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Anticholinesterasic Activity of Endosulfan in Wistar Rats

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Abstract The in vivo and in vitro effects of the pesticide endosulfan on the cholinesterase (ChE) activity were investigated in rats. ChE activity decreased in dams and in male pups within 65 days corresponding to 35% and 32% of inhibition respectively in the higher endosulfan dose (1.5 mg/kg). In vitro, the enzyme activity was found to be inhibited in a concentration dependent manner. The results suggest that endosulfan is able to inhibit the ChE activity and to cross the placental barrier and/or to be eliminated through milk affecting the enzyme activity in male rat pups.

Keywords Endosulfan · Cholinesterase · Offspring Wistar rats · Lactation period · Smooth muscle

The South American country of Brazil is home to extensive agricultural areas, which are used to grow food as well as materials for fibre production. Most of the cropping systems employed involve the regular usage of pesticides. These products, such as insecticides, are commonly used, not only in agriculture, but also in commercial industry and in private households. Some studies show that these chemicals have become so pervasive in Brazilian society, that accidents are now considered commonplace. Acute and chronic intoxication due to accidental contact with insecticides have become very frequent and reflect the dramatic increase in the use of these toxicants. Moreover, new pesticide compounds are frequently being introduced into this expanding market. The organochlorine endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzo-dioxathiepin-3-oxide), is no longer produced in the United States, however it has been detected in both fruits and vegetables (Araújo et al. 1999) as well as commercial milk (Ciscato et al. 2002), human placenta, and breast milk (Carreno et al. 2007; Shen et al. 2007). Endosulfan exposure has been shown to affect both the reproductive and endocrine systems of experimental animals and humans (Dalsenter et al. 2003). Impacts on the central nervous system, manifested as changes in normal behavioral, locomotory and neurotransmitter activity as well as the induction of seizures, have also been demonstrated (Zhenquan and Hara 2007).

While organophosphorus and carbamate compounds are the most widely known cholinesterase inhibitors, other compounds, such as heavy metals and detergents (Payne et al. 1996), have been identified as inhibitors in various species (Dutta and Arends 2003). Endosulfan is another compound that is being scrutinized for cholinesterase inhibiting properties. Gupta (1976) found a decrease in brain ChE activity in rats exposed to a single intraperitoneal injection of endosulfan. Naqvi and Vaishnavi (1993) showed an inhibition of cerebral and plasmatic cholinesterase in rats exposed to a dose of 4.6 and 27.2 mg/kg for 13 weeks.

The inhibition of ChE in experimental animals due to endosulfan exposure is still a debated topic in primary literature. The present study was designed to verify the possible transplacental passage of endosulfan and the potential repercussions on ChE activity.

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Materials and Methods

Endosulfan (CAS number: 115-29-7) was purchased from Defensa S.A. (Taquari, Brazil). The purity of the substance was 97%. Primigravidae female and male Wistar rats of the same age (90 days) were maintained under specific conditions on a constant day/night cycle (light from 7:00 am to 7:00 pm) at a room temperature of approximately 22°C. They received standard pellet food (Nuvital®, Curitiba/PR) and tap water was administered ad libitum for drinking. All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Female rats (n = 27) were randomly assigned to three groups, one as a vehicle control (sunflower oil), while the other two groups were treated with two different doses of endosulfan. The substance was dissolved in sunflower oil and administered to the dams orally by gavage in a volume of 5 mL/kg BW. The dams were treated daily with an oral administration of 0, 0.5 or 1.5 mg of endosulfan/kg starting 21 days prior to mating and continuing throughout the periods of mating, pregnancy, and lactation. The doses were chosen based on the maternal nontoxic dose and on the NOEL (no observed effect level) of 1.5 mg/kg for reproduction established by the WHO (1984). Endpoints were evaluated in the male pups of the treated dams.

The body weights of the dams were measured on a daily basis during the treatment period. Variables including litter size, birth weight, weaning rate, and weight on postnatal day 21 were also assessed. The male pups were randomly selected from each litter (2 pups/litter) that had been exposed to endosulfan during the gestational and lactational periods. The pups were weighed (one each) on postnatal days 65 and 140, representing puberty and adulthood, and were subsequently euthanized by decapitation. The liver and kidneys were removed and the absolute and relative weights were determined.

