Neurotoxicology and Teratology 50 (2015) 73–81

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

An avian model for ascertaining the mechanisms of organophosphate neuroteratogenicity and its therapy with mesenchymal stem cell transplantation

Adi Pinkas [a,b,*], Gadi Turgeman [c], Shay Tayeb [d], Joseph Yanai [a,e]

[a] The Ross Laboratory for Studies in Neural Birth Defects, Department of Medical Neurobiology, Institute for Medical Research-Israel–Canada, Israel
[b] The Hebrew University-Hadassah Medical School, Box 12272, 91120 Jerusalem, Israel
[c] Department of Molecular Biology, Ariel University, Ariel, Israel
[d] Department of Biotechnology, Hadassah Academic College, 9101001 Jerusalem, Israel
[e] Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA

ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form 16 June 2015
Accepted 21 June 2015
Available online 23 June 2015

Keywords:
Chick model
Chlorpyrifos
Mesenchymal stem cell therapy
Gene expression
Adult neurogenesis
Neurotransmitter systems
Pre-hatch exposure

ABSTRACT

Introduction: A fast and simple model which uses animals lower on the evolutionary scale is beneficial for progress in neuroteratological research. Here, we established this novel model and applied it in the study of the detrimental effects of pre-hatch exposure to chlorpyrifos on neurogenesis and several neurotransmitter systems in the chick and their reversal, using mesenchymal stem cell (MSC) transplantation.
Methods: Chicken eggs were injected with the organophosphate chlorpyrifos, 10 mg/kg eggs – a dose below the threshold for dysmorphology – on incubation days (ID) 0 and 5 and subsequently the embryos were subjected to intravenous transplantation of MSC on ID 13.
Results: After hatching (day 1) the expression of the neurogenesis-related genes DCX (also confirmed by immunohistochemistry), BDNF, MAP 2, FGF 2, SOX 2 and VEGF in the lateral striatum area was decreased in the exposed group ($p < 0.005$). Among the studied neurotransmitter systems (serotonergic, dopaminergic and cholinergic), increased gene expression was demonstrated for tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) with a corresponding decrease in serotonin receptor 1A (5HTR1A) ($p < 0.05$); no changes in gene expression of choline transporter, PKC beta and D2 were found following chlorpyrifos exposure.
Conclusion: Transplantation of MSC reversed all the neurogenic and serotonergic alterations ($p < 0.01$). The study of chick embryo exposure to insults with subsequent MSC therapy provides a fast and simple model for elucidating the mechanisms of both the neuroteratogenicity and the therapy, steps that are critical for progress toward therapeutic applications.

1. Introduction

The availability of a simple model could greatly contribute to the study of both the mechanisms and therapy of neurobehavioral teratogenicity. To this end, the present study was designed to develop a chick model for the study of the effects of the neuroteratogen chlorpyrifos on neurogenesis and neurotransmitter systems, and the reversal of the resulting defects with transplantation of mesenchymal stem cells (MSC). The chick model is ideal for the present goals because it represents a highly controlled model where confounding variables as maternal and litter effects are eliminated. In fact, the model was successfully applied in the past in neurobehavioral teratology research by various groups including our own (Izrael et al., 2004; Yanai et al., 2009; Slotkin et al., 2008a; Yamaguchi et al., 2011; Hamilton et al., 2005) but the methodology for reversal with cell transplantation remains to be developed.

Organophosphate pesticides are known neuroteratogens whose use has been recently limited. The neuroteratogenicity of chlorpyrifos, one of the most studied organophosphates, has been documented in the past and is expressed via cholinesterase inhibition in high levels of exposure (Mileson et al., 1998) and via other neurotransmitter systems in lower ones (Slotkin et al., 2008a; Slotkin and Seidler, 2005). Pertinent to the present study, chlorpyrifos was shown to impair neurogenesis as reflected in both in vitro and rodent in vivo models (Slotkin and Seidler, 2008; Slotkin and Seidler, 2010; Slotkin et al., 2008b), impairments which may be mechanistically related to chlorpyrifos-induced behavioral deficits (Sakamoto et al., 2014; Aimone et al., 2011).

