effect.

3.2. Toxicity in larvae

3.2.1. Alterations in the locomotor activity

Measurement of the locomotor activity allows the prediction of neurotoxic effects in zebrafish larvae. High or low activity respect to the control could be indicative of adverse effects on one or more components of the complex neuronal network that governs the early locomotor system (spinal cord and hindbrain). Therefore, alterations in spontaneous locomotor activity are related to the neurotoxicity of the dendrimers (Prieto et al., 2012; de Esch et al., 2012; Selderslaghs et al., 2013). Larvae were exposed to dendrimers at 4 dpf and locomotor activity was measured at 4, 24, 48 and 72 hpi (4, 5, 6 and 7 dpf).

DG4 0.5 μM induced a decrease in the swimming activity of larvae at 4, 24, 48 and 72 hpi, and the LAC_{50} was 0.485, 0.421 and 0.5 μM, respectively (Fig. 2). On the other hand, since DG4.5 caused no alterations in the swimming activity at any of the concentrations tested, LAC_{50} could not be determined (Fig. 2). Although there is no evidence of the effects on the swimming activity of free DG4 and DG4.5 in zebrafish larvae, it has been reported that DG4 has a concentration and time-dependent toxicity in zebrafish embryos, which correlates with the effect observed with this dendrimer in larval activity (Fako and Furgeson, 2009; Heiden et al., 2007).

The zebrafish embryos and larvae show numerous motor behavior stereotypes that vary with the pass of the time (Fig. 3) (Airhart et al., 2007; Brustein et al., 2003; Drapeau et al., 2002). The appearance of each is related to changes in cellular mechanisms that generate locomotion. The swimming starts when the pre-motor chemical synaptic unit integrates, and becomes sustained when serotonergic modulation is integrated. In addition, each movement requires a functional neuromuscular coordination. At 24 hpf of morphogenesis, the brain is divided into the forebrain (including the diencephalon and the telencephalon), the midbrain, the hindbrain and the spinal cord, while the first clusters of neurons are interconnected by axons (de Esch et al., 2012). At 4 dpf, larval activity may be inherently more variable than at other stages, because larvae are in the transition from an inactive to a fully inflated swim bladder (Colwill and Creton, 2011). At 5 dpf, when the swim bladder and sensory systems like vision are functional, the larvae begin to move and swim spontaneously (Drapeau et al., 2002; Colwill and Creton, 2011). Therefore, the incubation at 4 dpf allows not only the observation of neurological damage, because larvae are in full neuronal development, but also of other affections, such as uninflated swim bladder, arched body, ocular toxicity, alterations of the integrity of the musculature and alterations in the heart rate.

3.2.2. Alterations in the heart rate

Currently, there is no evidence of cardiotoxicity effects of free DG4 and DG4.5 in zebrafish larvae. As a criterion of cardiotoxicity, a statistically significant difference with respect to the control was considered as an abnormal heart rate. Larvae were exposed to dendrimers at 4 dpf and heart rate alterations were assessed at 24, 48 and 72 hpi (5, 6 and 7 dpf) (Fig. 4).

Lower concentrations of DG4 induced a slight increase in beats per minute at 24 and 48 hpi. Larvae treated with DG4 0.5 μM did not present heartbeat at 24 and 48 hpi. This result does not imply that all larvae were dead, because the larvae were taken randomly. A significant decrease in beats per minute (for all concentrations) was observed at 72 hpi. DG4.5 induced a significant decrease in the heart rate of larvae exposed to 10 μM along the days of treatment. This effect was also observed in larvae exposed to 5 and 20 μM at 48 hpi.

Treatment with DG4 and DG4.5 induced alterations in the heart rate. Both dendrimers induced a decrease in the beats per minute at high concentrations, but the effect of DG4 was concentration-dependent and stronger than that of DG4.5. No relationship was found between the heart rate and the locomotor activity; only an alteration in swimming activity with 0.5 μM DG4 (Figs. 2 and 4).

3.2.3. Morphological changes

Larvae were exposed to DG4 and DG4.5 at 4 dpf and the morphological effects were analyzed at 24, 48 and 72 hpi (5, 6 and 7 dpf) (Fig. 5).

