Toxicology Letters 217 (2013) 14–22

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

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

Chlorpyrifos and its metabolites alter gene expression at non-cytotoxic concentrations in D3 mouse embryonic stem cells under *in vitro* differentiation: Considerations for embryotoxic risk assessment

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HIGHLIGHTS

- Chlorpyrifos and its metabolites alter gene expression in mouse embryonic stem cells.
- Chlorpyrifos and its metabolites alter gene expression at non-cytotoxic concentrations.
- Chlorpyrifos and its metabolites alter pluripotency of mouse embryonic stem cells.
- Chlorpyrifos and its metabolites alter differentiation of three embryonal lineages.
- Chlorpyrifos might alter differentiation without detectable maternal toxicity.

ARTICLE INFO

Article history:
Received 26 September 2012
Received in revised form
27 November 2012
Accepted 29 November 2012
Available online 7 December 2012

Keywords:
Chlorpyrifos
Chlorpyrifos-oxon
Embryonic stem cell
Differentiation
Risk assessment

ABSTRACT

The effects of organophosphate insecticide chlorpyrifos (CPF) on development are currently under discussion. CPF and its metabolites, chlorpyrifos-oxon (CPO) and 3,5,6-trichloro-2-pyridinol (TClP), were more cytotoxic for D3 mouse embryonic stem cells than for differentiated fibroblasts 3T3 cells. Exposure to 10 μM CPF and TClP and 100 μM CPO for 12 h significantly altered the *in vitro* expression of biomarkers of differentiation in D3 cells. Similarly, exposure to 20 μM CPF and 25 μM CPO and TClP for 3 days also altered the expression of the biomarkers in the same model. These exposures caused no significant reduction in D3 viability with mild inhibition of acetylcholinesterase and neuropathy target esterase by CPF and severe inhibition by CPO. We conclude that certain *in vivo* exposure scenarios are possible, which cause inhibition of acetylcholinesterase but without clinical symptoms that reach high enough systemic CPF concentrations able to alter the expression of genes involved in cellular differentiation with potentially hazard effects on development. Conversely, the risk for embryotoxicity by CPO and TClP was very low because the required exposure would induce severe cholinergic syndrome.

Abbreviations: *Ache*, acetylcholinesterase (gene); AChE, acetylcholinesterase (protein); *Afp*, α-fetoprotein; CPF, chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate); CPO, chlorpyrifos-oxon (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphate); DEPC, diethylpyrocarbonate; DMEM, Dulbecco's Modified Eagle's Medium; ECx, concentration needed to cause a reduction in cell viability of *X%*; EST, Embryonic Stem cell Test; *Flk1*, foetal liver kinase 1; IC50, concentration needed to inhibit the enzymatic activity by 50%; LIF, leukaemia inhibition factor; *Mhc*, myosin heavy chain; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; *Nanog*, nanog homeobox; *Nefm*, neurofilament medium polypeptide; *Nes*, nestin; NTE, neuropathy target esterase; OP, organophosphorus compound; PBS, phosphate buffered saline; *Pnpla6*, patatin-like phospholipase domain containing 6; PV, phenyl valerate; qRT-PCR, quantitative real-time PCR; TClP, 3,5,6-trichloro-2-pyridinol; TLV, Threshold Limit Value.

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http://dx.doi.org/10.1016/j.toxlet.2012.11.026

1. Introduction

Chlorpyrifos (CPF) is an organophosphorus compound (OP) used to control insect pests which has been used for almost 40 years. CPF is not authorised for household applications by the USEPA since 2002 (USEPA, 2000) and by the European Commission since 2005 (European Commission, 2005). In the latter case however, it is included in Annexe I to Directive 91/414/EEC for plant protection products (European Communities, 1991).

The CPF metabolism consists in the oxidative desulphuration of the P=S group to form chlorpyrifos-oxon (CPO). This reaction is catalysed by cytochrome P450, mainly in the liver, and the oxidised form of OPs (in our case CPO) is always more powerful for inhibiting acetylcholinesterase (AChE)[1] and neuropathy target esterase (NTE) than the reduced form (in our case CPF) (WHO, 1986). CPO can be further chemically or enzymatically deactivated by hydrolysis with the release of diethylphosphate and 3,5,6-trichloro-2-pyridinol (TClP). Furthermore, the oxidative dearylation of CPF to directly release TClP and diethylthiophosphate can also occur in the liver and represents an alternative detoxification pathway (Sogorb and Vilanova, 2010).

CPF is able to induce the well-known cholinergic syndrome, caused by inhibition of AChE, and organophosphorus-induced delayed polyneuropathy, caused by inhibition of NTE (Jokanovic and Kosanovic, 2010). CPF also causes other neurological impairments, such as long-term spatial learning dysfunctions (Cañadas et al., 2005), which are probably related to loss of dendrite and spine processes in both the hippocampus and prefrontal cortex (Ruiz-Muñoz et al., 2011).

There is no consistent evidence for CPF's capacity to induce teratogenicity with exposures below those required to cause maternal toxicity (Eaton et al., 2008). However, there is contradictory information on the capability of CPF to cause neurodevelopmental toxicity since many *in vivo* studies have reported such effects on exposures causing decreases in brain AChE, while other studies have indicated effects at dose levels which do not cause any apparent AChE inhibition (Eaton et al., 2008). Indeed, Slotkin et al. (2006) have suggested that there is a complete dichotomy between the systemic toxicity of OPs and their capability to cause developmental neurotoxicity. These data suggest that it is necessary to assess the risk of exposure to CPF during development by especially considering that exposure occurs in the general (not only in an occupationally exposed) population as an epidemiological study has shown that CPF displayed a prevalence of 11% in meconium at a mean concentration of 53 ppm (Ostrea et al., 2002).

