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Transient modulation of acetylcholinesterase activity caused by exposure to dextran-coated iron oxide nanoparticles in brain of adult zebrafish

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ABSTRACT

Superparamagnetic iron oxide nanoparticles (SPIONs) are of great interest in nanomedicine due to their capability to act simultaneously as a contrast agent and as a targeted drug delivery system. At present, one of the biggest concerns about the use of SPIONs remains around its toxicity and, for this reason, it is important to establish the safe upper limit for each use. In the present study, SPION coated with cross-linked aminated dextran (CLIO-NH_2) were synthesized and their toxicity to zebrafish brain was investigated. We have evaluated the effect of different CLIO-NH_2 doses (20, 50, 100, 140 and 200 mg/kg) as a function of time after exposure (one, 16, 24 and 48 h) on AChE activity and *ache* expression in zebrafish brain. The animals exposed to 200 mg/kg and tested 24 h after administration of the nanoparticles have shown decreased AChE activity, reduction in the exploratory performance, significantly higher level of ferric iron in the brains and induction of *casp8*, *casp 9* and *jun* genes. Taken together, these findings suggest acute brain toxicity by the inhibition of acetylcholinesterase and induction of apoptosis.

1. Introduction

Magnetic nanoparticles (MNPs) have attracted great interest in recent years due to their unique physical and chemical properties and their potential applications in various biomedical fields (Lu et al., 2007). They consist of small domains (usually smaller than 100 nm), containing magnetic atoms such as iron, cobalt or nickel that can be easily manipulated using an external magnetic field (Wang et al., 2001). Among magnetic nanoparticles, the superparamagnetic iron oxide nanoparticles (SPIONs) are of particular interest on account of the strong magnetic response when the particles are exposed to an external magnetic field and, the lack of residual magnetization when the field is removed. In addition, these particles present biocompatibility, injectability, and may have a high rate of accumulation in the target tissue if adequate ligands are attached to their surfaces (Ito et al., 2005). SPIONs have found a great number of biomedical applications; for instance, as contrast agents in magnetic resonance imaging, in magnetic separation of cells and proteins, in drug and gene delivery, in anticancer treatments by hyperthermia and tissue engineering (Singh et al., 2010).

Surface characteristics such as size and shape affect the toxicological profile of the nanoparticles (NPs) and their overall *in vivo* behavior (Chouly et al., 1996). For this reason, it is important to establish the safe upper limit for each use. Diverse aspects of the *in vitro* toxicity including, cytotoxicity, genotoxicity and oxidative stress generation and some general aspects of the *in vivo* toxicity of SPIONs, were investigated (for review, see Mahmoudi et al., 2012). For instance, rats that had been intravenously injected with γ-Fe_2O_3 NPs (0.8 mg/kg) presented toxicity in liver, kidneys and lungs (Hanini et al., 2011). Acute oral exposure to Fe_2O_3-30 NPs caused more than 50% inhibition of total $Na^{(+)}$–$K^{(+)}$, $Mg^{(2+)}$, and $Ca^{(2+)}$-ATPase activities in brains of female rats and activation of the hepatotoxicity marker enzymes, aspartate aminotransferase and alanine aminotransferase in serum and liver (Kumari et al., 2013). In accordance, due to Fe_2O_3-30 NPs 28 days repeated oral dose,

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significant inhibition was observed in total $Na^{(+)}$–$K^{(+)}$, $Mg^{(2+)}$, and $Ca^{(2+)}$-ATPase activities in the brain of exposed rats (Kumari et al., 2012). Developmental toxicity causing mortality, hatching delay, and malformation were found in zebrafish (*Danio rerio*) embryos exposed to higher doses than 10 mg/L of iron oxide nanoparticles (Zhu et al., 2012).

In cholinergic neurotransmission, acetylcholine (ACh) promotes the activation of muscarinic and nicotinic cholinergic receptors. The maintenance of levels of ACh in the extracellular space is catalyzed by acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), by the hydrolysis of ACh into its component parts choline and acetate (Soreq and Seidman, 2001). It has been demonstrated that BuChE is not encoded in the zebrafish genome, but AChE is encoded by a single gene that has been functionally detected in zebrafish brain (Bertrand et al., 2001). The inhibition of AChE activity for assessment of the exposure of organisms to organophosphate and carbamate pesticides is well-known (for review see Van Dyk and Pletschke, 2011). However, other toxic compounds than organophosphate and carbamate pesticides both promoted AChE inhibition and AChE activation in fish. For instance, the inhibition of zebrafish brain AChE activity by neurotoxic compounds such as methanol (Rico et al., 2006), lithium (Oliveira et al., 2011), the heavy metals mercury and lead (Richetti et al., 2011), and the organochlorine pesticide Endosulfan (Pereira et al., 2012) has been demonstrated. Notwithstanding, AChE activation has also been demonstrated as a consequence of exposure to toxic substances such as ethanol (Rico et al., 2007), aluminum (Senger et al., 2011) and Microcystin-LR (Kist et al., 2012).

Thus, considering that (1) SPIONs have been developed for a number of applications; (2) it is crucial to establish the safe upper limit for each SPION's use; (3) the *in vivo* neurotoxic effects of SPIONs are not completely understood; (4) AChE activity is successfully used as a biomarker of brain injury, the aim of the present study was to evaluate the effects caused by exposure to dextran-coated SPIONs in the brain using adult zebrafish as the organism model.