Treatment with DG4 0.5 μM induced high mortality at 24, 48 and 72 hpi, while treatment with DG4 0.25 μM did it only at 72 hpi (data not shown). DG4 0.25 μM produced a decrease in the eye area at 24 hpi, but this alteration was reversed the following days (Fig. 6), in agreement with that reported by Heiden et al. (2007), who found that the treatment induced a size decrease in the head, eyes and growth of larvae incubated for 96 h, between 1 and 5 dpf. In addition, a decrease in the spinal cord length in animals treated with DG4 0.125 μM was observed at 72 hpi, which could be indicative of possible neurological alterations or low growth (Fig. 6). Concentrations lower than 0.125 μM

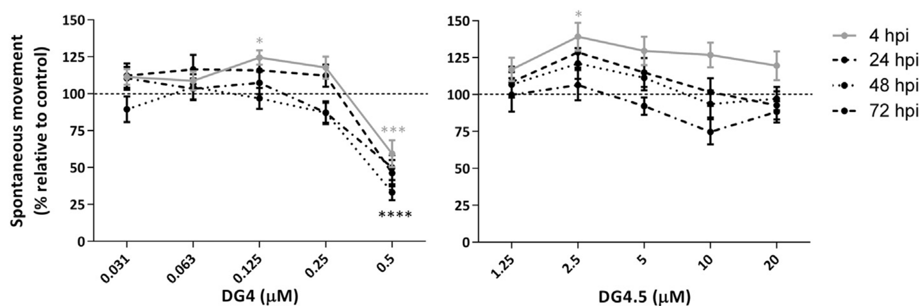

Fig. 2. Percentage of spontaneous movement respect to the control (untreated larvae) at 4, 24, 48 and 72 hour post-incubation of larvae exposed to different concentrations of DG4 and DG4.5. Data are shown as mean ± SEM (n = 24). Activity events were recorded for independent triplicates at room temperature with an automated infrared system. Statistical analysis was performed by ANOVA and the test of Dunnett comparing all samples to the control.

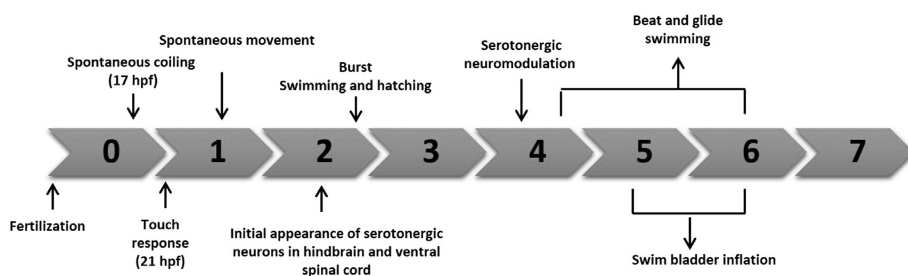

Fig. 3. Chronological sequence (in days post-fecundation) of steps during the development of the zebrafish locomotor network, adapted from Brustein et al. (2003) and Airhart et al. (2007). The sequence shows the appearance of motility patterns during the development of zebrafish, before and after hatching (52 hour post-fecundation).

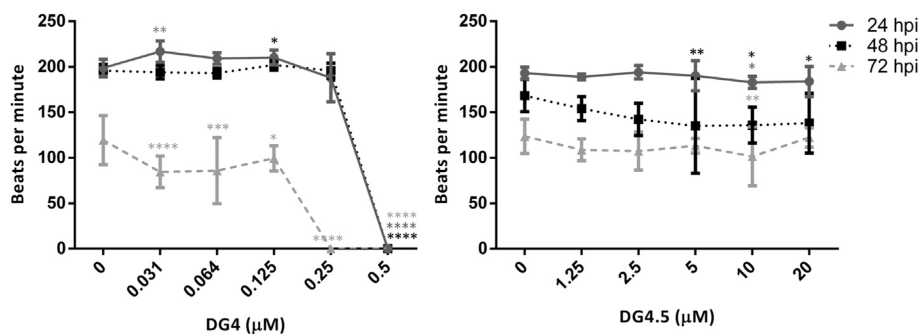

Fig. 4. Beats per minute at 24, 48 and 72 hour post-incubation of larvae exposed to DG4 and DG4.5. Data are shown as mean ± SD (n = 5). Statistical analysis was performed by ANOVA and the test of Dunnett, comparing all samples against the control.

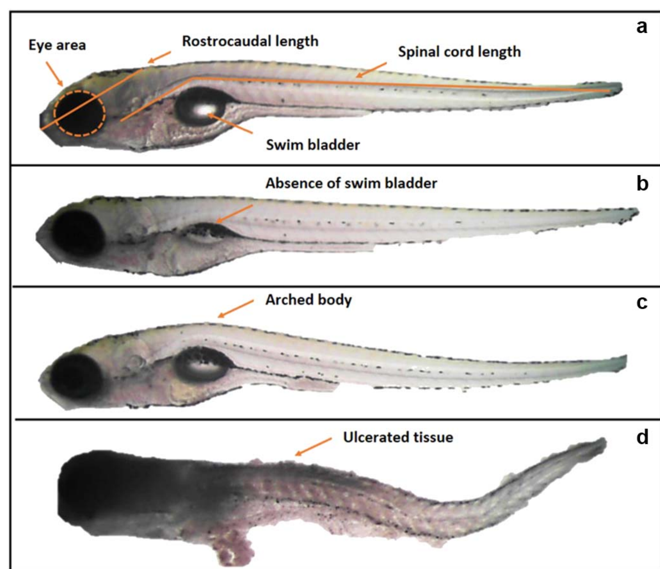

Fig. 5. Images of larvae exposed to DG4 and the morphological changes analyzed: (a) control, (b, c) intermediate concentration, (d) high concentration at 72 hour post-incubation.

