Effects of endosulfan sublethal concentrations on carp (*Cyprinus carpio*, Linnaeus, 1758): Morphometric, hystologic, ultrastructural analyses and cholinesterase activity evaluation

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Abstract

Endosulfan, an organochlorine pesticide, has been banned by most developed countries, although it is still produced, sold and used in developing countries. Used for control in crops, as well as for insect control in public health programs in some countries, its effects on the environment and its toxicity are still in discussion. For some researchers, its bioaccumulation in terrestrial organisms is considered irrelevant but for aquatic life it should be considered carefully. The present research work was to carry out an study on the effects of sublethal concentrations of endosulfan on the fresh water fish carp (*Cyprinus carpio,* Linnaeus, 1758). The fishes were exposed during 15 days to 0.001 mg/L of endosulfan using dimethylsulfoxide 0.1% (DMSO) as solvent. The acetylcholinesterase activity on the brain and axial muscle, as well as liver morphometric, histopathologic and ultrastructural analysis were studied. The hepatic somatic index and the livers weight showed smaller values when compared with the control groups, besides being also observed histopathological and ultrastructural alterations. It has not been observed significant alterations in the cholinesterase activity of both brain and striated muscle. These results suggest that the organochloride endosulfan caused toxic effects in the hepatic metabolism of the fish exposed to it in sub lethal doses.

Key words:
Endosulfan.
Fish.
Cyprinus carpio.
Cholinesterase.
Ultrastructural analyse.

Introduction

Persistent organic contaminants are compounds that because of their inner physicochemical properties are resistant in variable degrees to photochemical, chemical and biochemical degradation. Displaying considerable environmental mobility, those compounds accumulate in various species, their effects being magnified through the trophic chains.[1,2] In Brazil, endosulfan is being registered and liberated to be used only in soybean, cacao, cotton and coffee cultures. In spite of this fact, endosulfan is being found in aquatic environments as lakes and rivers, in fruits and legumes as well as in samples of pasteurized milk from State of São Paulo supermarkets.[3,4,5]

Considering that it is very scarce the knowledge on the endosulfan effects in regard to the environment and on its mechanism of action on both aquatic and terrestrial organisms [6], the main aim of the present research work was to carry out an experimental study on the hepatic tissue morphological and morphometric

alterations as well as on the brain and axial muscle acetylcholinesterase activity as effects of sublethal concentrations of endosulfan on the fresh water fish *Cyprinus carpio*.

Material And Methods

The experiments were conducted with carp (*Cyprinus carpio*) a fresh water fish of the grass variety. The fish used for the experiment were nearly 5 months old and were sexual immature, measuring from six to thirteen cm long.

Acclimatization

The specimens of fish used in the present experiment were raised at Project Tilapia, a pisciculture station from Toledo, State of Paraná, packed in plastic bags with 30% water and inflated with oxygen and transported to the Department of Pathology, Faculty of Veterinary Medicine and Zootechny, University of São Paulo (USP) and to the Laboratory of Environmental Toxicology, University Federal of Paraná, where they were subjected to a three weeks period of acclimatizing before the beginning of the experiments. The fish were maintained at 25°C ± 1°C in aquarium with 200 L of deionized water, under continuous aeration at a photoperiod of 11 hours day/13 hour's night, with the water pH from 6.0 to 7.3. The fish were fed once a day, in the morning, with ALCONÒ ration.

Determination of endosulfan CL_{50} - 96 hours

For the determination of the endosulfan CL_{50}, 16 groups of 5 fishes each were transferred to 20 L aquaria and maintained for 2 days period of adaptation in the same conditions as above mentioned except for the feeding that was suspended 24 hours before the beginning of the experiment. For the 96 hours acute toxicity experiment, 5 different logarithmic concentrations of endosulfan - 0.001 mg/L, 0.002 mg/L, 0.003 mg/L, 0.01mg/L 0.02 mg/L were used. Two control groups of fish were also established, one with water only and one with a 0.1% solution of dimethylsulfoxide (DMSO), the usual solvent for endosulfan.

Sublethal exposition to endosulfan

In order to study the possible effect on *Cyprinus carpio* displayed by endosulfan sublethal exposition, six groups of 35 fishes each were maintained in 80 L aquaria containing 0.001mg/L of this xenobiotic compound. After the period of incubation of 15 days, the fish was weighed, the standard length measured and then they were sacrificed by medullar transection. A total of 15 livers from each group were then dissected and subjected to fixation for histological and ultra structural studies. For the determination of acethylcolinesterase activity, axial muscle and brain were also collected and kept frozen.

