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ADHD-like behaviour in the offspring of female rats exposed to low chlorpyrifos doses before pregnancy

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The aim of this study was to investigate how chronic low-dose chlorpyrifos exposure of female Wistar rats before and during pregnancy affects behavioural parameters in their offspring. Four months before pregnancy, we exposed three groups of rats to chlorpyrifos doses of 5, 10, and 15 mg kg^{-1} body weight every day for 30 days, whereas one group received a single 30 mg kg^{-1} dose on gestational day 6. When the offspring of the exposed rats grew up, we studied their anxiety rate, motor activity, and cognitive abilities using the respective behavioural tests: open field test, dark/light box, and the extrapolation escape test. The offspring of rats exposed before pregnancy had significantly higher activity rate than controls, and even showed motor agitation and hyperactivity signs. The offspring of rats exposed to the single dose had difficulties solving the extrapolation escape test and showed poorer short- and long-term memory performance. This confirmed that even pre-pregnancy chlorpyrifos exposure can cause neurobehavioral consequences in offspring. Even though the mechanisms of the observed changes remain unclear and need further investigation, these data seem alarming and may serve as an important argument for revising the terms of safe pesticide use.

KEY WORDS: *neurotoxicity; organophosphate pesticides; anxiety; memory; motor activity*

Due to its neurotoxic properties chlorpyrifos (CPF) is one of the most common and most dangerous organophosphate pesticides used in agriculture, industry, and even at homes (1). It inhibits cholinesterase enzymes, causing acetylcholine overload in the cholinergic brain regions, over-excitation, and then disruption of synaptic transmission (2).

Another toxic effect it causes is oxidative stress (2). This effect can induce the death of isolated hippocampal neurons *in vitro* (3). One more recently discovered neurotoxic effect of CPF is that it affects the endocannabynoid system in the brain (4); even at low doses and without detectable AChE inhibition, CPF can lead to the accumulation of anandamide and a decrease in amidase activity. Moreover, CPF has been reported to affect the immune system; in a study by Nakamura et al. (5) perinatal exposure to CPF led to irreversible changes in T-lymphocytic response in mice. However, despite intense research, the mechanisms of CPF toxicity remain unclear.

Acute poisoning with CPF can lead to severe nerve damage, hypoxia, blood pressure decline, seizures, and even death (1). Behavioural manifestations of acute CPF poisoning observed in animal studies include a decrease in anxiety-related behaviour, motor hyperactivity or, conversely, reduced activity (6).

Chronic exposure to low doses of CPF may not cause any acute symptoms, but the subtle changes in CNS functioning induced by small amounts of the pesticide may lead to neurological damage. Subchronic exposure studies suggest a number of behavioural effects. Middlemore-Risher et al. (7) reported increased impulsivity and attention problems in rats. We too studied how subchronic low-dose CPF exposure affected rat behaviour (8). The exposed rats showed a decrease in long- and short-term spatial memory, and the anxiety rate increased rapidly on day 1 of exposure, compared to controls, but it dropped to control values within two weeks, showing adaptation to the toxicant.

In humans, chronic exposure to CPF may have severe neurological consequences, including Parkinson's disease (9). Recently, Rauh et al. (10) published alarming information that exposure to CPF in women (such as those working with pesticides on farms) may impair cognitive and behavioural development in their children. Bouchard et al. (11) found an IQ decline in children prenatally exposed to CPF, whereas other researchers associated it with the attention deficit hyperactivity disorder (ADHD) syndrome (12) and autism (13).

However, we found no information on the risks of CPF exposure before pregnancy for the neurological development of children, and it is still unclear whether CPF affects the developing foetus directly or through changes in the mother's body. We therefore designed an animal study whose aim was to compare behavioural and cognitive effects of CPF in the offspring of female rats receiving CPF

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subchronically before pregnancy and in the offspring of female rats that received a single CPF dose during pregnancy.

MATERIALS AND METHODS

Chlorpyrifos preparation

Chlorpyrifos (CAS No. 2921-88-2) (*O,O*-diethyl-*O*-3,5,6-trichloro-2-pyridyl phosphorothioate, purity 99.9 %) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). As CPF is poorly soluble in water, we dissolved it in refined sunflower oil at concentrations corresponding to doses given to each animal according to its body weight (b.w.) and group using oral probe.