of DG4 did not affect the rostrocaudal length of larvae (Fig. 6). An arched body and the ulceration in tissues increased along the days. An uninflated swim bladder was observed after treatment with DG4 in a concentration-dependent manner (Table 3). This effect was not observed with DG4.5.

The treatment with DG4.5 caused no alterations in the eye area, rostrocaudal and spinal cord length; no ulceration nor pericardial edema was observed (Fig. 6 and Table 3). However, numerous treated larvae presented an arched body.

Alterations in the eye area and in the rostrocaudal and spinal cord length may be related to the disturbance in the proliferation and differentiation of neuronal cells, and to alterations in early axial patterning events (Heiden et al., 2007). The arched body may be related to musculoskeletal disorders and agents that affect the central nervous system and neuromuscular junctions (Couch et al., 1977). Otherwise, the pericardial edema could be a consequence of cardiovascular abnormality, possibly due to an increase in the permeability of blood vessels (Hill et al., 2004).

In cell culture of previous studies, DG4.5 was less cytotoxic than DG4, due to the exposure of carboxylic groups to the surface. In contrast, DG4 exposes the positively charged amino groups on its surface. This could be a key feature of its toxicity both *in vivo* and *in vitro* (Heiden et al., 2007). Studies have reported that cationic PAMAM dendrimers generate holes *in vitro* in lipid bilayers of 1,2-dimyristoyl-sn-glycero-3-phosphocholine, and that dendrimers not only interact with the lipid membrane but also alter the protein conformation of the membrane (Duncan and Izzo, 2005; Hong et al., 2004). Besides, *in vivo* studies support the results obtained in cell culture. In particular, we have previously observed that the brain and muscle of Wistar rats are the main targets of intravenous dendrimers-sulfadiazine complexes (DG4 and DG4.5), the same organs that were affected with the DG4 or

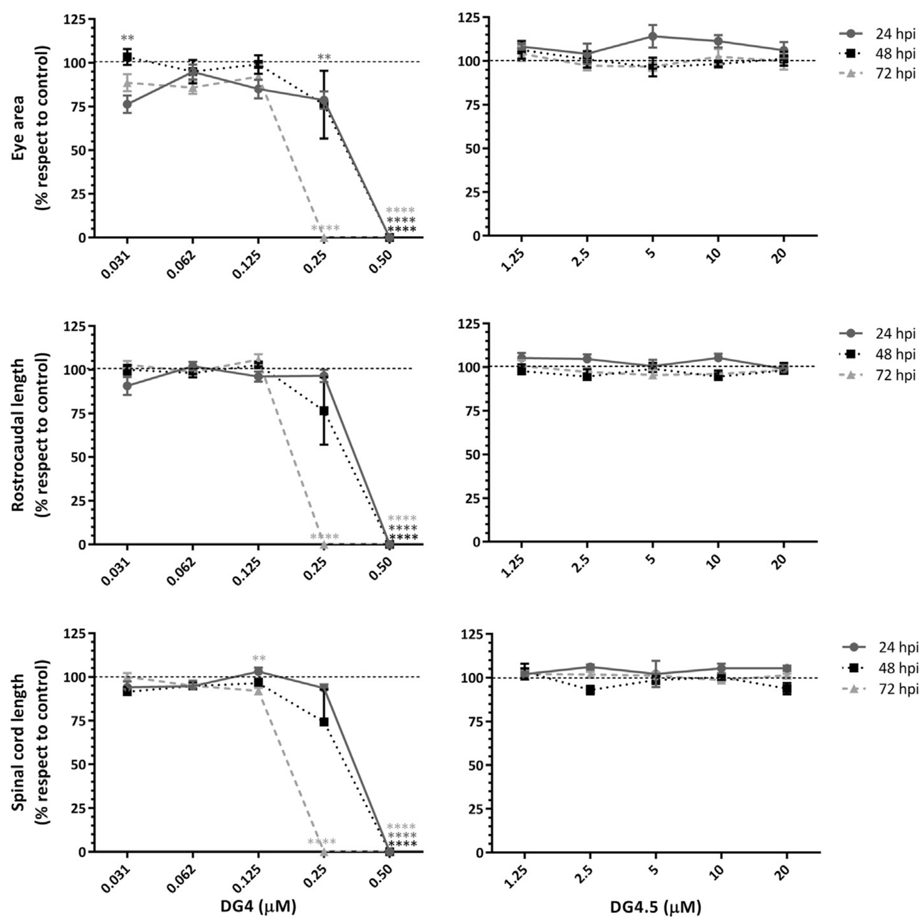

Fig. 6. Percentage of larval eye area, rostrocaudal length and spinal cord length compared with the control (untreated larvae) at 24, 48 and 72 hours post-incubation of larvae exposed to DG4 and DG4.5. Results are shown as mean ± SEM (n = 5). Statistical analysis was performed by ANOVA and the test of Dunnett comparing all samples against the control.

