NEUROTOXIC POTENTIAL OF TRICHLORFON TO MULTIPLE SUBLETHAL DOSES IN WISTAR RATS

Potencial neurotóxico del Triclorfón a dosis múltiples subletales en ratas wistar

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ABSTRACT
The organophosphates used for pest control induce sensory, motor and psychiatric disturbances after chronic exposure. The ester type is the cause of the intermediate syndrome and delayed neuropathy, in which the white and gray matter in the brain are severely affected. The aim of this study was to evaluate the effect of multiple sublethal doses of Trichlorfon on neurons, astrocytes and myelinated tissue in a rat model of brain neurotoxicity. Trichlorfon (metrifonate) was administered to adult Wistar rats at doses of 11 or 22 µg/kg by oral gavage every seven days for four or eight weeks (four experimental groups) and a control group (placebo). One week after the last dose, animals were euthanized and the brains perfused, removed and cut into coronal segments of 50 µm of thickness by using a vibratome. The sections were analyzed by immunohistochemistry, using markers of neuronal survival, astrocytic reactivity and the myelin basic protein. Neuronal and astrocytic reactivity were significantly reduced in Trichlorfon-treated animals relative to controls, whereas myelin reactivity was significantly increased, with abnormal distribution of myelin in white matter. The results suggest a neurotoxic damage of Trichlorfon on neuronal and astrocyte functional balance and abnormal myelin formation consequent to the cell damage.

Keywords: astrocytes, myelin, neurons, organophosphates, toxicology.

RESUMEN
Los organofosforados usados para control de plagas inducen trastornos sensoriales, motores y psiquiátricos por exposición crónica, siendo los de tipo éster, causa del síndrome intermedio y de la neuropatía retardada, que afectan severamente la sustancia blanca y gris del cerebro. El objetivo del presente trabajo fue evaluar el efecto del organofosforado Triclorfón sobre neuronas, astrocitos y tejido mielinizado en un modelo murino de neurotoxicidad encefálica a dosis múltiples subletales. Se suministró a ratas Wistar, triclorfón (metrifonato) a dosis de 11 y 22 µg/kg mediante sondaje esofágico, cada siete días durante cuatro y ocho semanas en cuatro grupos experimentales y un grupo control (placebo). Una semana después de la última dosis, los animales fueron sacrificados y los cerebros perfundidos, extraídos y cortados en segmentos coronales de 50 µm de grosor mediante vibratomo. Los cortes fueron analizados por inmunohistoquímica, usando marcadores de supervivencia neuronal, de reactividad astrocitaria y de la proteína base mielina. La reactividad neuronal y astrocitaria se redujo significativamente en los animales tratados con triclorfón en relación a los controles, mientras la reactividad de la mielina se incrementó significativamente, con distribución anormal en la sustancia blanca. Los resultados sugieren un daño neurotóxico del Triclorfón sobre el equilibrio
funcional neuronal y astrocitario, con formaciones anómalas de mielina consecuente al daño celular.

**Palabras clave:** astrocitos, mielina, neuronas, organofosforados, toxicología.

**INTRODUCTION**

Inappropriate use of pesticides is common in developing countries and approximately 3,000,000 cases of acute toxicosis are reported every year, with 220,000 deaths (Fenske *et al.*, 2002; Jaga and Dharmani, 2003; Barguil-Díaz *et al.*, 2012). The uncontrolled use of organophosphates (OPs) affects ecosystems and organisms that are not direct target of its action (Yavuz *et al.*, 2005; De Silva *et al.*, 2006).

OPs have been used in industry as antioxidants and plasticizers, as agriculture and household insecticides, and in the production of neurotoxic gases for warfare (Carod-Artal and Speck-Martins, 1999; Jaga and Dharmani, 2003). OPs are inhibitors of plasma and erythrocyte cholinesterase activity (Jaga and Dharmani, 2003); however, trichlorfon (TCF), chlorpyrifos (CPF) and other OPs, produce toxicity upon metabolic transformation into "oxons", which are less stable and up to three times more active as AChE inhibitors than the original compounds (Monnet-Tschudi *et al.*, 2000).

While acute toxicity of pesticides has been well documented, effects after chronic exposure are yet unknown (De Silva *et al.*, 2006). However, they have been reported to result in syndromes such as leukoencephalopathy induced by organophosphate (LEIO), delayed neurotoxicity induced by organophosphates (OPIDN) and chronic neurotoxicity induced by ester-type organophosphates (OPICN) (Carod-Artal and Speck-Martins, 1999; Abou-Donia, 2003). Kamanyire and Karalliedde (2004) describe the final stage of neuropathy caused by OPs after seven-to ten-days of exposure, which persisted by four weeks after eight weeks of continuous exposure, presumably caused by inhibition of Neuroathy Target Esterase (NTE) in a murine model of subchronic exposure (Aiouto *et al.*, 1993; Moretto and Lotti, 1998; Ray and Richards, 2001).