Experimental design

Figure 1 summarises the design of our experiment. Three groups of adult female Wistar rats (three in each group) were receiving CPF at the doses of 5 (group 1), 10 (group 2), or 15 mg kg^{-1} b.w. (group 3) every day for 30 days. As the LD_{50} range for rats varies between 90 and 270 mg kg^{-1} (1), our dose selection was based on literature data from similar research and on our previous work. The highest chronic dose of 15 mg kg^{-1} is the threshold for the visible effects of toxicity (such as porphyrine marks around the eyes). These rats were then kept under standard vivarium conditions without further exposure to CPF or other adverse factors for four months before pregnancy. Group 4 (three animals of the same age and weight) received a single dose of 30 mg kg^{-1} CPF on gestational day 6. This dose has been used in a number of studies as a sub-toxic acute dose that does not cause immediate symptoms of poisoning but can lead to some adverse effects (14, 15). Control females (group 5) received pure oil.

Each rat was weighed and numbered, and all were housed in standard conditions with the 12-hour light/dark cycle and free access to standard feed and water.

After weaning on postnatal day 21, rat pups were divided by experimental groups; rats of each group were housed in separate cages and numbered. When they reached maturity at two months of age, the offspring rats were put to the following behavioural tests: open field, dark/light box, and extrapolation escape (see Figure 2). The open field and extrapolation escape tests were repeated after 10 days. The dark/light box test was performed once, as its results clearly confirmed the ones of the open field test, and it seemed unnecessary to repeat it.

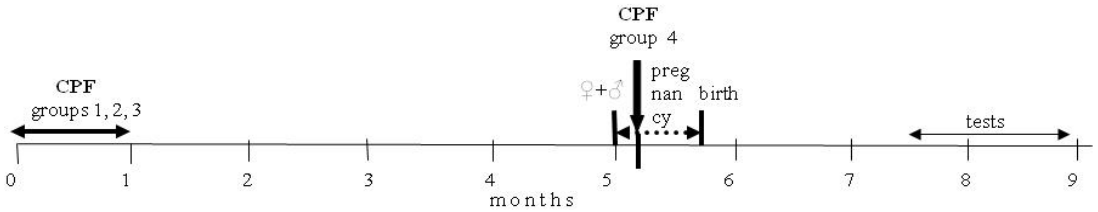
To avoid artefacts, the animals were kept in silence under dim light for two hours before the tests. No feeding, grouping, or other manipulations were performed at that time. All testing was carried out at the same time of the day, under the same lighting and temperature conditions, without disturbing odours and noise.

All procedures followed the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (16) and General Ethical Principles of Experiments using Animals (17).

Open field test

The open field test was introduced by Hall and Ballachey (18) in 1932 and is commonly used to assess animal emotionality, exploratory activity, and anxiety rate. It is based on the natural exploration drive of animals, rodents in particular, when placed in a new environment.

We used the test apparatus shown in Figure 2. One rat at a time was placed in the test arena for 3 min, and its behaviour was video-recorded. We looked for inner and outer horizontal activity, vertical activity (free and wall-supported), long and short grooming, number and total time of freezing reactions, number of defecations, and number of times the rats sniffed the holes. After each test, the arena



Group	CPF dose (mg kg^{-1} b.w.)	Time and duration of treatment	Number of pregnancies	Number of offspring
1	5	every day for 30 days, 4 months before pregnancy	1	14
2	10	every day for 30 days, 4 months before pregnancy	1	12
3	15	every day for 30 days, 4 months before pregnancy	1	10
4	30	a single dose on gestational day 6	2	21 (12+9)
Control	0	none	1	13