Table 3
Morphological changes in larvae at 24, 48 and 72 hpi treated with DG4 and DG4.5. Uninflated swim bladder, presence of arched body, ulceration of tissue and pericardial edema were determined. The percentage of larvae with adverse effects was scored as 80–100% (+ + + +), 60–80% (+ + +), 40–60% (+ +), 20–40% (+) and 0–20% (−).

	Concentration [μM]	Uninflated swim bladder			Arched body			Ulcerated tissue			Pericardial edema		
		24 hpi	48 hpi	72 hpi	24 hpi	48 hpi	72 hpi	24 hpi	48 hpi	72 hpi	24 hpi	48 hpi	72 hpi
DG4	0.031	−	−	−	−	+ +	+ + +	−	−	−	−	−	−
	0.062	−	−	+	−	+	+ +	−	−	−	−	−	−
	0.125	+ +	+	−	−	+	+ +	−	−	−	−	−	−
	0.250	+ + + +	+ + +	+ + + +	−	+	+ + + +	−	−	+ + + +	−	−	−
	0.500	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	−	−	−
DG4.5	1.25	−	−	−	−	−	+ +	−	−	−	−	−	−
	2.50	−	−	−	−	+ +	+ + +	−	−	−	−	−	−
	5	−	−	−	−	+ +	+ +	−	−	−	−	−	−
	10	−	−	−	−	+ + + +	+ + +	−	−	−	−	−	−
	20	−	−	−	−	+	+ +	−	−	−	−	−	−

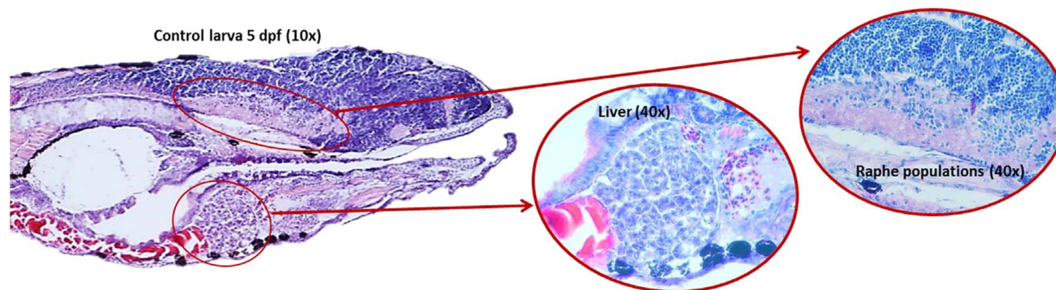

Fig. 7. Hematoxylin-eosin staining of control larvae at 5 days post-fecundation (10 ×). Liver and raphe populations are pointed in the control larvae and amplified (40 ×). Both are the study regions for histological analysis.

