UDK 612.017:632.95:611.81

TOXIC EFFECT OF CHLORPYRIFOS ON HIPPOCAMPAL NEURONS *IN VITRO*

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Taking into account the widespread use of different pesticides in the world, it is very important to perform studies evaluating their potential effects on the central nervous system In the present study, an in vitro primary cultures of rat hippocampal neurons was used to assess whether exposure to chlorpyrifos — one of the most commonly used organophosphorus pesticides in the world could induce cell damage and cell death. An attempt was made to find the level of toxic effect of low doses of chlorpyrifos at young neurons in conditions of cell culture. It has long been known, that chlorpyrifos is acetylcholinesterase inhibitor and that high sensitivity of the cholinesterase's inhibitors makes them highly toxic to the central nervous system. In the past five years there have been some innovations in the question concerning mechanisms of chlorpyrifos neurotoxicity, specifically it was repeatedly demonstrated that chlorpyrifos toxicity is not limited to cholinesterase inhibition alone but can act by other mechanisms. Our results show the toxic influence of 20 μM chlorpyrifos concentration on young hippocampal neurons in cell culture.

Key words: BRAIN, CELL CULTURE, CENTRAL NERVOUS SYSTEM, CHLORPYRIFOS, HIPPOCAMPUS, NEURON, NEUROTOXICITY, ORGANOPHOSPHATE PESTICIDES

Chlorpyrifos (CPF) is one of the most commonly used organophosphate pesticides for domestic, agricultural and industrial purposes. Despite recent restrictions on home use in certain countries, it remains a popular pesticide throughout the world. For example, only in United States of America currently there are over 850 registered chlorpyrifos products [1]. Chlorpyrifos is moderately toxic to humans but simultaneously it may be estimated that CPF causes thousands of deaths per year worldwide [2–4]. Poisoning from CPF may affect the central nervous system, the cardiovascular system, and the respiratory system. CPF is a well-known acetylcholinesterase (AChE) inhibitor. Emerging evidence, obtained largely through the use of rodents, suggests that acute or prolonged exposure to CPF and/or its metabolic product(s) may overtly injure the central nervous system or produce marked changes in neuronal function that persist after exposure has ceased, particularly during the early postnatal period [5, 6]. At the same time, it seems that either developmental toxicity may be unrelated to AChE inhibition, or that even a brief period of AChE inhibition is sufficient to disrupt development [4, 7]. These aspects occupy great deal of attention. Studies are in progress to evaluate the importance of various factors and it is the object of a very thorough study. A lot of scientist repeatedly demonstrated that CPF toxicity is not limited to cholinesterase inhibition alone but can act by other mechanisms. For example, *in vitro* and *in vivo* studies at three levels of development from DNA to the cell and the whole animal revealed that CPF is far more toxic than previously thought because of this wider range of activity [8]. CPF impairs the binding to DNA of nuclear transcription factors (AP-1 and Sp1) that modulate cell replication and differentiation. Therefore, CPF targets mammalian brain development through a combination of effects directed at cholinergic receptors and intracellular signalling cascades that are involved in cell differentiation. There are data that CPF and CPF-oxon, but not 3,5,6-trichloro-2-pyridinol (TCP; the breakdown product of CPF and CPF-oxon), induce apoptosis in primary cortical neurons cultured from embryonic day 17 or newborn rats [9]. It is generally agreed that chlorpyrifos-oxon is approximately three orders of magnitude more potent than chlorpyrifos in the brain AChE inhibition.

While much is known about the lethality and neurotoxicity produced by acute CPF exposure, relatively there is not a great deal known about the means by which chronic exposure to this compound, particularly low concentrations of CPF, may adversely affect neuronal function [10]. The objectives of this study were to determine if CPF induces cell damages and cell death in

primary cultured central nervous system (CNS) neurons and to ascertain amounts of these phenomena.