Figure 1 *Scheme of the experimental design and animal groups*

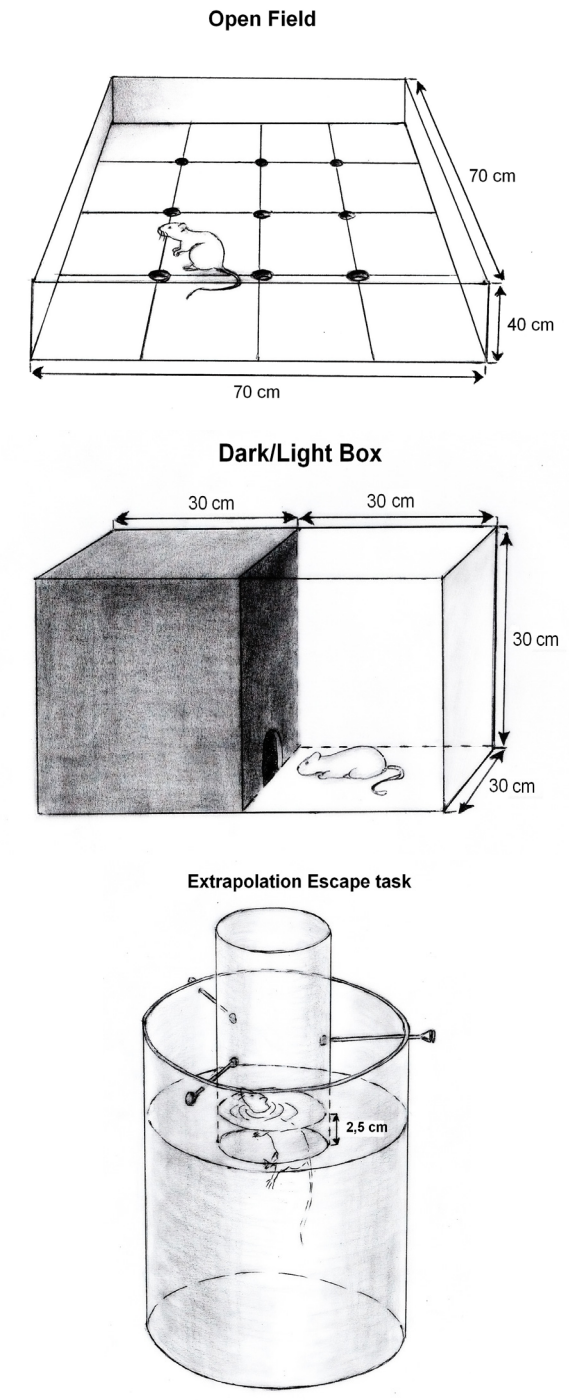


Figure 2 *Test apparatuses*

was cleaned up to avoid the influence of other animals' odour marks.

Dark/light box test

The dark/light box test is another behavioural test to assess animal emotionality, exploratory activity, and anxiety rate, especially in laboratory rodents. We used the test apparatus as described by Bourin and Hascoe (19) (for details see Figure 2), which was a plastic box divided to a dark and a light chamber by a partition with a hole in it. Each animal was put into the light chamber for 3 min and its behaviour observed. As rodents prefer dark and enclosed places, they tend to hide in the dark chamber, but also like to peek out and sometimes return to the light chamber to investigate it. We measured the time spent in the light chamber before entering the dark one, the number of times the animal peeked out from the hole, the number of times the animal returned to the light chamber, and total time spent there.

Extrapolation escape test

The extrapolation escape test serves to assess the cognitive function in rodents (20). The animals were placed under acute stress and had to find a way to escape and then to recall the successful strategy when they faced the same stress again 30 minutes later. The test apparatus consisted of a Plexiglas water container and a Plexiglas cylinder in its centre (Figure 2). The lower edge of the cylinder was immersed 2.5 cm below the water surface. The rats were put in the cylinder, and the only way to escape was to dive below the lower edge of the cylinder. If a rat failed to do so, we took it out after 2 min. We calculated the ratio of successful and failed escape attempts and measured the time before diving and the duration of initial immobility. Also, we calculated the "learning effectiveness" parameter that is the difference between the time before diving in first and second trials of each animal in each test session.

Statistical analysis

The obtained data were analysed with multifactorial ANOVA and ANOVA for repeated measures using Statistica 12 software (StatSoft Inc., Tulsa, OK, USA). The value of $p<0.05$ was considered statistically significant. ANOVA has been used in many studies with behavioural tests similar to our own (21-23).

RESULTS AND DISCUSSION

The results obtained by the open field and dark/light box tests had similar tendencies. In the open field test group 3 showed significantly higher horizontal and vertical activity and the number of hole sniffing actions than control and other experimental groups (Table 1).