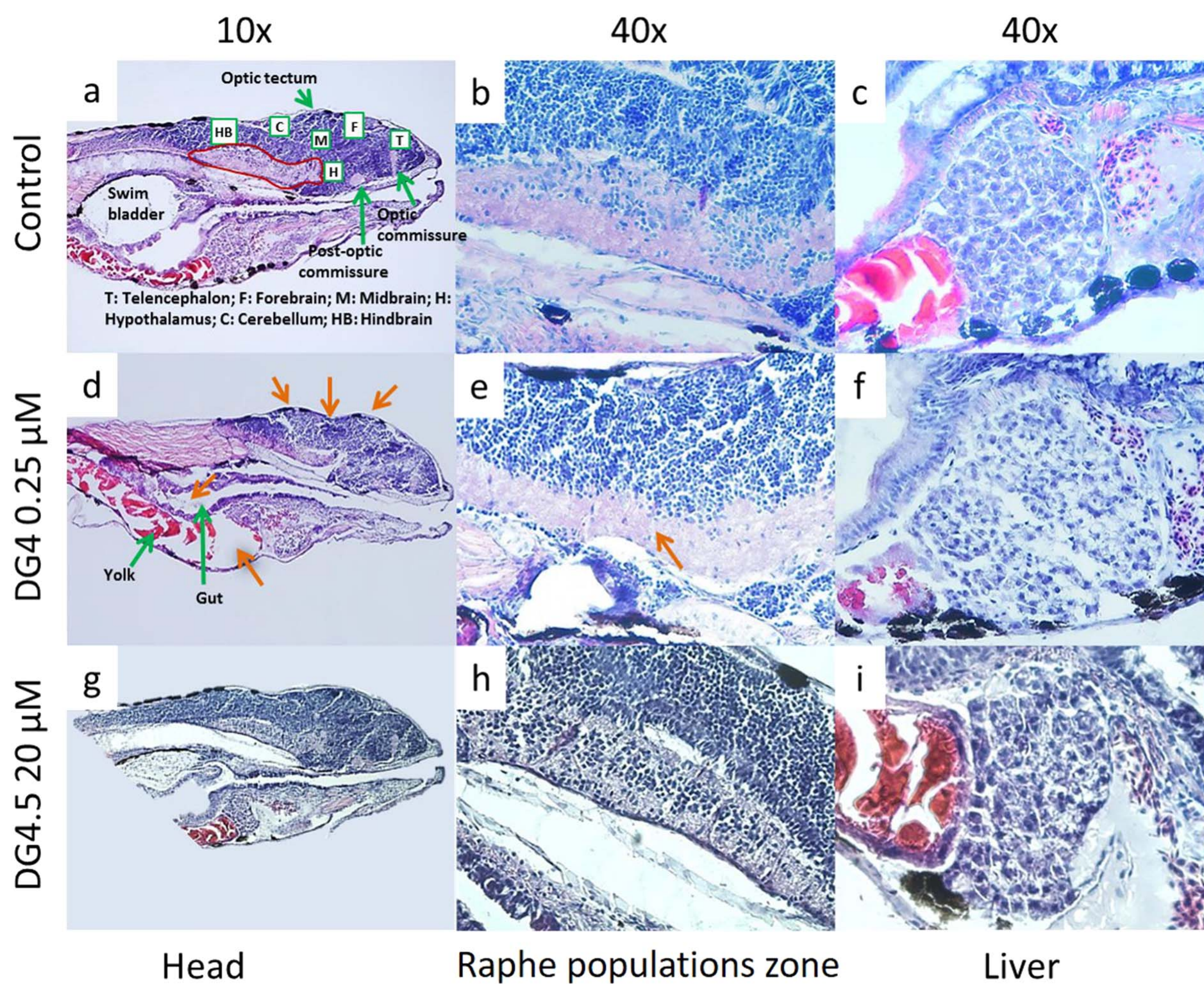

Fig. 8. Histological sections of 10 μm in thickness of 5 days post-fecundation larvae stained with hematoxylin-eosin; (a–c) untreated controls, (d–f) and (g–i) 24 hour post-incubation larvae treated with DG4 0.25 uM and DG4.5 20 μM, respectively. (a), (d) and (g) show the head and abdomen of the larva (10 ×); (b), (e) and (h) show cells of the raphe populations (40 ×); and (c), (f) and (i) show the liver (40 ×). In figure (a), the red line indicates the raphe population in the brain; in figure (d), the arrows show a sinking in the region of the optic tectum and an elevation of the adjacent areas, in addition to the delayed development of the digestive system and the swim bladder. Figure (e) shows a decrease in nucleated areas in the raphe populations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DG4.5 treatment in zebrafish larvae (Duncan and Izzo, 2005; Prieto et al., 2006, 2008). Within the systemic circulation, cationic dendrimers possibly interact with blood components, destabilize cell membranes, induce cellular lysis and cause tissue damage (Aillon et al., 2009). Greish et al. (2012) have demonstrated that PAMAM dendrimers have a surface charge and functional group-dependent toxicity profile, and that anionic dendrimers are less toxic than cationic ones when administered at 50-fold or higher doses in mice.

In the present study, the decrease in locomotor activity of larvae treated with DG4 was possibly related either to the uninflated swim bladder, observed in the morphological analysis (Fig. 2 and Table 3), or to the increase in the death rate (data not shown), which was concentration-dependent. With DG4.5 an arched body was observed, but this did not affect the swimming activity of the larvae (Fig. 2 and Table 3).

3.2.4. Histological analysis

To analyze toxic effects in the brain, spinal cord and liver, histological slides and staining with hematoxylin-eosin of zebrafish larvae were performed at 24 and 72 hpi (5 and 7 dpf) (Fig. 7). Larvae were incubated at 4 dpf with DG4 (0.25 μM) and DG4.5 (20 μM). The concentration was selected on the basis of the concentration that induced an alteration in spontaneous locomotor activity, but did not induce high mortality or severe morphological changes. For DG4.5, the higher concentration was selected because of its low toxic effects.