Materials and methods

Primary cultures of rat hippocampal neurons and neuronal transfections. Neurons from 18 days Wistar rat embryos hippocampi were dissociated using 0,25 % trypsin for 15 min at 37 °C and plated on coverslips coated with poly-ethylenimine at a density of 70 000 cells cm^2 in minimal essential medium (MEM) supplemented with 10 % NU serum (BD Biosciences, Le Pont de Claix, France), 0,8 % glucose, 1 mM sodium pyruvate, 1,5 mM Hepes, and 10 IU ml−1 penicillin–streptomycin as previously described [11]. On days 7, 10 and 13 of culture incubation, half of the medium was changed to MEM with 2 % B27 supplement (Invitrogen).

We used green fluorescent protein (GFP) for neuron transfection. At 9 DIV, mixed hippocampal cultures were transiently transfected using a Magnetofection Kit (OZ Biosciences, Marseille, France) and lipofectamine 2000 as described [11]. Magnetofection — is a novel transfection technology based on the delivery of DNA-coated magnetic nanobeads and it can be used to transfect primary hippocampal neurons. Following transfection, the cells were used for time lapse analysis. Cultures showing low rate of transfection (<10 neurons per coverslip) were withdrawn from experiments.

Drug treatment. Chlorpyrifos (99,9 pure; Sigma) was dissolved in ethanol for the final concentration of 0,05 % (this level of ethanol is not cytotoxic) and freshly prepared for each experiment of CPF.

Life imaging of cultured hippocampal neurons. Time lapse imaging of developing neuronal cells was performed using Metamorphe software on inverted Nikon-TE300 microscope equipped with CO_2 and temperature control units (Princeton Instruments). Images of same 20–30 transfected neurons per condition were taken during 3 consecutive days starting 3 days after transfection. After each imaging cultures were returned to the CO_2 incubator. Neurons with normally developing dendrites (smooth distribution of GFP) were considered as alive, whereas neurons with clustered GFP or neurons where GFP disappeared were considered as dead.

Statistical analysis. All population data were expressed as mean ± SEM. The Student's τ test was employed to examine the statistical significance of the differences between groups of data.

Results and discussion

To examine the neurotoxic effect of CPF, neurons from rat embryos hippocampi were first treated to various concentrations of it. Treatment with 20 μM concentration of CPF was selected for the investigation. Exposure of in vitro primary cultures of rat hippocampal neurons to chlorpyrifos for 24, 48 and 72 hours starting 3 days after transfection had significant toxic effect on neuronal cells (Fig. 1). In control conditions the majority of neurons normally developed for more than 6 days after transfection.

