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Effects of the neurotoxic thionophosphate pesticide chlorpyrifos on differentiating alternative models

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HIGHLIGHTS

- There are controversial opinions about the effects, at low doses, of Chlorpyrifos on neurodevelopment.
- We exposed to a wide range of CPF concentrations three models compatible with the 3Rs Strategy.
- We evaluated the effect of CPF on cholinesterase activity, growth and differentiation.
- We revealed that developing organisms are sensitive to CPF also at the doses found in food for children.

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ABSTRACT

Studies by researchers worldwide have revealed that, even in industrialised nations, people, infants and the aged in particular, are even more exposed to neurotoxic drugs as a consequence of the increased quantity of pesticide residues in food. This phenomenon, as underlined by The Worldwatch Institute (2006), is linked to the exponential increase in the use of these toxic compounds over the last 40 years, up from 0.49 kg per hectare in 1961 to 2 kg in 2004, with the result that these substances are found in the daily diet.

Many studies have demonstrated how the assumption of pesticides in the neonatal period and early infancy can alter the development and function of the nervous, immune, endocrine and reproductive apparatuses. Moreover, the unequivocal relationship between brain tumours, infant leukemia and pesticides are well recognised.

On the basis of the above information, the effects of the neurotoxic thionophosphate pesticide chlorpyrifos (CPF) have been tested, considering biomarkers of toxicity and toxicity endpoint, on the biological models *Dictyostelium discoideum*, *Paracentrotus lividus*, and NTera2 Cells, as they are compatible with the 3Rs strategy (Reduction, Replacement, and Refinement in animal experiments). Our results have revealed that developing organisms are particularly sensitive to the toxic effects of CPF.

1. Introduction

For several years, there has been a growing awareness of the developmental role of molecules related to the cholinergic system. In particular, acetylcholinesterase (AChE, EC 3.1.1.7), the lytic enzyme that removes ACh from its receptors, has been demonstrated to be involved in regulating a number of events related to embryonic development, from fertilisation, to cell proliferation, gastrulation movements, neurogenesis and growth. In these events, AChE is involved in the modulation of cell proliferation (Angelini et al., 2004), cell-substrate interaction, messages mediated by ion changes (Aluigi et al., 2005), and apoptosis (Aluigi et al., 2010b). For these reasons, there has been an increase in attention to the possible negative effects of pollutants on cholinergic molecules and therefore on human and, particularly, child health (Eskenazi et al., 1999). The reported outcomes shed a new light on the mechanisms of damage exerted by neurotoxic pesticides, i.e. cholinesterase inhibitors (namely organophosphorus and carbamate compounds) (Aardema et al., 2008) present in the environment and food from agricultural sites (Abdel Rasoul et al., 2008). Many studies have demonstrated how the assumption of pesticides in the neonatal period and early infancy can alter the development and function of the nervous, immune, endocrine and reproductive apparatuses (Glynn et al., 2008; Grandjean et al., 2008; Borchers et al., 2010), and how children exposed to chlorpyrifos while in the womb have an increased risk of delays

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in mental and motor development and an increased occurrence of pervasive developmental disorders such as Attention Deficit Hyperactivity Disorder (ADHD) (Rauh et al., 2006). Overexposure of the infant to pesticides can also increase the risk of developing allergic pathologies (Proskocil et al., 2008). Finally, the unequivocal relationship between brain tumours, infant leukemia and pesticides are well recognised (Proskocil et al., 2008).

Infants are in contact with pesticides during early development through the maternal blood, and this pre-load is increased by direct exposure once they are born, of varying degrees, depending on the family lifestyle and location (urban or agricultural sites) (Berry, 1997; Akland et al., 2000; Curl et al., 2003; Rauh et al., 2006; Abdel Rasoul et al., 2008). According to the European Food Safety Authority (EFSA) (Tucker, 2008) it is now clear that there is a need to adopt a tiered approach to the toxicological evaluation and intake estimation of these pollutants. A harmonised consumption survey has also been identified as an important outstanding task. In addition EFSA and the EU Member States are continuing to cooperate to develop new methods to meet the challenges of cumulative risk assessment; for example in the implementation of more representative residue surveillance schemes.

In this frame, the need for new models for toxicity tests emerges, in order to establish a correlation between benchmark doses and effects which should be cost effective, bioethically compatible, high throughput and at different degrees of complexity, to identify either the action mechanisms of old and new pesticides, or the general effects at a systemic level (EFSA Scientific Committee, 2009).

In order to be able to establish reference doses (BMD doses), as recommended by EFSA for genotoxic and carcinogenetic substances, we propose using chlorpyrifos (CPF), a neurotoxic pesticide widely used all over the world, whose toxicity is well-known. In fact, the carcinogenetic activity of CPF (Blair et al., 2011) may be due to the depression of AChE activity and mutation on AChE gene (Perry and Soreq, 2004) exerted by organophosphate compounds. In addition, there are controversial opinions about the effects, at low doses, of CFP on neurodevelopment (Eaton et al., 2008).