In vertebrates, serotonin plays a key role in the modulation of locomotor movements (Airhart et al., 2007; Jacobs and Fornal, 1997; Lillesaar, 2011; Airhart et al., 2012). In zebrafish, serotonergic neurons in the raphe nuclei and projections appear early at 2 dpf, and innervate motor neurons projecting to axial muscles. The entire spinal cord is innervated by raphe axons at 4 dpf, a moment that corresponds to the exposure of larvae to the nanomaterials studied (Prieto et al., 2012; Airhart et al., 2012; McLean and Fetcho, 2004). Only the raphe serotonergic neurons project to the spinal cord (Airhart et al., 2007). Therefore, changes in the raphe populations and alterations in spontaneous locomotor activity events reflect the neurotoxicity of the different treatments (de Esch et al., 2012; Selderslaghs et al., 2013). In addition, raphe serotonergic neurons of zebrafish have apparent phenotypic resemblance with those of mammals (Gaspar and Lillesaar, 2012). In mammals, most serotonergic cells are located in the hindbrain, while zebrafish have at least three clusters of serotonergic neurons, but only the cell groups in the hindbrain (raphe populations) and medullary cells are also found in tetrapods (Maximino et al., 2013).

The histological analysis showed that DG4 had a higher toxic effect than DG4.5. Larvae treated with DG4 0.25 μM at 24 hpi presented an increase in non-nucleated zones and a decrease in the cellular organization in the raphe populations zone (Fig. 8e). Moreover, severe craniocerebral abnormalities were observed at the optic tectum, where the