Morphometric evaluation

Morphometric evaluation liver has been carried out according to Weibel et al.[7], as the ratio between the fish body and the liver weights, expressing the Liver Somatic Index (LSI) and Conditional Factor (CF) as follows:

LSI = liver weight (g) X 100/ body weight (g)

C F = 100 X body weight (g)/ Body length (cm)

Histological analysis

After 5 hours step of fixation by means of Carnoy solution, 4 livers were subjected to dehydration in crescent series of ethanol, turned diaphanous with xilene and then embedded and included in paraffin and sliced in 2 to 4 m-m slices, the resulting slides being colored by hematoxylin eosin dyes. After this step, the slides were mounted with synthetic resin and photographed at an optical microscope.

Transmission Electronic Microscopy

Livers were fixed for 24 hours by Karnowsky solution and then washed 5 minutes three times with 0,2M cacodylate

buffer. The post fixation step was carried out with osmiun tetraoxyde for 30 minutes and the inclusion of the samples performed in araldite epoxy resin containing capsules. For the resin polymerization the capsules were maintained for about 24 hours in an oven at 60^0C. After this step the blocks were sliced in nm slices by means of an ultramicrotome, the contrast of the tissues and cells structures being given by means of the uranil citrate solution and the Reynold solution. Photomicrographies from all the samples were carried out by means of a Zeiss electronic microscopy.

All this procedure has been carried out at the Laboratory of Electronic Microscopy, Department of Pathology, Faculty of Veterinary Medicine and Zootechny, University of São Paulo (USP).

Cholinesterase activity

For the determination of cholinesterase activity preparations of carp axial muscle and brain were carried out as follows. Fragments of 150 to 250 mg of the carp axial muscle were added with 2 ml of 0.1M phosphate buffer, pH 7.4, homogenized in a Potter-Ehlvehjem homogenizer and spun down 10 minutes at 10,000 g at 4º C in an Eppendorf 5403 centrifuge, the supernatant being collected for enzymatic analysis. Brains from 60 specimens of fish from each group, weighing between 0.021 and 0.035 g each were excised and washed out from the excess of hemoglobin with 0.1M pH 7.4 phosphate buffer and then added with 2 ml of the same buffer and homogenized in a Potter-Elvehjem homogenizer, spun down 10 minutes at 10,000 g in an Eppendorf 5403 centrifuge, the supernatant then collected for enzymatic analysis.

Cholinesterase activity was assayed by the method of Ellman et al.[8], according to Sturm, Silva de Assis and Hansen[9], by the spectrophotometric determination of the color reaction developed by DTNB 5,5'-dithiobisnitrobenzoate and thiuocholine. Determination of protein concentration was carried out according to the method of Bradford[10].

Statistical analysis

For the determination of the endosulfan CL_{50} statistical analysis was carried out by the method of Trimmed Sperman-Karber [11]. Morphometric analysis and enzyme activity were evaluated by ANOVA, and the Tukey test ($p < 0.05$).

Results

Endosulfan lethal concentration

The endosulfan lethal concentration-CL_{50} - has been established in 0.002mg/L. In spite of the fact that this concentration of endosulfan did not cause the death of the fish during the experiments, it was observed that the fish exposed to sublethal concentration of endosulfan of 0.001mg/L for 15 days, displayed hyperactivity with rapid swimming movements in regard to the fish of both the control and DMSO groups.

Morphometric analysis

According to the table 1 results, no significant differences were found between control, DMSO and endosulfan groups of fish in regard to the body weight, body length and the conditional factor (CF). However, a significant difference ($p < 0.001$) has been observed between the liver weight of the control group of fish and the endosulfan treated group of fish. In regard to the liver somatic index (LSI) no significant difference has been found between the control group of fish and the DMSO treated ones. However, a significant reduction ($p < 0.001$) has been displayed between the endosulfan treated group of fish and the control one.