The aim of this study was to evaluate the effect of multiple sublethal doses of Trichlorfon on neurons, astrocytes and myelinated tissue in the brain of Wistar rats.

**MATERIALS AND METHODS**

**Animals**

Twenty male Wistar rats with an average weight of 200 ± 10 g were used. Animals, from the vivarium of the University of Tolima, were kept on dark/light cycle (12:12-h) and received food and water ad libitum. The treatments were made in the biotechnology and toxicology laboratories at the University of Tolima. The rats were handled according to Colombian standards (Law 84 of 1989), European Union guidelines (86/609/EEC) and the experiments were conducted upon approval of the Local Ethics Committee (Act No. 8 of June 2, 2010).

**Experimental Design**

The rats were distributed into four experimental groups T1, T2, T3, T4 (n = 16) with four rats per group and a control group (n = 8). Trichlorfon (Dimethylphosphonate of 2,2,2-trichloro-1-hydroxyethyl) powder 97% (Bayer ®) was prepared in solution at 1:1000 (1 mg/mL) in corn oil as vehicle. The rats of the groups T1 and T3 received a weekly dose of 11 µg/kg of TCF for four or eight weeks, respectively. The animals of the groups T2 and T4, received a weekly dose of TCF (22 µg/kg) for four and eight weeks, respectively. The calculated doses were administered by gavage and the rats of control group received the same volume of TCF-free corn oil (1 mL) via the same administration route, frequency and duration that the experimental groups. Doses were established from the acceptable daily intake (ADI Acceptable Daily Intake) reported by the WHO (Lu, 1995) for trichlorfon (ADI = 0.011 mg/kg weight) and after a previous test with 4 dose (55 - 5.5 - 0.55 and 0.055 mg/kg weight) to establish which of them did not triggered TCF cholinergic syndrome. The average lethal dose reported as LD50 in rats is 450-650 mg/kg (Karademir-Catalgol *et al.*, 2007).

**Extraction and Preparation of Brain Tissue**

Seven days after the last treatment, the animals were anesthetized with sodium pentobarbital 60 mg/kg (Penthal 6.48 %, Invet, SA) and xylazine 10 mg/kg 2 % Bayer SA) intraperitoneally (i.p.) The brains were perfused intracardially with NaCl 0.9 % N (200 mL) using aortic advance at moderate positive pressure and subsequently fixed with paraformaldehyde (PFA) 4 % (200 mL). The brains were extracted and post-fixed (PFA 4 % at 4 °C/24 hours) for subsequent cutting into 50 µm coronal sections (Vibratome 1500) and conservation in a cryopreservative.

**Immunohistochemistry**

Immunohistochemistry was carried out following the protocol described in Current Protocols in Neuroscience (Volplicelli-Daley and Levey, 2003) with modifications as follows: Inhibition of endogenous peroxidase (Methanol:PBS 1:1 - 1 % H2O2), washed with PB 0.1 M, pre-incubation (PB 0.1M - Triton 100X 3 % - BSA 1 %) for 60 minutes and incubation at 4 °C overnight in the primary antibodies (anti-NeuN A 60 µg/mL - BSA 0.02 %). The sections were put on slides, covered with cover-
slips and sealed with resinous solution (Shandon Consult-Mount®, Kalamazoo, Mi. USA). Photomicrographs were taken by using optic microscope (Motic Microscopes BA 210 NY, USA) and digital camera (Moticam 2000 2.0M Pixel, NY, USA) and the digitalized images (10X) were taken in the hippocampal CA1, internal capsule, striatum and the paraventricular zone (PVZ), then analyzed by densitometry with the Fiji-Image J software (v-1.45 - NIH). The brain sections were prepared in parallel for immunohistochemistry, so that incubation with the specific antibodies, the complex avidin / biotin and DAB, were made simultaneously for all groups in each replica. Similarly, the image capture and processing were done under the same optical parameters in all experiments to avoid biases. The setting scale, calibrate parameters, background subtracting, equal filters, homogenization and others utilities of the software, were used to calculate and measure the signal in all cases; however, some images which appear to contain background were corrected in densities for quantification in relative units by subtraction of background and thresholding to the same rank of signal detection, through use of Image J software.