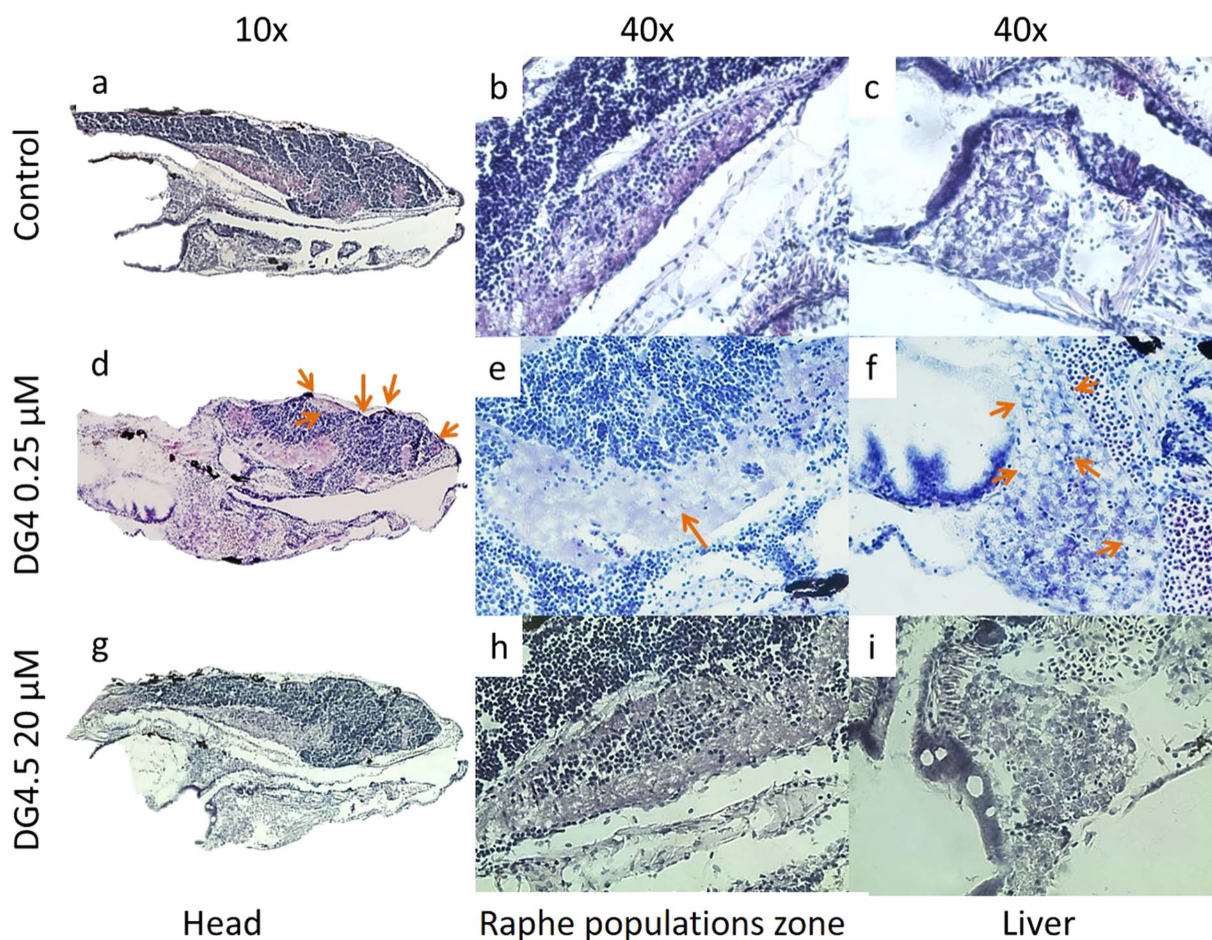

Fig. 9. Histological sections of 10 μm in thickness of 7 days post-fecundation larvae stained with hematoxylin-eosin; (a–c) untreated controls, (d–f) and (g–i) 72 hour post-incubation larvae treated with DG4 0.25 μM and DG4.5 20 μM, respectively. (a), (d) and (g) show the head and abdomen of the larvae (10 ×); (b), (e) and (h) show cells of the raphe nuclei (40 ×); and (c), (f) and (i) show the liver (40 ×). In figure (d), the arrows show a sinking in the region of the optic tectum and an elevation of the adjacent areas. In addition, an increase in non-populated areas can be observed in the region of the telencephalon, forebrain and optic tectum. Figure (e) shows a decrease in nucleated areas in the raphe populations and (f) vesicles in the liver are indicated.

cleavage sinking that separates the forebrain from the hindbrain was more pronounced, with an increase in non-nucleated zones (Fig. 8d). On the other hand, larvae treated with the highest concentration of DG4.5 (20 μM) did not present alterations respect to control (Fig. 8g and h).

Larvae treated with DG4 0.25 μM at 72 hpi presented a low proportion of nucleated zones in the raphe populations, with a low cellular organization (Fig. 9e). Larvae showed non-nucleated zones in the dorsal area of the optic tectum and the forebrain (Fig. 9d). In addition, a decrease in the number of nuclei in the frontal area of the telencephalon and post-optic commissure, together with a cleavage sinking that separates the forebrain from the hindbrain, were observed at 72 hpi (Fig. 9d). DG4.5 20 μM only caused a decrease in the number of nuclei, and therefore, an increase in the size of the post-optic commissure (Fig. 9g and h).

The craniofacial abnormalities and brain changes observed suggest that the neuroanatomy of zebrafish was affected due to the incubation with DG4 0.25 μM, although these alterations would not be sufficient to modify the locomotor activity of larvae. Otherwise, DG4.5 only caused alterations in the post-optic commissure, which did not alter the activity of larvae. These results suggest that, during the treatment, the dendrimers crossed the larval developing blood-brain barrier, whose maturation occurs between 3 and 10 dpf (Fleming et al., 2013). A previous study has also demonstrated that DG4 can cross an *in vitro* blood-brain barrier model, and supported that DG4 activates the immune cell response and thus causes neuro-inflammation (Bertero et al., 2014).

In the present study, the larvae treated with DG4 presented a yolk sac larger than control and larvae pre-treated with DG4.5, and the development of the digestive system was retarded in comparison to that of the controls, at 24 hpi (Fig. 8d). This could be in relation with a lower food intake and a retarded growth, but did not alter the swimming activity of larvae at 24 hpi, as would be expected for the lower energetic consumption. However, this condition was reversed at 72 hpi (Fig. 9d). In addition, the space occupied by the swim bladder was not clearly seen (Fig. 8d). This corroborated the uninflated swim bladder observed in the morphological analysis of larvae treated with DG4 (Table 3). In contrast, DG4.5 did not cause alterations in the progress of the development of the organs and the yolk sac (Fig. 8g).

Neither DG4 nor DG4.5 altered hepatocytes, which maintained a normal size, prominent nuclei, preserved cytoplasm, well-defined cellular polygonal shape and close cell-cell contacts at 24 hpi (Fig. 8f and i). Nevertheless, the larval liver treated with DG4 had a normal development, but presented several vesicles at 72 hpi (Fig. 9f). This alteration could be an evidence of liver damage (He et al., 2013). In contrast, treatment with DG4.5 did not alter liver physiognomy (Fig. 9i).

We have previously observed that dendrimers-sulfadiazine complexes accumulate mainly in the brain as well as in the liver of Wistar rats, after 4 h by intravenous administration (Prieto et al., 2008). Although these results were not compared with free dendrimers, the dendrimer-drug complex caused an increase in the accumulation in