To carry out this task, we will use the following biological models chosen on the basis of their compatibility with the 3Rs Strategy (Replace, Reduce and Refine animal testing) (Russell and Burch, 1959), which has been adopted by ECVAM as the basis for the development of new toxicity tests (Atterwil et al., 1994).

Dictyostelium discoideum (Protozoa) included in the eight bioassay alternatives to vertebrate models for the study of human disease by the U.S. National Institute of Health (Williams et al., 2006). *Paracentrotus lividus* (Echinodermata), included in the five bioassay alternatives to mammalian models, for neurotoxicity studies in the nervous system's embryogenesis by the European Centre for the Validation of Alternative Methods (ECVAM). NTera2 cells-clone D1 (NT2), pluripotent cells able to develop in cholinergic nervous cells and generally deemed an ethical substitute for germinal neurogenic stem cells.

In previous works we detected the presence and the role of a pseudocholinesterase, named propionylcholinesterase (PrChE), in the cell to cell interactions of *D. discoideum*, (Falugi et al., 2002; Amaroli et al., 2003), and how this enzyme can react to environmental stress in the same way as the molecules of macroinvertebrate and vertebrate models (Delmonte Corrado et al., 2005, 2006; Amaroli, 2011). Moreover, our previous results showed how pesticides have serious effects on the acetylcholinesterase activity of *P. lividus* (Pesando et al., 2003; Aluigi et al., 2010a) and how the exposure to diazinon affects the balance between cell viability/apoptosis in NT2 cells (Aluigi et al., 2010b).

2. Material and methods

2.1. Chlorpyriphos (CPF)

The pesticide CPF is a crystalline thionophosphate insecticide which inhibits AChE activity and is used to control insect pests.

This pesticide was chosen for the experiments because it is one of the most active anti-cholinesterase (ChE) agents (Aluigi et al., 2005), and its metabolites were found in very high concentrations in the blood and urine of young children fed with non-organic fruit and vegetables (Lu et al., 2008). In addition, the CPF is an organophosphate insecticide which as a lipophilic molecule, can easily pass through the cell membrane into the cytoplasm (Uzun et al., 2010). Purified CPF was obtained from PESTANAL, through Sigma purchase.

The stock solution of CPF was obtained by a dilution of the pesticide in dimethyl sulfoxide (DMSO). The toxicity of this solvent was assessed by exposing the cells and the organisms to DMSO at the maximum final concentration employed in the experiments [$<10^{-3}$] (Sciarrino and Matranga, 1995). DMSO at that concentration had no effect on the cells and the organisms tested in this work.

2.2. D. discoideum growth and differentiation

The life cycle of *D. discoideum* includes two phases: the reproductive and the developmental phase. The reproductive phase consists of growth and multiplication by binary fission of single-cell amoebae feeding on bacteria. Starvation triggers the developmental phase (the streaming, mound, first finger, and mexican hat stages), and results in the formation of the fruiting body anchored to the substratum.

The reproduction phase, used in this work, was axenically induced by inoculating the fruiting bodies in Falcon flasks containing AX-2 axenic medium as described in Amaroli et al. (2006). The developmental phase was induced by transferring some drops of the AX-2 culture, onto a B2 *Escherichia coli* monolayer growing on a nutrient agar-N plate (Amaroli et al., 2003). The plates were incubated in a moist chamber for 3 d at 25 °C (Swan et al., 1977) to allow the cells to exhaust the supply of bacteria (reach starving conditions) and migrate and aggregate. When the fruiting bodies had developed, the plates were kept at 4 °C.

2.3. P. lividus growth and differentiation

Embryos and larvae of the sea urchin *P. lividus* were reared from fertilisation in ultra-filtered, pasteurised pelagic sea water, 1 larva mL^{-1} sea water, in tanks of the same size (100 mL), shape (cylindrical) and material (borosilicate glass). The pelagic sea water was collected from the water column and maintained for at least one month in a glass tank of 100 L at 20 °C. The salinity of this water was 3.7%, and the pH 8.00. The sea urchin spermiotoxicity test (ISO 2007) showed that the quality of our pelagic sea water was higher (15% ± 2.3) than artificial sea water. The pelagic sea water was collected far enough off the coast that it was not affected by agricultural pesticides, and kept in a glass tank for 1 month to allow the decantation of heavy metals. Before our rearing experiments, the water was ultra-filtered through a filter with 0.2 μm pores.

The larvae were fed with *Cricosphaera elongata* microalgae from the end of the vitellophagic phase (48 h) according to appropriate protocols (Fenaux et al., 1994), and maintained at a temperature of 18 °C. Metamorphosis was obtained according to standard procedures, by exposing 20-day-old larvae to the presence of stones freshly taken from clean sea sites.