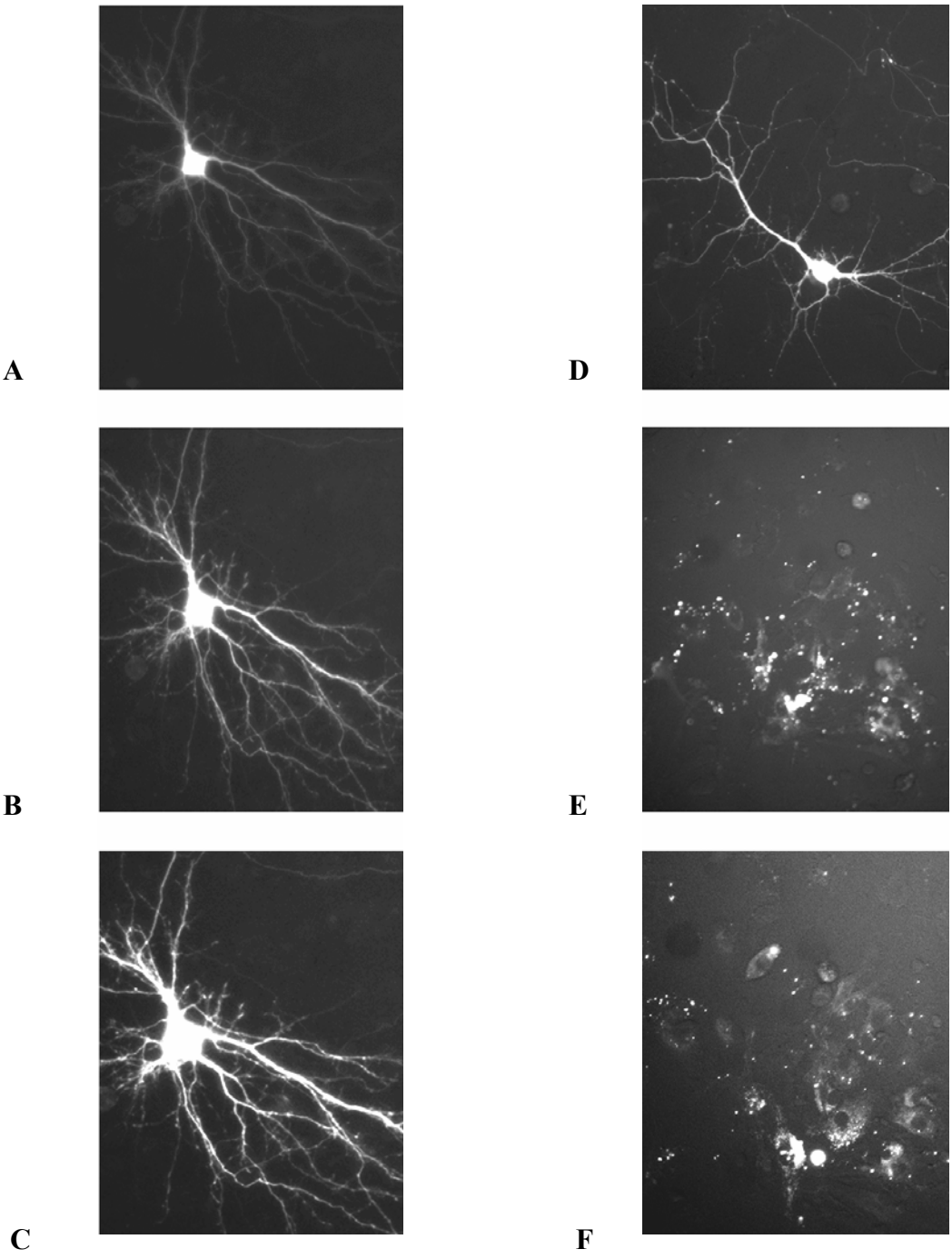

Fig. 1. Neurons survival in cultures transfected with green fluorescent protein in control conditions (A, B, C) and under the CPF applicatioin (D, E, F). A, B, C and D, E, F — images of the same neurons taken on 24 (A, D), 48 (B, E) and 72 (C, F) hours after the experiment beginning. In normal conditions the majority of neurons (left column) normally developed for more than 6 days after transfection. By contrast, near 40 % of neurons incubated in the medium with addition of CPF-fully degraded (right column)

By contrast, approximately 11 % of neurons incubated in the medium with addition of CPF, degraded on the second experimental day and near 40 % of investigated neuronal cells fully degraded on the third day of experiment with 20 μM concentration of CPF exposure in to the cell culture medium. We found significant differences in these indexes (Fig. 2). Consequently the CPF induced cell damage and cell death.