CPF undergoes intensive biotransformation in live organisms, mainly the liver. Therefore, it remains unclear if the chemical responsible for the above-stated effects is the same CPF or some of its metabolites (CPO or TClP). *In vitro* studies have demonstrated that oxidised forms of OPs, such as CPO, display greater capability (up to 1000 times) to disrupt a number of neurodevelopmental processes such as neuronal proliferation and differentiation, gliogenesis and apoptosis through direct interference with the morphogenic activity of AChE, receptors and cell signalling molecules and cytoskeletal proteins (Flaskos, 2012).

Embryotoxicity can be studied *in vitro* using the Embryonic Stem cell Test (EST). This methodology is successfully validated and, as endpoints, it employs the relationships between the cytotoxicity induced by the assessed compound in 3T3 mouse fibroblasts (used as a differentiated cells model) and in D3 mouse embryonic stem cells and the capability of the compound to alter the spontaneous differentiation of D3 cells into beating cardiomyocytes (Genschow et al., 2004). Nevertheless, the EST needs to be improved by using molecular endpoints for studying alterations in differentiation (Spielmann et al., 2006). In this way, gene expression has proved a good endpoint to be implemented within conventional EST (Romero et al., 2011). Specifically, α-fetoprotein (*Afp*) (Romero et al., 2011; Pamies et al., 2010a) and nestin (*Nes*) have already been found suitable for testing embryotoxicity by monitoring their expression, but there are other genes like the patatin-like phospholipase domain containing 6 (*Pnpla6*), the codifying gene for NTE, which seem to play a prominent role in the differentiation of D3 cells (Pamies et al., 2010b) and might, therefore, be good candidates for embryotoxicity testing.

In this work, we studied the effects of CPF and its metabolites, CPO and TClP, on D3 mouse embryonic stem cells under spontaneous differentiation after short- (12 h) and mid- (3 days) term exposure using the gene expression of biomarkers of differentiation of the three main embryonic lineages and the gene expression of biomarkers of pluripotency. Most tested genes have previously proven their capability to predict embryotoxicity (Romero et al., 2011; Pamies et al., 2010a). Our main aim was to estimate the risk of embryotoxicity to exposure to CPF and we conclude that in certain scenarios, exposure to CPF could be enough to become a matter of concern.

2. Materials and methods

2.1. Chemicals

CPF (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) and TClP (3,5,6-trichloro-2-pyridinol) were purchased form Sigma–Aldrich Spain with purities of 99.5% and 98.8%, respectively. CPO (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphate) was obtained from Dr. Ehrenstorfer with a purity of 98%. All the other chemicals, materials for cell culture or molecular biology managements were attained from Sigma–Aldrich Spain, Roche or local suppliers, and were of analytical grade.

2.2. Cell cultures and exposures

Cells were exposed to all chemicals during either 12 h or 3 days. Twelve hours was chosen since this is the earliest point were the expression of the biomarkers genes could be measured with acceptable accuracy. Indeed, the expression of most of the tested genes could not be detected after only 10 h of differentiation (data not shown).

2.2.1. D3 mouse embryonic stem cells

D3 mouse embryonic stem cells were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were firstly isolated from the inner cellular mass of a 129/Sv mouse blastocyst on day 4 of gestation. D3 cells were grown on monolayers in an undifferentiated state on 75-mm plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% heat-inactivated foetal calf serum, 1% non-essential amino acids, 50 units of penicillin/ml, 100 μg streptomycin/ml, 0.1 mM β-mercaptoethanol and 1000 units of leukaemia inhibition factor (LIF)/ml. Undifferentiated cells were incubated at 37 °C in an atmosphere with 1.5% CO_2 and 95% humidity. For culturing D3 cells under spontaneous differentiation, LIF was removed from the medium culture and the CO_2 concentration was increased to 5%.

D3 undifferentiated cells were seeded in P100 Petri dishes at a density of 2×10^6 cells/dish (to record gene expression) or in 96-well plates at a density of 2×10^5 cells/well (for cell viability tests and to test the effects on NTE enzymatic activities). Afterwards, LIF was removed from the media in order to trigger differentiation, and fresh CPF, CPO and TClP ranging between 10 and 1200 μM were added to the media. The exposure performed under differentiation conditions was prolonged to 12 h.

Similar experiments were performed by prolonging differentiation and exposure for 3 days and by seeding cells at a density of 4×10^5 cells/dish in P100 Petri dishes (to record gene expression) or 3.5×10^4 cells/well in 96-well plates well (for cell viability tests and to test the effects on AChE and NTE enzymatic activities). The CPF, CPO and TClP concentrations ranged between 20 and 300 μM, the media of the cells exposed to CPO and their correspondent controls were renewed daily, and the media of the cells exposed to CPF and TClP was left unchanged for 3 days.

[1] In this manuscript we quote the abbreviations for the genes italicised with the first letter in the upper case and all the other letters in the lower case. Therefore, the term *Ache* (italicised) is used to quote the gene codifying for the protein acetylcholinesterase, while the term AChE (non-italicised with the upper and lower case in the word) is used to quote the protein acetylcholinesterase or its enzymatic activity.