In the repeated test, group 3 also showed significantly lower anxiety rate than controls (long and short grooming, defecation, and freezing). Forty to sixty crossed squares (*vs* 10-20 in controls), no freezing, no defecation, and no short grooming at all constitute abnormal behaviour. In fact, these results suggest hyperactivity disorder, and confirm that exposure of mothers to OP pesticides may lead to ADHD in children (24).

Table 2 shows the results of the dark/light box test. Group 3 significantly differed from control in the number of peeks out of the hole, once more demonstrating a decrease

Table 1 *Open field test results*

Test session	Group	Outer horizontal activity	Inner horizontal activity	Vertical activity	Long grooming	Short grooming	Defecation	Hole sniffing	Freezing time (s)	Freezing quantity
1	1	28.50±2.73	1.50±0.68	10.50±0.82	0.38±0.26	0.50±0.19	0*	1.13±0.27	0	0
	2	21.63±3.57	0.75±0.49	7.75±1.88	0.25±0.16	0.25±0.16	2.25±0.84	0.75±0.25	30.25±18.50	0.38±0.18
	3	49.00±4.69**	4.00±1.35	14.75±0.85**	0	0.50±0.29	0.50±0.50	2.25±0.75**	0	0
	4	29.75±3.29	1.08±0.23	10.00±1.33	0.25±0.13	0.75±0.25	2.25±0.75	1.33±0.28	5.83±5.83	0.16±0.16
	Control	16.13±3.27	1.63±0.80	10.00±2.21	1.00±0.46	1.38±0.38	2.75±0.59	0.25±0.16	2.63±2.63	0.13±0.13
2	1	18.38±5.14	0.38±0.26	5.25±1.51	0.25±0.16	0.25±0.16	0.50±0.50	1.38±0.63	48.38±15.20	0.88±0.30
	2	14.88±4.51	1.13±0.88	4.50±1.38	0.50±0.19	0*	1.25±0.45	0.88±0.64	60.38±22.14	0.88±0.35
	3	42.50±6.50**	1.00±0.71	12.75±2.06**	0.25±0.25*	0*	0*	2.50±1.04**	0**	0**
	4	22.83±4.37	2.67±1.28	9.08±2.25	0.42±0.14	0.67±0.26	1.08±0.57	1.83±0.39	27.17±15.04	0.42±0.19
	Control	11.75±4.46	0.25±0.16	5.63±1.89	0.63±0.26	0.63±0.26	1.88±0.77	1.00±0.38	66.13±19.45	1.13±0.30

**significantly different from control (p≤0.05)*
***significantly different from control (p≤0.01)*

Table 2 *Dark/light box test results*

Group	Hiding time (s)	Peeks out	Returns time (s)	No. of returns
1	19.48±5.18	5.63±0.56*	11.38±6.72	0.63±0.32
2	35.6±24.15	4.14±1.24	0.97±0.97	0.14±0.14
3	13.70±3.57	6.25±0.63*	10.63±6.13	0.5±0.29
4	34.13±13.89	5.00±0.77*	21.13±9.23	0.83±0.30
Control	45.70±19.75	1.75±0.67	0	0

** significantly different from control (p≤0.05)*

Table 3 *Extrapolation escape test results*

Test session	Group	Trial 1		Trial 2		Successful attempts ratio	Learning effectiveness
		Immobility time (s)	Diving time (s)	Immobility time (s)	Diving time (s)		
1	1	4.20±2.30	61.76±14.50	4.07±1.42	30.35±11.89	0.75±0.16	21.30±7.83
	2	0.64±0.64	45.96±13.86	5.59±2.00	80.20±19.25	0.86±0.14	-20.70±25.07
	3	0	75.64±15.12	3.07±1.62	36.79±14.05	0.75±0.25	51.98±19.85
	4	1.70±1.15	67.98±13.19	14.97±9,74	78.99±14.67	0.67±0.14	-1.47±13.35
	Control	1.99±1.32	38.65±17.89	4.19±2.21	49.65±16.01	0.75±0.16	7.61±23.77
2	1	2.95±1.53	40.46±17.23	5.04±2.05	23.20±13.90	0.88±0.13	5.27±1.50
	2	1.76±1.31	66.67±21.40	25.37±15.87	76.23±20.85	0.43±0.20*	3.97±2.84
	3	0	23.66±9.11	3.62±2.09	5.91±1.53	1.00±0	5.28±2.94
	4	2.64±1.22	69.45±13.98	5.84±2.25	75±27±15.97	0.41±0.15*	3.72±1.48
	Control	1.99±1.32	38.65±17.89	6.64±2.12	50.95±17.39	0.75±0.16	-1.29±15.11

**significantly different from control* ($p \leq 0.05$)
***significantly different from control* ($p \leq 0.01$)

in anxiety-like behaviour and increased motor activity. Other differences between the groups were not statistically significant.