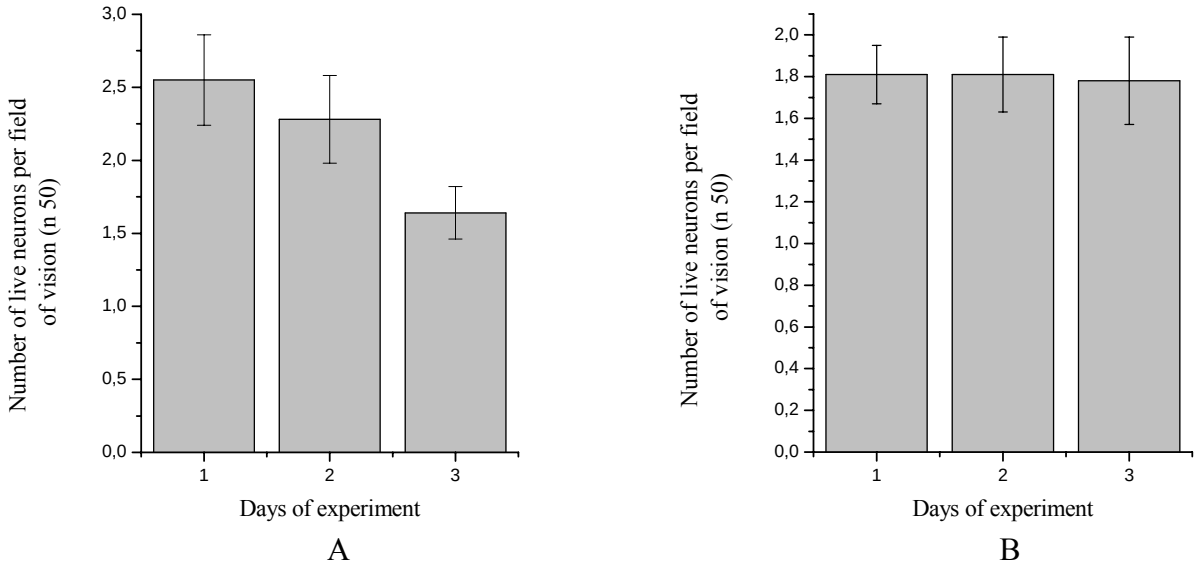

Fig. 2. Neurons survival in cultures transfected with green fluorescent protein and under the CPF applicatioin (A) and in control conditions (B)

Discussing our results, it is necessary to underline the fact that as scientists probed deeper into the activity of CPF, a wealth of information surfaced from laboratory studies about its effects on the development and function of the brain and nervous system in embryos, fetuses, and young animals. Although many of the studies were performed on rats and there are differences in the ontogeny of specific parts of the brain between rats and humans, the development of the rat brain through postnatal day (PND) 21 provides a model for the development of the human brain through to birth [12]. Investigations of neurons from rat embryos hippocampi are very interesting and important because hippocampus is the key limbic structure responsible for associative learning and memory. It is known that building of the neuronal network in the rat hippocampus starts around the birth and the two first postnatal weeks are characterised by intensive neuronal growth and synaptogenesis.

Concerning possible mechanisms of CPF neurotoxicity a number of hypotheses have the right to be in existence. For example, recently Dr Anne Caughlan from the University of Washington has showed that CPF activates the ERK 1/2 and p38 MAP kinases. Surprisingly, blocking ERK 1/2 activation by the MEK inhibitor SL327 caused a small but statistically significant inhibition of apoptosis, while blocking p38 with SB202190 significantly accelerated apoptosis induced by chlorpyrifos. This suggests a pro- and anti-apoptotic role for ERK 1/2 and p38, respectively [9]. In our previous investigations we have studied changes in activity of the antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase in hippocampus, cerebellum and cerebral cortex of growing rats subjected to low doses of CPF. We observed age-dependent and brain region-dependent changes in antioxidant enzymes activity. The data concerning effect of daily CPF intake by rats during one month at the dose 15 mg/kg on indexes of superoxidedismutase, glutathionperoxidase and catalase at the tissues of hippocampus, cerebellum and cerebral cortex were obtained. One other aspect of the problem was illuminated by these facts — CPF may induce an oxidative stress that may be one of the reasons of cell damage and finally cell death. Further work is in order to settle this and contiguous questions. Simultaneously we plan continue to study the effect of the CPF action on maturation of hippocampal pyramidal cells and interneurons *in vitro* (cell culture). This includes study of the effects of CPF on the alterations and developmental changes in the neuronal and synaptic connections properties by measuring morphometric parameters of neurons including somatical, axonal and dendritic development, using for visualization different fluorescent proteins.