**Statistical Analyzes**
The data was analyzed using descriptive statistics, by dimensional exploratory analysis, including mean, standard deviation, standard error of the mean and coefficient of variation. We used a scheme [\[Y_{ij} = \mu + t_i + e_{ij}\]], where \(\mu\) is the mean, \(t_i\) the treatment effect and \(e_{ij}\) (i) experimental error. The response variable was densitometry in relative units. Statistical analyses were carried out using ANOVA (\(p < 0.05\)) and multiple comparisons between treatment means (Tukey), previously to homogeneity of variances and normality tests. Data were analyzed using Prism 5.01 version (Graph Pad Software, Inc. 2007 California, USA).

**RESULTS**

**Effect of Trichlorfon on NeuN Immunoreactivity**
The immunoreactivity of neuronal protein NeuN was decreased in CA1 hippocampal area, paraventricular zone (PVZ) and lateral striatum body of rats exposed to 11 or 22 \(\mu g/\text{kg}\) of TCF for four or eight weeks compared to the controls (Fig. 1a). The decrease of NeuN immunoreactivity was evident at low dose (Fig. 1 - B, G, L) and high dose (Fig. 1 - D, I, N) compared to controls (Fig. 1 - E, J, O). By densitometric analysis, significant differences were observed between T3, T4 (***\(p < 0.001\)) and T2 (*\(p < 0.05\)) relative to the control group in the hippocampal CA1 area (Fig. 1b), and likewise, significant differences were obtained for T1 vs. T3 (###\(p < 0.001\)), T1 vs. T4 (##\(p < 0.001\)) and T2 vs. T3 (##\(p < 0.001\)) and between T2 and T4 (###\(p < 0.001\)) as shown in the same figure. In lateral striatum body, significant differences were observed between T1 vs. T4 (**\(p < 0.05\)) and T4 vs T2 (*\(p < 0.05\)) and T4 vs. Control (**\(p < 0.05\)) and highly significant differences between T4 and T3 (\(\pm \pm \pm \ p < 0.001\)) (Fig. 1c). In the PVZ, no significant differences between any of the treatments, neither of these with respect to control group, were observed (Fig. 1d).

**Effect of Trichlorfon on GFAP Immunoreactivity**
In the hippocampal CA1 area (Fig. 2a, AE), reactivity of the GFAP was significantly reduced in the T2, T3 and T4 groups relative to the control group (***\(p < 0.001\)). Additionally, T2 vs. T1 (***\(p < 0.001\)), T3 vs. T1 (###\(p < 0.001\)) and T4 vs. T1 (##\(p > 0.001\)) (Fig. 2b) differed significantly, while T1 did not show any change in GFAP reactivity compared with control rats. There were also significant differences between groups T4 and T2 (**\(p < 0.01\)), but T2 showed less reactivity of astrocytes. Similarly, in the lateral striatum body (Fig. 2a, FJ), treatments T2, T3, T4 showed a highly significant reduction of GFAP immunoreactivity, T2 vs. T1 (***\(p < 0.001\)), T3 vs. T1 (###\(p < 0.001\)) and T4 vs. T1 (##\(p < 0.001\)) as well as T2 vs. T4 (***\(p < 0.001\)) with highly significant differences between T3 vs. control group (**\(p < 0.01\)) and T4 vs. Control (**\(p < 0.001\)) (Fig. 2c). In PVZ also observed the effect of treatments on the reactivity of GFAP (Fig. 2a, KN) in comparison with the untreated control group (Fig. 2a O) with highly significant differences among T2 and Control (**\(p < 0.01\)) and T3 - T4 vs. control (**\(p < 0.001\)) (Fig. 2d). Moreover, T2 significantly reduced the astrocytic reactivity compared with T1 (***\(p < 0.001\)), likewise T3 vs. T1 (###\(p < 0.001\)), while T4 showed a highly significant reduction in GFAP immunoreactivity (Fig. 2d) compared to T1 (##\(p < 0.001\)).

**Effect of Trichlorfon on Mature Myelin (Mb) in Cerebral White Matter**
In the lateral striatum body there was a significant increase in MBP reactivity in T2 (**\(p < 0.05\)) and a highly significant increase in T3 (***\(p < 0.001\)) (Fig. 3a-C) and T4 (***\(p < 0.001\)) (Fig. 3a-D) with respect to control group (Fig. 3a-E), particularly, the T4 evidenced greater reactivity than all other treatment groups and relative to the control (Fig. 3b). In the internal capsule, there was a significant increase in the protein MBP reactivity of T1 vs. control (***\(p < 0.001\)) and a highly significant increase in T2, T3 and T4 with respect to control group (**\(p < 0.001\)) as shown in Figure 3c. In addition, changes in the distribution of MBP immunolabeling in all treated groups T1, T2, T3 and even more markedly in T4 compared to control (Fig. 3a-I) with differences highly significant (##\(p < 0.01\)) compared with T1 (F and 3a-3c).