For the cholinesterase assay, the dams were euthanized on day 21 of lactation and their blood was collected in a test tube containing heparin, centrifuged at 2,000 × *g* for 5 min. This same method was used to collect blood from the pups euthanized on days 65 and 140. After centrifugation, for the samples from both the dams and pups, the plasma was frozen until analyses were performed. The cholinesterase activity was measured spectrophotometrically according to Ellman et al. (1961) and Evans and Wroe (1978), modified for microplate. The tested samples were diluted (1:100) in phosphate buffer 0.1 M pH 7.5. A negative and a positive sample (plasma exposed to an organophosphate compound) were also measured. Propionylthiocholine iodide 30 mM was used as a substrate and 5,5′-dithio-bis (2-nitrobenzoic) acid (DTNB), 0.75 mM as a color reagent. The optical density at 415 nm was measured during 5 min using an ELISA plate reader (TECAN). ChE activity was expressed in KU/L (degradation of 1 mol of substrate/plasma liter).

For the in vitro test, segments of jejunum (1–1.5 cm long) were removed from the abdomen of the rats and were dissected free of mesentery and fat. The lumen was flushed with Tyrode solution (mM: NaCl 136.9, KCl 2.68, $CaCl_2$ 1.8, $MgCl_2$ 0.53, NaH_2PO_4 0.33, $NaHCO_3$ 11.9, D-glucose 5.6). The organs were set up in a 10-mL chamber, bathed in Tyrode solution at 37°C, and aerated. A tension consisting of 1.0 g was applied and isometric recordings were obtained using a Kymograph. Blood from the femoral artery was collected in a test tube containing heparin (50 μL heparin for 4 mL of blood) and centrifuged to separate plasma. An acclimation period of 30 min ensued, during which the physiological solution was changed every 15 min. After this period a cumulative dose–response curve for acetylcholine (ACh) (1 nM to 1 mM) was recorded. The EC50 was determined for each experiment and two consecutive responses to ACh EC50 were recorded for 90 s at 30-min intervals. In order to analyze the cholinesterase (ChE) activity in vitro, 0.4 mL plasma was incubated with twice the volume of ACh EC50 at room temperature for 5 min. Subsequently, the volume of the mixture was adjusted to 1 mL with saline solution. Half of this solution (0.5 mL) was administered in the bath and the effect was recorded for 90 s. The tissue was washed every 15 min for 30 min. To evaluate the anticholinesterase activity, endosulfan was prepared at a concentration of 400 mg/mL. It was dissolved in 300 μL of DMSO and a volume of 0.4 mL plasma was incubated with 50, 100, and 150 μL of the endosulfan solution, twice the volume of ACh EC50, and the solution was incubated at room temperature for 5 min. After this incubation period, an experimental procedure similar to the previous one was used. The concentrations of 1, 2, and 3 mg/mL of endosulfan were obtained. The tissue was washed every 15 min for 1 h, the acetylcholine cumulative dose–response curve was recorded and the endosulfan reversion effect was tested.

The data were expressed as mean and standard error of the mean (M ± SEM) and analyzed by analysis of variance (ANOVA) followed by the Bonferroni test. Differences were considered to be statistically significant when $p < 0.05$.

Results and Discussion

A reduction in the maternal body weight of the dams exposed to endosulfan during the periods of pregnancy and lactation was not observed (data not presented), indicating that these doses did not induce maternal toxicity. The body weight of the rat pups exposed to 1.5 mg/kg of endosulfan

was higher at birth, but at weaning on postnatal day 21, they weighed significantly less than the control pups. However, the low body weight did not interfere with the normal development of the pups. No significant difference was observed with the administration of 0.5 mg/kg of endosulfan (Table 1). The body weight and relative organ weights of the pups treated with 0.5 and 1.5 mg/kg of endosulfan did not differ significantly from those of the control group (Table 2). Although endosulfan is metabolized in the liver and one of these metabolites is approximately three times as toxic as the beta-isomer, neither the induction of microsomal enzymes nor histopathological effects have been observed in most chronic experiments. In other studies, examining the kidneys, histopathological alterations such as dilatation of renal tubules with degeneration of epithelium have been noted (Singh and Pandey 1989), however the relative kidney weight was not affected.

