Neurobehavioral evaluation of adolescent male rats following repeated exposure to chlorpyrifos

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HIGHLIGHTS

- Chlorpyrifos (CPF) exposure is suggested to be related with mood disorders.
- CPF exposure leads to despair behavior.
- CPF exposure has no effects on locomotor.
- CPF exposure facilitates the development of learned helplessness.

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ABSTRACT

Chlorpyrifos (CPF), a highly effective organophosphate pesticide (OP), is extensively used worldwide. However, its agricultural use was extensively reduced in 2001. Several studies have suggested an association between mood disorders and CPF exposure in humans, especially in children, a subgroup that is highly susceptible to xenobiotics. We investigated the hypothesis that repeated CPF exposure in animals would elicit depressive-like behavioral alterations that reflected depression-related symptoms. Adolescent male rats were subcutaneously injected with either olive oil or 2.5, 5, 10, or 20 mg/kg CPF from postnatal day 27 to 36, then were followed by a series of neurobehavioral evaluation. Our studies revealed depressive-like alterations that were manifested by increased despair behavior in the forced swimming test, increased escape failure in the learned helplessness test, and altered approach-avoidance conflict in the novelty-suppressed feeding test. There was no effect on locomotor activity in the open-field activity test. This study indicates that repeated exposure to CPF elicits depressive-like behavioral alterations in adolescent male rats.

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1. Introduction

Recent studies have demonstrated the potential risks of organophosphate pesticide (OP), including its neurotoxic effects on developing organisms. Although the restriction of their agricultural use in the United States and European Union was implemented in 2001, they are still widely used by agriculture and industry in the other parts of the world [5].

Chlorpyrifos (CPF) is an OP pesticide that remains one of the most extensively used insecticides worldwide because of its relatively low cost and its effectiveness against a wide variety of pest species. Even so, the association between emotional disorders and CPF exposure in humans is a major environmental health issue, especially in children, a subgroup that is highly susceptible to environmental hazards.

Populations that are regularly exposed to pesticides have been found to be more susceptible to depression [12]. A three-year follow-up study found that pesticide poisoning was significantly associated with depression [2]. Moreover, suicide rates are higher in farming populations [13], and workers in the farming, fishing, and forestry industries have the highest lifetime risk for major depression [21]. Most importantly, inherent biological vulnerabilities and certain characteristic behaviors make children particularly susceptible to the neurotoxic effects of pesticides [9]. For example, they exhibit unique activity and dietary patterns [7] that result in greater exposure via the dermal and oral pathways [8]. The potential...
neurotoxic effects of acute and chronic CPF exposure were investigated in developing organisms [1]; however, the effects on adolescents have not been thoroughly investigated.

It is extremely important to understand the mechanisms underlying CPF-induced mood disorders in adolescents. Young animals are generally more sensitive to the lethal effects of cholinesterase-inhibiting pesticides, and young rats (postnatal day 17, PND 17) have shown similar behavioral changes and ChE inhibition at a five-fold lower dose than that in adult rats [17]. Furthermore, our previous studies have found that repeated exposure to CPF induces behavioral and cellular alterations in adolescent male rats, providing additional evidence to support CPF's neurotoxic effects in young animals [3,4]. Based on these observations, we hypothesized that during adolescent period, repeated exposure to lower doses of CPF elicits depressive-like alterations that resembling core symptoms of depression.

2. Materials and methods

2.1. Animal

All experiments on animals were carried out in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1996). Four-week-old (started at PND 22) adolescent male Sprague-Dawley rats (Beijing Vital River Laboratory) were housed in an environmentally controlled room. There was a 12-h light/dark cycle (with the light cycle beginning at 8:00 AM) and animals had free access to food and water. On arriving, the animals were allowed to accommodate to the housing conditions for five days and then were randomly assigned to either the control or CPF-treated groups.