Morphological and ultra structural alterations

The structural organization of the liver from the control group fish (Figure 1) and the 0.1% solution of DMSO treated livers from the experimental group fish (Figure 2) possessed an hexagonal shaped lobule with the hepatoxytes displaying arrangements around the central vein and, the glycogen being distributed in an homogeneous way

Table 1 - Morphometric analysis of the fish *Cyprinus carpio* from the control group and from the DMSO and endosulfan groups

	Control	DMSO	Endosulfan
Body weight (g)	2,22 ± 0,08	2,18 ± 0,06	2,03± 0,07
Body length (cm)	5,93 ± 0,07	6,0 ± 0	6,05 ± 0,11
Conditional factor (FC)	37,34± 1,19	36,45± 1,08	33,51± 0,8
Liver weight (g)	0,065± 0,006	0,043± 0,008 *	0,021± 0,003* *
Liver somatic Index (LSI)	2,964± 0,28	2,008 ± 0,44	1,050 ± 0,17* *

The data shown are in regard to the average and standard error. ANOVA followed by the test of Tukey (*p< 0,05; **p < 0,01)

along the hepatic tissue (Figures 4 and 5).

At the liver of the endosulfan treated fish at the concentration of 0.001 mg/L (Figure 3), the hepatic lobule displayed some lost of its hexagonal conformation and of the cells delimitation, the synusoidal spaces being not very well defined, the cells being more agglomerated. Furthermore, it has observed a depletion of the hepatic glycogen (Figure 6) and an increase in the amount of the endoplasmic reticulum (Figure 7).

Levels of acetylcholinesterase

Levels of acetylcholinesterase activity

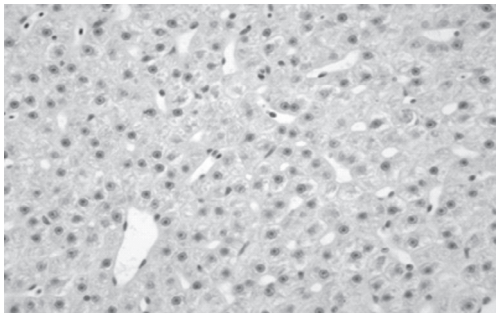

Figure 1 - Hystological aspect of the carp liver from the control group. (Colour: H.E; magnifying; 165x)

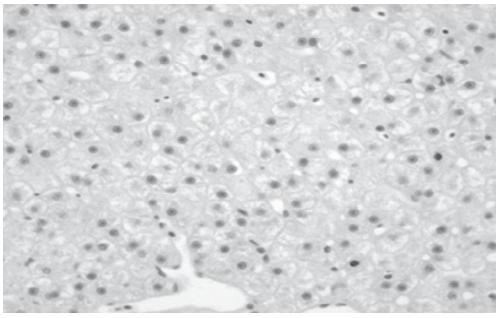

Figure 2 - Hystological aspect of the carp liver from the experimental group of fish exposed to 0.1% DMSO. (Colour: H.E.; magnifying: 165x)

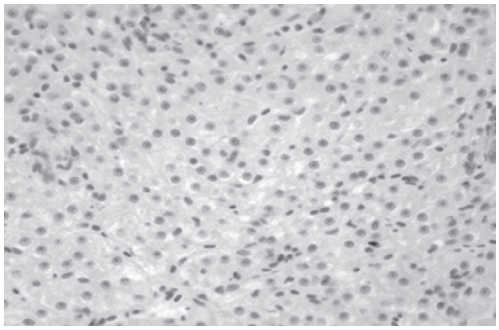

Figure 3 - Hystological aspect of the carp liver from the experimental group of fish exposed to endosulfan in the concentration of 0.001mg/L. (Colour: H.E.; magnifying: 165x)

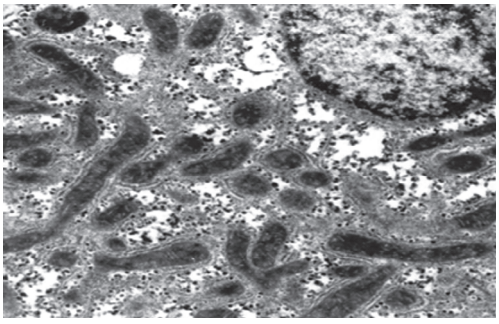

Figure 4 - Photomicrograph of the carp liver from the control group of fish (magnifying: 14,000 x)