Table 1 Pregnancy outcome of dams

Parameter	Control	Endosulfan (mg/kg)	
		0.5	1.5
Litters (n)	9	8	10
Postnatal day 1			
Live births (%)	100	100	100
Litter size (n)	9.8 ± 0.73	10.1 ± 0.35	10.1 ± 0.17
Birth weight (g)	5.8 ± 0.08	5.8 ± 0.09	6.1 ± 0.07**
Postnatal day 21			
Weaning rate (%)	98.3	97.6	97.2
Body weight (g)	32.0 ± 0.50	31.9 ± 0.36	30.0 ± 0.72*

The litters were used as statistical unit

Data are presented as means ± SEM

* $p < 0.05$; ** $p < 0.01$ compared to control group (ANOVA, Bonferroni test)

Table 2 The body weight and relative organ weights of the male rat pups

Variables	Day	Endosulfan (mg/kg)		
		0	0.5	1.5
Body weight (g)	65	254 ± 10	253 ± 5.6	255 ± 8.3
	140	362 ± 6.0	345 ± 5.3	367 ± 7.6
Relative liver weight (%)	65	4.82 ± 0.16	4.64 ± 0.10	4.45 ± 0.07
	140	4.05 ± 0.06	4.00 ± 0.09	3.98 ± 0.09
Relative kidney weight (%)	65	0.42 ± 0.01	0.42 ± 0.01	0.41 ± 0.01
	140	0.35 ± 0.01	0.37 ± 0.01	0.35 ± 0.01

Eight animals/group/day were investigated at postnatal days 65 and 140

Data are presented as means ± SEM

$p > 0.05$ – no statistically significant (ANOVA, Bonferroni test)

The ChE activity decreased significantly only in dams treated with 1.5 mg/kg of endosulfan (Fig. 1). In the male pups, an inhibition of cholinesterase on Day 65 was observed at high doses of endosulfan; this effect was not observed at day 140 (Fig. 2). Endosulfan induced an inhibition of cholinesterase activity in both dams (35%) and pups (32%) on day 65 at the higher dose. This anticholinesterasic effect of endosulfan may be due to an ability to cross the placental barrier and/or to be passed on to the pups through lactation. This has serious implications for human health as endosulfan residues have been detected in both raw and pasteurized bovine milk (Ciscato et al. 2002) as well as human breast milk (Sanghi et al. 2003; Carreno et al. 2007). Kathpal et al. (2001) reported that 15 out of 100 bovine milk samples collected from different locations in India contained endosulfan residues, the concentrations of which were above the maximum residue limit (MRL). Nag and Raikwar (2007) also reported that of 83 bovine milk samples collected from locations in the Bundelkhand region of India, 26 were contaminated with endosulfan. Cerrillo et al. (2005) found concentrations of endosulfan metabolites in human milk. Botella et al. (2004)

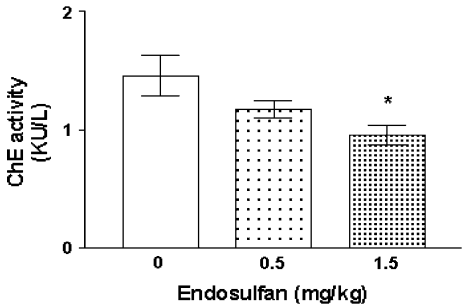

Fig. 1 Cholinesterase activity in dams treated with 0 (N = 9), 0.5 (N = 10), and 1.5 mg (N = 8) endosulfan/kg. *$p < 0.05$ compared to control group (ANOVA, Bonferroni test)

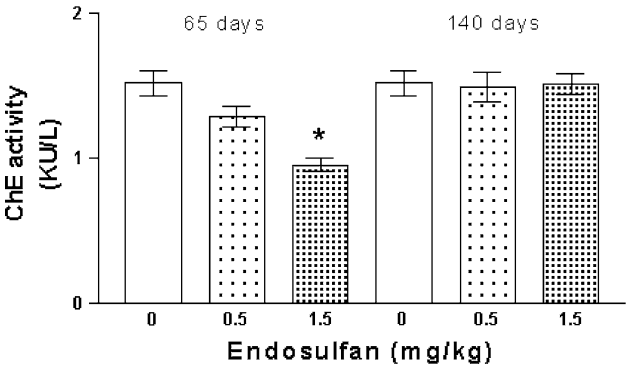

Fig. 2 Cholinesterase activity at postnatal days 65 and 140 in male pups (N = 8/group/day) exposed during gestation and lactation periods to 0, 0.5, and 1.5 mg endosulfan/kg. *$p < 0.05$ compared to control group (ANOVA, Bonferroni test)