2. Materials and methods

2.1. Animals

Adult wild-type zebrafish (*D. rerio*, Cyprinidae) of both sexes (6–9 months-old) were obtained from a specialized supplier (Redfish Agroloja, RS, Brazil). Animals were kept at a density of up to five animals per liter in 50 L housing tanks with tap water that was previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mg O_2/L) at 26 ± 2 °C, under a 14/10 h light/dark controlled photoperiod. Animals were acclimated for at least two weeks before the experiments and were fed three times a day with TetraMin Tropical Flake fish food®. The fish were maintained healthy and free of any signs of disease and were used according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. All procedures in the present study were approved by the Animal Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS), protocol number 12/00288.

2.2. Chemicals

Trizma Base, ethylene-dioxy (EDTA), ethylene glycol bis (beta amino ethylether)-N,N,N′,N′-tetraacetic acid (EGTA), sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Aldrich Chemical Co (St. Louis, MO, USA). TRIzol® reagent, ImPROm-II Reverse Transcriptase® (Promega, Madison, Wisconsin, USA), Platinum® Taq DNA Polymerase and GelRed® were purchased from Invitrogen (Carlsbad, CA, USA).

2.3. Dextran coated SPION synthesis and characterization

Iron oxide (Fe_3O_4) nanoparticles coated with cross linked aminated dextran (CLIO-NH_2) were synthesized by the co-precipitation method in an alkaline environment, based on the procedure described previously (Wunderbaldinger et al., 2002). The synthesis was made by dissolution of dextran (T10, pharmacosmos) in an aqueous medium and mixed with salts of $FeCl_3.6H_2O$ and $FeCl_2.4H_2O$ (Merck) with a molar ratio of 2:1, in a cold environment and N_2 flux. NH_4OH (25%, Merck) was added slowly in the solution and stirred at 75–85 °C for 90 min. To eliminate the excess dextran, the mixture was centrifuged in Amicon® filters with a molecular mass cutoff of 50 kDa. Cold 5 M NaOH (Merck) was added slowly and stirred for 15 min and then epichlorohydrin (Fluka) was added for the crosslinking of the dextran chains. For amination of the dextran coating, NH_4OH (25%, Merck) was added in the NP solution and stirred for additional 24 h. After that, the remaining NH_4OH was eliminated by dialysis, using cellulose membranes (Spectra/Por®) submerged in distilled water under continuous magnetic stirring. Water was exchanged several times in this process. The resulting CLIO-NH_2 nanoparticles were dispersed in sodium citrate buffer (10 mM $Na_3C_6H_5O_7$/150 mM NaCl) at pH 8 and stored at 4 °C.

For characterization, all samples were initially sonicated (40 kHz) and stirred in a vortex and then the desired aliquots collected. Iron concentration was determined by UV–vis spectroscopy (Lambda 35, Perkin Elmer), using the absorbance at 410 nm. The concentration was obtained interpolating the absorbance value of the NP solution in a calibration curve made from Fe atomic spectroscopy standards. The [Fe] of the stock solution was approximately 10 mg/mL. The hydrodynamic diameter of the NPs in the aqueous solution was measured with a Nano-ZS Zetasizer (Malvern). The elemental composition of the dried NPs on Si substrates was measured by Rutherford backscattering spectroscopy (RBS), using a 2 MeV He beam and a detection angle of 165° and by RX energy dispersion spectroscopy.

The nuclear magnetic relaxation properties of the particles on water protons were obtained in a 3T clinical magnetic resonance scanner (SIGNA XDXT, G&E), imaging a phantom containing NP solutions with eight different concentrations, using spin eco or inversion recovery sequences.

2.4. Animal procedures

Intraperitoneal (i.p.) injection was adopted as the administration route for the *in vivo* protocols to ensure that exposure concentrations are in line with the target values. Intraperitoneal injections were conducted using a 3/10-mL U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16″) × 31G Short Needle (Becton Dickinson and Company, NJ, USA) according to the protocol established by Phelps et al. (2005). Briefly, the volume injected into the animal (mean injection volume of 10 μL) was adjusted to the fish bodyweight (mean mass of the animals was 0.5 ± 0.06 g/mean ± S.E.M.) to achieve 200 mg/kg. The animals of the control group received the same volume of saline solution and the animals of the buffer control group received the same volume of sodium citrate buffer. Anesthesia of the animals prior to the injection was obtained by immersion in a solution of tricaine (0.01%) until the animal showed a lack of motor coordination and reduced respiratory rate. The anesthetized fish was gently placed in a water-soaked gauze-wrapped hemostat with the abdomen facing up and the head of the fish positioned at the hinge of the hemostat (the pectoral fins were used as a landmark on the abdomen). The needle was inserted parallel to the spine in the midline of the abdomen posterior to the pectoral fins. The injection procedure was conducted in such a way as to guarantee that the animal did not spend more than 10 s out of the water. After the injection, the animals were placed in a separate tank with highly aerated dechlorinatedtap water (25 ± 2 °C) to facilitate recovery from the anesthesia. Saline solution was used as control. All the animals that recovered within 2–3 min following the injection continued in the experiment, while the animals