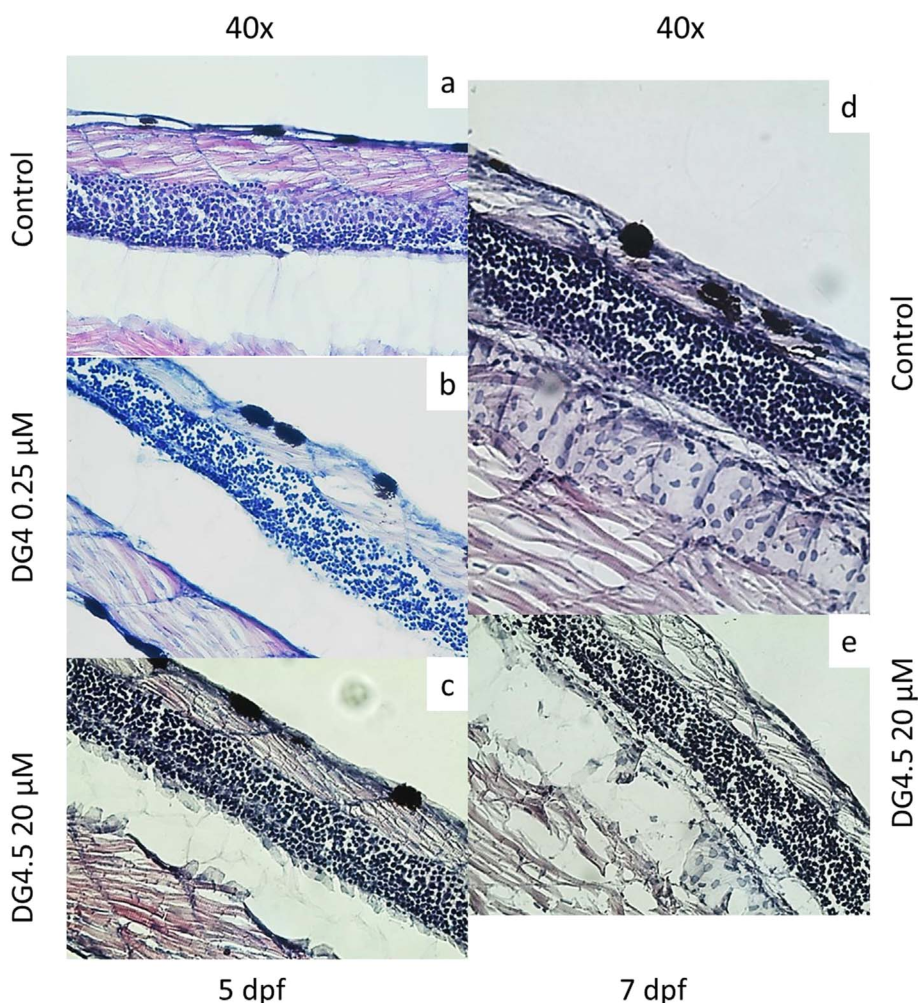

Fig. 10. Histological sections of 10 μm in thickness of spinal cord (40 ×) stained with hematoxylin-eosin. Figures (a–c) correspond to larvae of 5 dpf, (d) and (e) correspond to larvae of 7 dpf. Figures (a) and (d) correspond to the untreated controls; (b) and (c) correspond to 24 hpi larvae treated with DG4 0.25 μM and DG4.5 20 μM, respectively. Figure (c) corresponds to a 72 hpi larva treated with DG4.5 20 μM.

those organs in comparison with the free drug (Prieto et al., 2008). Moreover, it has been reported that 1 h after intravenous and intraperitoneal administration of cationic dendrimers labeled with I^{125} in rats, the liver shows high radioactivity, between 60 and 90% of the dose, while the liver treated with anionic dendrimers shows radioactivity values between 25 and 70% of the dose (Duncan and Izzo, 2005; Malik et al., 2000). Also, it has been reported that DG4 accumulates in the mouse liver, probably due to the opsonization of the dendrimer by blood proteins, because of the surface charge (Greish et al., 2012). In contrast, anionic PAMAM dendrimers of similar size are retained longer in the blood and slowly excreted by urine (Greish et al., 2012). Greish et al. (2012) supported that the liver accumulation of PAMAM dendrimers with amine terminal groups is mainly a consequence of the surface charge over the size of the nanoparticle.

Neither treatment led to alterations in the spinal cord, where cells maintained a normal spatial disposition at 24 and 72 hpi (Fig. 10).

4. Conclusions

The *in vivo* toxicity of dendrimers is highly influenced by their nature. DG4, which has cationic amine terminal groups, was more toxic than DG4.5, which has anionic carboxylic acid terminal groups, both in embryos and larvae. Larvae were less sensitive than embryos to the lethal effects of DG4. DG4 and DG4.5 induced teratogenic effects in larvae, but DG4 also induced lethal effects in zebrafish embryos and larvae. This platform of assays allowed obtaining valuable and reproducible information of toxic and teratogenic effects of dendrimers. This study has demonstrated the zebrafish embryo-larva as a promising model for prediction of adverse effects of nanoparticles that may be useful as for exploratory assessment prior to *in vivo* toxicological evaluations in preclinical animal species.

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