Conclusions

1. Chlorpyrifos (CPF) possess the toxic influence on young hippocampal neurons in conditions of cell culture.

2. In control conditions the majority of neurons normally developed for more than 6 days after GFP transfection. By contrast, near 40 % of neurons incubated in the medium with addition of CPF (20 μM concentration) — fully degraded on the 3rd day of experiment.

Perspectives of further inquiry. It is important to explore more profoundly the mechanisms underlying the actions of CPF and its impact on the developing brain. Agree with professor Slotkin from USA, the finding of a novel set of mechanisms underlying the developmental neurotoxicity of CPF sparked a wider degree of interest in the issue of organophosphate pesticides and brain development. A definitive demonstration that CPF exerts direct effects on neurodevelopment requires control over the cellular environment, and at the same time investigations on cellular and molecular level. The main hypothesis of future investigations is that CPF neurotoxicity predominantly is independent of AchE inhibition and that CPF induces apoptosis of developmental neurons. This suggestion is based on number of recent studies performed in well-known laboratories but this important and difficult task still confronts us [4, 9].

Acknowledgements. This work was partially supported by the short term scientific fellowship declared by French Embassy in Ukraine (EGIDE 693489G). We are grateful to Dr I. Medina from the Institute of Neurobiology (INMED/INSERM, France) for advice on cell culture experiments.

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ТОКСИЧНИЙ ВПЛИВ ХЛОРПІРИФОСУ НА НЕЙРОНИ ГІПОКАМПУ *IN VITRO*

Р е з ю м е

Беручи до уваги широке використання у світі різноманітних пестицидів, дуже важливими є дослідження з метою з'ясування їх можливого впливу на центральну нервову систему. У представленій роботі *in vitro* культура клітин нейронів гіпокампу щурів була використана для того, щоб встановити чи викликає дія хлорпірифосу — одного з найпоширеніших у світі фосфорорганічних пестицидів, клітинні пошкодження і клітинну смерть. Було зроблено спробу встановити рівень токсичної дії низьких доз хлорпірифосу на молоді нейрони в умовах клітинної культури. Давно відомо, що хлорпірифос є інгібітором ацетилхолінестерази і, що висока чутливість холінестераз до фосфорорганічних інгібіторів робить ці інгібітори надзвичайно токсичними для центральної нервової системи. За останні п'ять років з'явилися деякі нововведення у питаннях, що стосуються механізмів нейротоксичності хлорпірифосу, зокрема, було неодноразово продемонстровано, що токсичність хлорпіпифосу не лімітується інгібуванням холінестерази, а може мати інші механізми дії. Наші результати показали токсичний вплив 20 μM концентрації хлорпірифосу на молоді нейрони гіпокампу в культурі клітин.

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ТОКСИЧЕСКОЕ ВЛИЯНИЕ ХЛОРПИРИФОСА НА НЕЙРОНЫ ГИППОКАМПА *IN VITRO*

А н н о т а ц и я

Учитывая широкое использование в мире разнообразных пестицидов, очень важными являются исследования с целью определения их возможного влияния на центральную нервную систему. В представленном исследовании *in vitro* культура клеток нейронов гиппокампа крыс была использована для того, чтобы установить вызывает ли действие хлорпирифоса — одного из самых распространенных в мире фосфорорганических пестицидов, клеточные повреждения и клеточную смерть. Была предпринята попытка установить уровень токсического эффекта низких доз хлорпирифоса на молодые нейроны в условиях клеточной культуры. Давно известно, что хлорпирифос является ингибитором ацетилхолинэстеразы и, что высокая чувствительность холинэстераз к фосфорорганическим ингибиторам делает эти ингибиторы в высшей степени токсичными для центральной нервной системы. За последние пять лет появились некоторые новшества в вопросах, касающихся механизмов нейротоксичности хлорпирифоса, в частности, было неоднократно продемонстрировано, что токсичность хлорпирифоса не лимитируется ингибированием холинэстеразы, а может обладать другими механизмами действия. Наши результаты

показали токсическое влияние 20 μM концентрации хлорпирифоса на молодые нейроны гиппокампа в культуре клеток.

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