In contrast to the open field, the differences from control in the extrapolation escape test were less prominent (Table 3). Groups 2 and 4 showed significantly fewer successful attempts than controls, and those who were successful spent longer time in the cylinder before diving, which points to poorer cognitive performance. In contrast, Group 3 showed significantly better diving time in the repeated test than all other groups, including control, and all escape attempts were successful. Although the learning effectiveness in group 3 may seem higher than in other groups, the difference was not significant due to statistical error caused by a wide variation of data within the group. It is also worth noting that in the first test, group 3 did not show any immobility at all, which points to motor hyperactivity.

Our findings suggest that chronic CPF exposure of mothers before pregnancy could cause hyperactivity and reduce anxiety in their offspring, but does not seem to affect the cognitive function, which points to symptoms similar to the ADHD syndrome, as reported elsewhere (12). Acute prenatal exposure, however, seems to affect the cognitive function.

The limitation of our study is the number of animals we used. Due to high mortality of pups (particularly in group 3, as only four animals reached maturity and were tested), data within the groups varied strongly and caused high statistical error, which rendered some of the observed differences (mostly in the extrapolation escape test) non-significant. This is why we plan to use more animals in future research. Moreover, future research should benefit from including mice and other species as well as from including other behavioural tests, such as T-maze, Vogel conflict test, and beam-walking.

Our study has produced intriguing data on the adverse effects of maternal exposure to CPF even before fertilisation. It opens a number of questions about the underlying mechanisms and tissue accumulation of CPF (being a non-persistent pesticide), as it remains unclear how the pesticide caused the observed changes in young rats that have not been exposed to it either pre- or postnatally. Future research should address these questions, specifically ADHD and other behavioural abnormality risks in children exposed to chronic low doses of pesticides through their mothers and the mechanisms that increase these risks.

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Ponašanje nalik ADHD-u u potomaka ženki štakora izloženih niskim dozama klorpirifosa prije trudnoće

Cilj je ovog istraživanja bio ispitati kako izloženost ženki Wistar štakora niskim, kroničnim dozama klorpirifosa prije i tijekom trudnoće utječe na parametre ponašanja njihovih potomaka. Četiri mjeseca prije trudnoće tri su skupine štakorica 30 dana primale klorpirifos u dnevnim dozama od 5, 10 i 15 mg kg^{-1} tjelesne mase, a jedna je skupina primila jednokratnu dozu od 30 mg kg^{-1} šestog dana gestacije. Kad je mladunčad odrasla, bihevioralnim testovima otvorenog polja, testom tamne/svijetle komore i ekstrapolacijskim testom bijega izmjerili smo njihovu razinu tjeskobe, motoričke aktivnosti i kognitivne sposobnosti. Mladunci ženki izloženih prije trudnoće iskazali su značajno više razine aktivnosti od kontrolne skupine, napose motoričku agitaciju i znakove hiperaktivnosti. Mladunci ženki izloženih jednokratnoj dozi imali su poteškoća u rješavanju ekstrapolacijskoga testa bijega te su iskazali slabije kratkoročno i dugoročno pamćenje. Naši su rezultati pokazali da izloženost klorpirifosu prije trudnoće može uzrokovati neurobihevioralne poremećaje u mladunčadi. Premda istraživanjem nismo uspjeli utvrditi mehanizme uočenih promjena, ova su saznanja uznemirujuća i mogu poslužiti kao snažan argument za ponovno promišljanje o ograničenjima u primjeni pesticida.

KLJUČNE RIJEČI: *motorička aktivnost; neurotoksičnost; organofosforni pesticidi; pamćenje; tjeskoba*