Abbreviations: chlorpyrifos, CPF; mesenchymal stem cells, MSC; central nervous system, CNS; sub-ventricular zone, SVZ; neural stem cells, NSC; protein kinase C, PKC; brain-derived neurotrophic factor, BDNF; fibroblast growth factor 2, FGF 2; SRY-sex determining region Y-box 2, SOX 2; doublecortin, DCX; microtubule-associated protein 2, MAP 2; vascular endothelial growth factor, VEGF; insulin-like growth factor 1, IGF-1; 5-hydroxytryptamine receptor 1A, 5HTR1A; tryptophan hydroxylase 2, TPH2; dopamine receptor D2, D2; tyrosine hydroxylase, TH; protein kinase C beta, PRKCB; choline transporter, SLC5A7; glyceraldehyde 3-phosphate dehydrogenase, GAPDH; dimethylsulfoxide, DMSO; 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, DiI.

* Corresponding author.
E-mail address: adi.pinkas@gmail.com (A. Pinkas).

http://dx.doi.org/10.1016/j.ntt.2015.06.004
0892-0362/

Prenatal exposure to organophosphates, including chlorpyrifos, induced alterations in various neurotransmitter systems, among them are the cholinergic, serotonergic and dopaminergic systems (Izrael et al., 2004; Yanai et al., 2009; Slotkin et al., 2008a; Slotkin and Seidler, 2007; Chen et al., 2011), some of which were shown in a mouse model to be reversed by stem cell transplantation. Whether a similar therapy could be applied in an avian model remained an open question.

MSC offer numerous benefits within the realms of stem cell research (Chen et al., 2003; Crigler et al., 2006; Hardy et al., 2008). The application of MSC for the reversal of defects, including neurobehavioral defects, is common and new models are frequently being established (Tfilin et al., 2010; Mahmood et al., 2004). However, no avian model was established for the reversal of neurobehavioral teratogenicity with MSC or any other cells. Recently, we have shown that neural stem cells (Dotan et al., 2010) and MSC (Pinkas and Yanai, 2013) injected into blood vessels in the incubating egg reach the embryonic brain.

The rodent model for neurobehavioral teratogenicity has inherent methodological shortcomings from confounding indirect variables related to maternal effects (Sastry, 1991; Sobrian et al., 1999), such as maternal care and mother-offspring interaction (Fernandez et al., 1983; Navarro et al., 1988; Riley and Barron, 1989; Barron et al., 1991), and disruption of maternal endocrine status, all which affect behavioral outcomes. Obviously, the avian model avoids these confounds, since the teratogen is injected into the egg, so that the embryo is directly exposed to a defined concentration; the exposure thus resembles the zebrafish model (Levin et al., 2003), in which eggs are placed in a constant concentration in the medium (water) and are thus exposed to a uniform level of teratogen. Similarly, the rodent model exhibits a "litter effect" (Spear and File, 1996), a limitation that is absent in the chick model; every individual offspring represents an independent sample. Finally, chick eggs are cheap, abundant, and easy to maintain in large numbers, and thus are more suitable for higher near complementary screening than mammalian models. Ultimately, we believe it is most advantageous to work simultaneously with both chicks and rodents to reap the benefits of each model.

In the present study, chick embryos were exposed to chlorpyrifos pre-hatch and were subsequently transplanted with MSC. The effects of the teratogen with or without stem cell therapy was evaluated by ascertaining the expression of genes related to neurogenesis and to several neurotransmitter systems pertinent to the known behavioral deficits related to chlorpyrifos neurobehavioral teratogenicity.