**DISCUSSION**
The trichlorfon increases acute toxicity by dearylation metabolic reactions, desulfurization and alkylation (Flaskos, 2012) with the consequent generation of metabolites highly harmful to the organism. The OPs inactivate the AChE by...
Figure 1. Representative coronal sections of the NeuN immunoreactivity in different brain regions of rats exposed to multiple doses of TCF. Hippocampal CA1 area (Fig. 1a, A-E, 1b), PVZ (Fig. 1a, F-J, 1d) and lateral striatum body (Fig. 1a, K-O, 1c). T1: TCF 11 µg/kg each week for four weeks (A, F, K), T2: TCF 22 µg/kg each week for four weeks (B, G, L), T3: TCF 11 µg/kg each week for eight weeks (C, H, M) and T4: TCF 22 µg/kg each week for eight weeks (D, I, N) in comparison with the control group CTR (E, J, O). NeuN marking decreased in a dose- and time of exposure-dependent way. n= 16 (10X). Values are expressed in units of relative density. Scale bar 100 µ.
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phosphorylation of the serine hydroxyl group (Aluigi et al., 2005) and directly interact with other molecules such as membrane channels, molecular receptors and neurotransmitters producing structural and functional cellular changes that interfere with neurotransmission (Yousefpour et al., 2006). Due to the inhibition of AChE, trichlorfon has been
used as a therapeutic agent against Alzheimer disease (Liu et al., 2002; Becker et al., 2009), nevertheless, some studies aware about the toxicity of this therapy including the inhibition of other enzymes affected by the organophosphate class of drugs, by tolerance of low doses allowing too rapid dose escalation and irreversible enzyme inhibition producing cumulative drug effects (Rakonczay, 2003; López-Arrieta and Schneider, 2006; Becker and Greig, 2008; Becker et al., 2009; Becker and Greig, 2010).

**Effect of TCF on Neuronal and Astrocytic Population**

Several studies have demonstrated the deleterious potential of OPs on cell populations (Pohanka et al., 2011) including neurons and glial cells (Carlson et al., 2000; Yousefpour et al., 2006; Flaskos et al., 2007, Liu et al., 2009). Other studies have shown that metabolites derived from Trichlorfon, like dichlorvos, can induce a neurotoxic effect even greater than the starting compound. In addition, chronic exposure to trichlorfon affects the cerebral glucose metabolism and may induce an acidosis state (Poindessous-Jazat et al., 1998; Liu et al., 2009). The exposure to trichlorfon decreases the neuronal and glial viability in a dose-dependent manner (Liu et al., 2009), which agree with the findings of the present study, that evidenced a decrease in immunoreactivity of neurons and astrocytes in CA1 hippocampus and lateral striatum body; but in PVZ, just astrocyte marker was reduced. It has been reported that the decrease in these cell populations is an apoptosis-mediated processes with the high toxic effect attributed to the secondary metabolite dichlorvos (Carlson et al., 2000; Liu et al., 2009).
The OPs induce oxidative stress (Kaur et al., 2007) and cell death in animals exposed to trichlorfon or its oxon metabolites (Guizzetti et al., 2005). The overproduction of free radicals involved in the glial activation are typical in clinical progress of neurodegenerative diseases by exposure to toxic (Astiz et al., 2012) and this can result in apoptotic cell death, which is consistent with a marked reduction in the expression of neuronal protein immunoreactivity. Several studies have been shown the involvement of astrocytes in neuroprotection and neurorepair of nervous tissue after exposure to the toxins, at blood-brain barrier (BBB) level (Giordano et al., 2008; Sofroniew and Vinters, 2010). It has been described that sublethal doses of OP decrease protein markers for astrocytes (Garcia et al., 2002) and the total number of glial cells (Roy et al., 2004), that is compatible with this study, where the GFAP protein immunoreactivity was found significantly reduced in the hippocampus, lateral striatum body and PVZ in all treated groups at both doses of TCF (11 and 22 µg/kg). Similarly, exposure to the oxon forms, such as chlorpyrifos oxon, significantly decreases the glutamine synthetase activity (a marker for astrocytes). This effect may be mediated by direct toxicity on astrocytic cells and can directly interfere with the cellular replication (Qiao et al., 2001; Flaskos, 2012).