CPF (Sigma-Aldrich Chemical) at doses of 2.5, 5, 10, or 20 mg/kg was dissolved in olive oil and administered subcutaneously at 2 ml/kg body weight. The injections were administered once daily for 10 consecutive days, from PND 27 to 36, which is the accepted adolescent period in rats [24]. The control animals received equivalent injections of olive oil. The design of behavioral assessments after the last CPF exposure is outlined in Fig. 1. Based on our previous studies [3,4], the doses were chosen to avoid evident weight loss or mortality.

2.2. Behavioral assessments

2.2.1. Forced swimming test (FST)

A FST was performed following the methods described in our previous study [3]. Briefly, the rats were placed individually in glass cylinders (40 cm high and 18 cm in diameter). The water was maintained at 28 ± 1°C and was 22 cm deep. The pre-swimming and swimming tests were separated by 24 h. During the pre-swimming test, the rats were allowed to swim in the cylinders for 15 min, and then were removed from the cylinders and dried with towels. The next day, rats were retested under identical conditions for 5 min and their immobility time was measured.

2.2.2. Novelty-suppressed feeding test (NSFT)

Forty-eight hours prior to the behavioral testing, all food was removed from the home cages (while water was freely accessible). At the time of the testing, eight equal-sized food pellets, weighing approximately 4 g each, were placed in the center of a novel plastic box (76 cm × 76 cm × 46 cm). The feeding latency during a 5-min period was recorded. Immediately after the test, the animals were transferred to their home cages and their food consumption during the first 5 min was measured.

2.2.3. Open-field test

Locomotor activity was measured with an open-field test [11]. The apparatus consisted of an arena 80 cm in diameter with a white, opaque wall (30 cm). The arena was divided into 18 equal sectors. The rats were placed in the center of the arena, and their locomotor activity, including the number of crossings and rearings, was recorded for 5 min.

2.2.4. Learned helplessness (LH) test

The procedure for the LH test was modified from that previously described [6,22]. The rats were tested using a modular shuttle box (MED-APA-D1R, Med Associates Inc, USA). This apparatus consisted of 8 automated shuttle boxes, each with 2 equal-sized (20 cm × 6.5 cm × 20 cm) compartments that were separated by a wall, with a central door that remained open and with a tungsten lamp at the top of each compartment. Each shuttle-box was connected to a computerized shock controller that could electrify the stainless steel grid floors. The experiment was carried out over the course of 5 days and utilized 2 paradigms. The first paradigm (on day 1) was helplessness induction by subjecting rats to inescapable shock (IS). Rats were placed in the right side of the shuttle box. A programmable shocker delivered 60 scrambled, randomized inescapable electric foot shocks (with a 0.5 mA intensity, a 15 s duration, and an intershock interval of 45–75 s) to both compartments. Starting the next day, the rats were subjected to a conditioned avoidance test for 4 consecutive days (days 2–5). For the conditioned avoidance test, the rats were allowed a 5 min habituation period in the shuttle box prior to the testing. Each test session consisted of 30 trials lasting no longer than 6 s with 29 inter-trial intervals of 24 s duration. A light was presented during the first 3 s of each trial. If the animal did not enter the other compartment, they were subjected to a 3 s shock (0.8 mA). The rats could avoid (cross during the first 3 s of light), escape (cross during the 3 s of light + shock), or fail to escape (remain on the same side throughout the 6 s trial) the shock. The number of avoidance and escape responses was automatically recorded by the computer. The escape failures, a measure of helplessness behavior, were determined using the following formula:

Number of escape failure = 30 – (number of avoidance + number of escape)

2.3. Statistical analysis

The data are expressed as the means ± S.E.M. and were analyzed using a one-way analysis of variance (ANOVA), with treatment (Veh and CPF) as an intergroup factor. Student’s T-test was used to compare differences between two groups, while an ANOVA followed by Dunnett’s T-test was used to compare the differences among three or more groups. The significance level for the main treatment effects was set at p < 0.05.