2. Materials & methods

2.1. Teratogen treatment: chlorpyrifos

All animal procedures were conducted in accordance with the protocol approved by the Hebrew University Institutional Animal Care and Use Committee, ethics approval number MD-13-13620-2. Teratogen was introduced as previously described (Izrael et al., 2004). Briefly: fertile heterogeneous stock eggs (60 ± 3 g) of the Cobb I chicken broiler strain (*Gallus gallus domesticus*) were obtained from a commercial source. In this commercial source, eggs are collected 5 times a day. Immediately upon collection, the eggs are put in a refrigerator, set to 16 °C. Collected eggs are delivered to the lab on collection day; immediately upon arrival they are put back in a 16 °C refrigerator. On the following day, the eggs are injected with the teratogen and incubated (day 0). A good indicator for the synchronized developmental stages of the embryos is the time of hatching: all eggs hatch within a 10 hour time frame. Chlorpyrifos was injected to the eggs (10 mg/kg of egg) on incubation days 0 and 5, the period of time during which most of the brain structures develop (Hamburger VaH, 1951; Pearson, 1972); teratogen dosage is based on previous studies which include dose–response evaluations (Izrael et al., 2004). Control eggs received equivalent volumes (510 μL/kg of egg) of dimethylsulfoxide (DMSO; sigma). Twenty four hours post-hatch the brains were removed and taken for either real time qPCR or immunohistochemical analysis. To control for the possible effect of DMSO per se on the variable studied, a comparison was made between control eggs that were injected with saline and with DMSO.

2.2. MSC derivation

MSC were isolated from the bone marrow of newly hatched chicks, 24 h post-hatch, as previously described (Khatri et al., 2009). Briefly, bone marrow was extracted from tibias and femurs of chicks and suspended in Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum (Biological Industries),100 units ml^{-1} of penicillin (Biological Industries), 100 mg ml^{-1} of streptomycin (Biological Industries) and 2 mM of L-glutamine (Biological Industries). Marrow cells were separated and suspended by repeated passage through 19, 20, 21, 23 and 25G syringe needles. Non-adherent cells were removed at 24 and 48 h after plating. MSC were expanded in culture for 3–8 passages.

2.3. MSC transplantation

The procedure is an adaptation of our previously described protocol for NSC (Dotan et al., 2010) transplantation. Suspended MSC were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Sigma) fluorescent dye to trace the migration of the cells in the brain: trypsinized MSC were suspended in phosphate buffered saline (100 cells/μL) in the presence of DiI at a final concentration of 1 μg/μL and incubated for 5 min at 37 °C followed by 15 min at 4 °C and finally washed three times with PBS. Eggs containing E13 chick embryos were briefly removed from the incubator and carefully cut from the blunt end in order to reveal the air cell and the blood vessels attached to the chorio-allantoic membrane. Each embryo received a single IV injection of 200,000 cells in 100 μL PBS using a 30G needle, according to previously described procedures (Dotan et al., 2010; Taizi et al., 2006): a small window is cut open above the air sac; the egg is candled in a dark room so the blood vessels are clearly seen; the needle, bent to an "L" shape, is inserted into the largest blood vessel and the liquid is injected. After transplantation, the cut was covered with transparent adhesive tape and the eggs were returned into the incubator until E19.

2.4. Immunohistochemistry

Brains of chicks were removed 24 h post-hatch and immunohistochemistry was carried out as previously discussed (Dotan et al., 2010). Briefly: brains were frozen and cut serially in the cryostat (Leica, Wetzlar, Germany) into 25 μm thick slices; one section out of every 10 was kept in 0.01% sodium azide solutions until examined. To identify neurogenesis, tissues were washed and blocked with 1% BSA in PBST for 30 min. Primary antibody was then administered, 1:5000 doublecortin (sigma) diluted in blocking solution, overnight at 4 °C. This was followed by washings and administration of the secondary antibody, 1:1000 goat anti-mouse IgG FITC conjugated (Abcam) diluted in 1% BSA-containing PBS, for 2 h at room temperature in the dark. After washings, tissues were mounted on slides with DAPI-containing mounting solution, coverslipped and taken for immunofluorescent evaluation; histology of brain regions was determined using the stained lateral striatum, the shape of the lateral ventricles and the intermediate medial mesopallium (IMM), with an online brain atlas for guidance (www.brainmaps.org). Neural differentiation of transplanted MSC was evaluated using the same procedure, with different antibodies: for neurons, the primary antibody was mouse anti-MAP 2 (Millipore, 1:1000) and the secondary antibody was goat anti-mouse IgG FITC conjugated (Abcam, 1:1000); for astrocytes, the primary antibody was rabbit anti-GFAP (sigma, 1:5000) and the secondary antibody was goat anti-rabbit (Abcam, 1:1000). Tissues were examined with a fluorescence microscope (eclipse 90i, Nikon, Tokio, Japan); DCX immunoreactive area was measured using Image J software (NIH, Bethesda, USA).