The TCF and its metabolite dichlorvos easily cross the BBB and inhibit both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Pohanka et al., 2011). In the present study, the decreased immunoreactivity of GFAP in the para-ventricular area, which corresponds to a border area in close contact with the ventricles, the BBB can be seriously compromised by the decrease in the population of astrocytes. Parran et al., (2005) and Song et al., (2004) showed that exposure to OPs affects the integrity of the BBB and alters their functionality.

**Effect of TCF on Myelination Status**

In this study, the reactivity of the MBP protein was increased by TCF in a dose and time dependent manner with higher reactivity to 22 µg/kg only after eight weeks of exposure. This increase in MBP reactivity may be indicative of myelination or remyelination processes resultant to tissue damage induced by the OP, which contrasts with that reported by Flaskos (2012) who found a decrease in cyclic nucleotide phosphohydrolase (a marker for oligodendrocytes). Exposure to OPs alters cytoskeleton-associated neurofilaments leading to the destruction, which in turn leads to destruction of the axon as well as myelin sheaths. This process also seems to be mediated by increased levels of intracellular calcium (Abou-Donia, 1993; Song et al., 2009). Also, it has been described that OPs, particularly oxon-type, can produce a disruption of neuronal processes by detriment of growth factors and inhibition of its receptors, inducing cell death. At the ultrastructural level, mitochondrial dilation, disruption of rough endoplasmic reticulum, production of lysosomal lipid vacuoles, neuronal degeneration and necrosis, and intracytoplasmic myelin forms (Yousefpour et al., 2006).

In another study related with the *in vitro* effect of Chlorpyrifos (CPF) on glia, it was established that elicited disruption in glial development. Furthermore, since astrocytes and oligodendrocytes (the myelin-forming cells) arise from one common glial precursor, myelination can be affected by chlorpyrifos (Garcia et al., 2002), suggesting that exposure to the CPF is related to the reduction in the levels of myelin associated glycoprotein (MAG) mRNA, inhibition of DNA synthesis in undifferentiated oligodendrocytes and astrocytes as well as alterations in the expression of GFAP and MBP (Saulsbury et al., 2009).

The hyperreactivity of MBP in the present study after TCF exposure shows not only an increase in MBP marker density, but a distribution of abnormal forms in comparison with the control group. Because MBP protein was evaluated as a marker of mature myelin in white matter-rich areas (internal capsule and striatum body) where there is also a large population of radiated astrocytes, is possible that these hyperreactive forms may be involved in the proliferation of oligodendrocytes or astrocytes within a tissue repair process or remyelination. Seems that glial cells, astrocytes and oligodendrocytes, are essential for neuronal differentiation, myelination, the propagation of synaptic impulses and the maintenance of homeostasis (Saulsbury et al., 2009), thus any disruption of these cells can lead to serious functional disturbances.

In mice mutant for the gene encoding the synthesis of GFAP, abnormal myelination in white matter, suggest the involvement of GFAP as a link between astrocytic function and myelination. GFAP and MBP have been used as biomarkers in glial alterations, suggesting that the effect on glia may contribute to the late onset of neuronal damage (Roy et al., 2004) and can be used as biomarkers of neurotoxicity induced by OPs. Although clinical signs in patients with leukoencephalopathy induced by organophosphate (LEIO), delayed neurotoxicity induced by organophosphates (OPIDN) and chronic neurotoxicity induced by ester-type organophosphates has been reported, we have not observed any clinical signs, probably for low doses used in this investigation; maybe it is a dose dependent effect. In the other hand, the aim of this study was to evaluate the effect of Trichlorfon on neurons, astrocytes and myelinated tissue in a rat model of brain neurotoxicity to multiple and sublethal doses which may help to elucidate the cellular effects that accompany referring syndromes in humans and can help in the research of therapeutic drugs and pathophysiological study of neurological diseases by pesticide and other toxic chemicals.

**CONCLUSIONS**

Our results indicate that TCF exposure to low sublethal doses of 11 and 22 µg/kg for four and eight weeks in Wistar rats, was sufficient to generate cell damage in neurons and astrocytes in the hippocampus (CA1), striatum and PVZ, while...
MBP hyperreactivity in white matter with anomalous shapes and changes in the distribution of mature myelin, suggest modification in the remyelination process subsequent to injury by TCF. Anti-NeuN, anti-GFAP and anti-MBP antibodies are proposed as sensitive neurotoxicity biomarkers of OP oxon type exposure or by chronic toxicity to these.

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