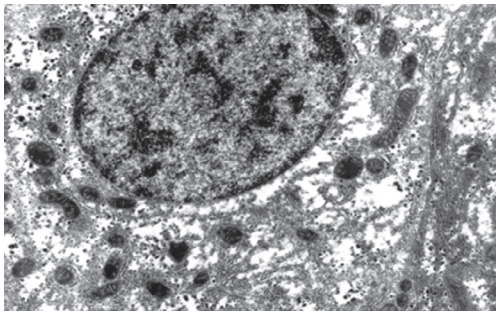

Figure 5 - Photomicrograph of the carp liver from the DMSO treated experimental group of fish (magnifying: 14,000 x)

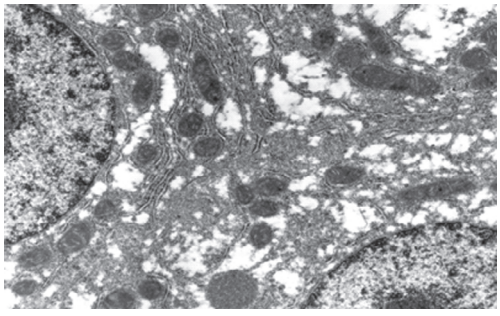

Figure 6 - Photomicrograph of the carp liver from the endosulfan experimental group of fish (magnifying: 14,000 x)

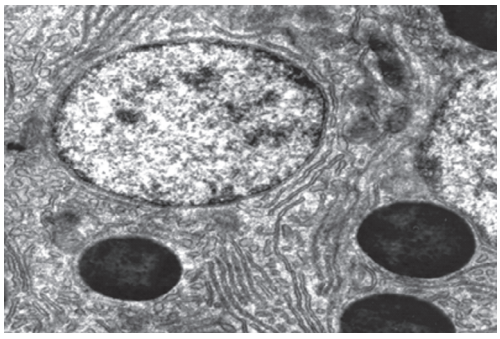

Figure 7 - Photomicrograph of the carp liver from the endosulfan experimental group of fish (magnifying: 14,000 x)

Table 2 - Cholinesterase activity of the axial muscle from carps (*Cyprinus carpio*) exposed to endosulfan in the concentration of 0.001mg/L

Groups	N	Cholinesterase activity ($nmol.min^{-1}. mgprotein^{-1} \pm ep$)
Control	20	341 ± 41,64
DMSO	20	550,6 ± 68,29
Endosulfan	20	477,4 ± 79,35

Data regarding the average values and the standard error from 20 fishes of each group. ANOVA ($P > 0.05$)

Table 3 - Cholinesterase activity in a pool of brains from *Cyprinus carpio* exposed to endosulfan in the concentration of 0,001mg/L

Groups	N (group)	Cholinesterase activity ($nmol.min.^{-1} mgprotein^{-1} \pm ep$)
Control	3	371,2 ± 11,80
DMSO	3	371,2 ± 11,80
Endosulfan	3	397,4± 10,35

Data from average and standard error of several pools of brain From 60 fish from each group. ANOVA ($p>0.05$)

in carp axial muscle and brain are shown in tables 2 and 3, respectively, the assay being carried out in the control, DMSO and endosulfan groups.

Discussion And Conclusion

In the present research work morphometric, hystopathologic, ultra structural and enzymologic parameters have been used aiming the evaluation of the sublethal effects caused by endosulfan on carp (*Cyprinus carpio*).

For the morphometric analysis parameters as body weight, body length, liver weight and hepato-somatic index were established. As a result it has been observed significant alterations in the somatic index and in the liver weight from the animals exposed to endosulfan in the concentration of 0.001mg/L (Table 1). Those results support the findings by Arnold, Pluta and Braunbeck[12], in regard to the effects induced by endosulfan (50 ng/L) causing significant alterations in the somatic indexes and in the liver weight of the rainbows trout (*Onchorhyncus mykiss*), while the CF remained unaltered as in the present study.

In regard to the morphological and ultra structural analysis of the hepatic cells from the fish exposed to endosulfan (Figures 6 and 7) it has been observed depletion on the concentration of liver glycogen and an apparent proliferation of the endoplasmic reticulum. In studies by electron microscopy, with rainbow trout a similar observation has been made by Arnold, Pluta and Braunbeck[12], when observed an increase in the size of the cell nucleus and depletion in the concentration of hepatic glycogen in the experimental group of fish treated with endosulfan and disulphoton. They also observed an increase in the hepatocytes volume and diameter and a proliferation of the endoplasmic reticulum, a possible indication of mixed-function oxygenase (MFO) induction in different species of fishes as the Indian catfish, exposed to 1.5 mg/L of endosulfan no alterations of hepatic glycogen has been observed, however, there was a significant increase in the blood glucose levels.[13] Gill, Tewari and Jaishree[14] observed a decrease in hepatic glycogen in the fish *Barbus conchonius* exposed to endosulfan. In *Anguilla anguilla* exposed to endosulfan in the concentrations of 8.2 and 4.1 mg/L for 12, 24, 48, 72 and 96 hours. Gimeno et al.[15] observed an increase in the blood glucose levels besides a depletion in muscle glycogen that occurred during some periods of the experiment.