investigated the distribution of parent compounds and metabolites in the fat and serum of humans and reported that the distribution of endosulfan metabolite residues depended upon their lipophilicity. Although this research did not focus on the nervous system, previous studies have reported that its development can be affected due to endosulfan exposure. In mammalian species, there is a perinatal period during which the brain undergoes rapid growth. In humans, this period begins during the third trimester and continues for the next 2 years of life (Eriksson 1997). During this time, the brain undergoes rapid growth and forms structures, which will become permanent, however it lacks defensive mechanisms. It is assumed that chemicals may move freely to the perinatal brain due to the incomplete development of the blood–brain barrier (NRC 1999). Most importantly, exposure of adults to endosulfan will often result in short-term symptoms, whereas perinatal exposure can engender more long-term and insidious effects. Previous studies have shown the inhibition of cholinesterase activity in rats exposed to endosulfan (Naqvi and Vaishnavi 1993) while others have demonstrated inhibition of the activity in both fishes (Dutta and Arends 2003) and prawns (Bhavan and Geraldine 2001). However, no prior study has examined the rat pups ChE inhibition after exposure during pregnancy and lactation.

Pushpanjali et al. (2005) also found an inhibition of ChE activity, indicating that endosulfan, similar to organophosphorus compounds, is a potent neurotoxic compound to embryonated chicks. The exact mechanism by which endosulfan, an organochlorine, inhibits ChE is not known. In the in vitro assay, an isometric contraction of rat jejunum was induced by acetylcholine (ACh EC50), before and after plasma incubation, in the absence and in the presence of 1, 2, and 3 mg/mL of endosulfan (Fig. 3). Results were analyzed based on the EC50 of ACh response (7.89×10^{-7} with confidence Intervals 6.536×10^{-7} to 9.526×10^{-7}). The three tested concentrations of endosulfan, 1, 2 or 3 mg/mL, inhibited the plasmatic cholinesterase activity by 0.3 ± 0.3 (0.6%), 23.5 ± 5.1 (46%) and 42 ± 3.07 mm (80.9%) respectively. The smooth muscle contraction, which was inhibited by the plasmatic cholinesterase, was induced by EC50 of ACh (51.4 ± 0.7 mm), and the inhibitory action was almost totally nullified in the presence of the endosulfan (percentile of the response in the presence of endosulfan compared with ACh EC50). Endosulfan prevented the degradation of acetylcholine by the plasmatic cholinesterase, suggesting an anticholinesterasic activity. The rat plasma cholinesterase was inhibited in a concentration-dependent manner.

These results confirm the inhibition of the enzymatic activity in in vivo experiments, indicating that endosulfan is able to affect cholinesterase activity. Further, it has been demonstrated that male rat pups may be affected due to exposure during pregnancy and lactation.

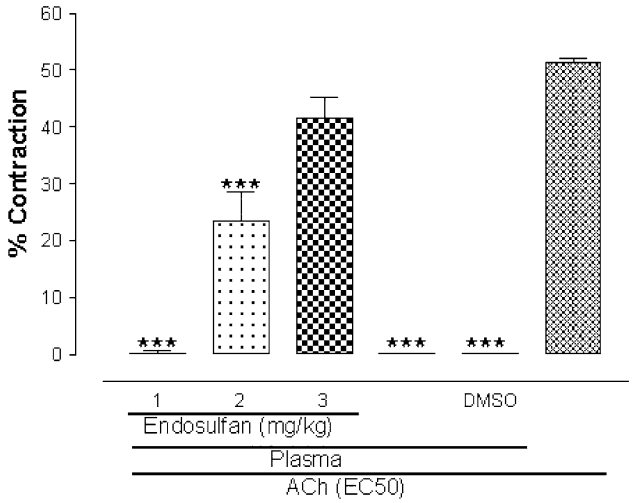

Fig. 3 Anticholinesterase activity of endosulfan (1, 2 and 3 mg/mL). This result was determined in vitro, through the effect on the contraction of the jejunum induced by acetylcholine (*ACh*) in the absence or presence of plasma (N = 6). ***$p < 0.001$ compared to EC50 group values (ANOVA, Bonferroni test)

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