In regard to the axial muscle and brain cholinesterase activity no significant differences were found between the fish from the control groups and the fish treated with DMSO 0.01% and with endosulfan in the concentration of 0.001mg/L, a result similar to the one found by Devi et al.[16], in an experiment carried out with the fish *Channa punctatus*. On the other hand, in research carried out by Naqvi and Vaishnavi[17] they have found inhibition of brain acetyl cholinesterase in rats (*Ratus novergicus*) exposed to 4.6 mg/kg/day of endosulfan. The concentration of endosulfan may also influence such inhibition by not being a molecule structuraly specific to the enzyme active sites as it is the case of carbamates and organophosphate compounds, as it is also the case of heavy metals as cadmium and lead.[14,18] In fish there is still much controversy in regard to the inhibition of acetyl cholinesterase by other compounds if not by carbamates and organophosphates as it is the case of endosulfan.[19,20] According to Sturm et al.[21], the concentrations of acetyl cholinesterase inhibitors should be relatively high in comparison with the inhibitory concentrations displayed by carbamates and organophosphate compounds.

Beyond those results, some behavioral alterations such as hyperactivity and quick swimming motions were observed in the animals exposed to endosulfan. These same alterations as well as spastic muscle contractions were found by Carlson et al.[22], in experiments with the rice fish medaka (*Oryzias latipes*) in studies on the neurological effects on startle response and scape from predation when exposed to endosulfan, a result suggestible of

neurotoxicity [23]. However, these observed behavioral alterations cannot be related only to a supposed inhibitory effect of cholinesterase activity caused by endosulfan. Another mechanism through which endosulfan may act is by blocking the site of picrotoxine liason on GABA receptors, decreasing the Cl- ions influx and consequently the threshold for depolarization, resulting in hyperexcitability.[22,24,25]

Conclusion

In spite of its environmental stability, endosulfan possesses a high potential for endangering aquatic life because of its high toxicity towards fish. The results of the present research suggest that endosulfan produced toxic effects on *Ciprinus carpio*, causing functional and morphological alterations to the liver as well as some behavioral disturbs.

Efeitos do endosulfano em concentrações subletais em carpas (*Cyprinus carpio*, Linnaeus, 1758): Análises morfométricas, histológicas e ultraestrutural e avaliação da atividade da colinesterase

Resumo

Endosulfano, um pesticida organoclorado, tem sido banido pela maioria dos países desenvolvidos, embora seja ainda produzido e utilizado deliberadamente em países em desenvolvimento. Utilizado no controle de pragas, assim como no controle de insetos em Programas de Saúde Pública em alguns países, seus efeitos no meio ambiente e sua toxicidade continuam em discussão. Para alguns pesquisadores a bioacumulação nos organismos terrestres é considerada irrelevante, mas não para a vida aquática. O objetivo da presente pesquisa foi estudar os efeitos das concentrações subletais do endosulfano em peixes de água doce *Cyprinus carpio,* (Linnaeus, 1758). Os peixes foram expostos durante 15 dias, a uma concentração de 0,001mg/L de endosulfano utilizando o dimetilsulfóxido (DMSO) a 0,1% como solvente. A atividade da acetilcolinesterase do músculo axial e cerebral assim como a morfometria, histopatologia e a ultraestrutura do fígado desses peixes foram avaliadas. O índice somático hepático e o peso dos fígados mostraram valores menores quando comparados ao grupo controle, observando-se também, alterações histopatológicas e ultraestruturais. Nenhuma alteração significante na atividade da acetilcolinesterase muscular e cerebral foram observadas. Os resultados sugerem que o organoclorado endosulfano causou efeitos tóxicos no metabolismo hepático dos peixes expostos a doses subletais.

Palavras-chave:
Endosulfano.
Peixes.
Cyprinus carpio.
Colinesterase.
Análise Ultraestrutural.

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