3. Results

Generally, in the current experimental settings, no significant changes in viability, weight, or general growth was found, and no signs related to cholinergic intoxication were observed. Results of neurobehavioral evaluations were listed as follows.
3.1. Forced swimming test

Similar to our previous reports, we observed a significant increase in immobility time at 10 mg/kg (p < 0.01 vs. the vehicle), while this effect was not significant at 5 mg/kg (p = 0.082 vs. the vehicle). An ANOVA revealed a significant effect of the CPF treatment (p < 0.05) (Fig. 2), indicating that the repeated CPF exposure led to depressive behavioral alterations.

3.2. Novelty-suppressed feeding test

Fig. 3 shows the effects of repeated CPF exposure on feeding latency in the NSFT. The CPF produced an evident effect on the latency (p < 0.03). At lower doses (2.5 and 5 mg/kg), the CPF increased the feeding latency, whereas at higher doses (10 and 20 mg/kg) the animals exhibited latency decreases. There were no significant differences in home-cage food consumption among the groups (data not shown).

3.3. Open-field test

An ANOVA revealed no significant CPF-exposure effect on the numbers of crossings or rearings (Table 1). These results suggest that for the doses used in this study (2.5–20 mg/kg), there was no effect on CNS or locomotor activities; they also exclude the possibility of false positives on the behavioral tests.

<table>
<thead>
<tr>
<th>Drug dose (mg/kg/day)</th>
<th>Crossings</th>
<th>Rearings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>58.7 ± 8.5</td>
<td>23.8 ± 5.0</td>
</tr>
<tr>
<td>CPF</td>
<td>73.0 ± 4.7</td>
<td>27.3 ± 2.2</td>
</tr>
<tr>
<td>5</td>
<td>69.6 ± 6.2</td>
<td>19.0 ± 2.5</td>
</tr>
<tr>
<td>10</td>
<td>73.0 ± 5.8</td>
<td>25.6 ± 3.8</td>
</tr>
<tr>
<td>20</td>
<td>65.7 ± 9.5</td>
<td>22.0 ± 2.8</td>
</tr>
</tbody>
</table>

The values represent the number of crossings and rearings within the 5 min data collection period. The data are represented as the mean ± S.E.M. An ANOVA model did not reveal a significant effect compared to the vehicle (control).

3.4. Learned helplessness test

As shown in Table 2, repeated CPF exposure significantly increased the number of escape failures after the 75-min period of IS on days 2 to 5, suggesting that the CPF-exposed rats were more vulnerable to LH. An ANOVA detected no significant effect from the CPF treatment on day 2, although this effect was significant on days 3, 4 and 5 (p < 0.04, p < 0.02, and p < 0.005, respectively). These findings illustrate that repeated exposure facilitates the development of LH.

4. Discussion

Previously, we demonstrated that repeated exposure to CPF elicits evident emotional behavioral alterations in adolescent male rats, as assessed by various behavioral tests: forced swimming test, novelty-suppressed feeding test, elevated plus-maze test, and Vogel’s conflict test. However, these emotional perturbations could have been driven by either AChE inhibition or by other noncholinergic mechanisms. In the present study, therefore, we chose lower doses that avoided cholinergic intoxication, based on our previous report, and we observed no signs of acute cholinergic toxicity [5]; meanwhile, another core aspect implicated in major depression, learned helplessness [18], was also assessed in this study. Specifically, we found that CPF exposure elicits effects on immobility time in the FST, on latency to feed in the NSF test, on the number of escape failures in the LH test, without altering locomotor activity in the open-field test.

So far, scarce studies had been reported that repeated exposure to CPF induces depressive-like alterations in adolescents. It was suggested that neonatal CPF exposure produces persistent changes in serotonergic systems and induces alterations in 5-hydroxytryptamine (5-HT)-related behaviors, displayed increased time spent in the open arms of an EPM test and increased working and reference memory errors in a radial-arm maze training test [1]. However, this study did not employ the most widely used behavioral tests in depression research: the forced swimming and learned helplessness tests.

<table>
<thead>
<tr>
<th>Drug dose (mg/kg/day)</th>
<th>Number of escape failure in 30 trials</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>Vehicle</td>
<td>9.8 ± 3.2</td>
</tr>
<tr>
<td>CPF</td>
<td>8.2 ± 2.7</td>
</tr>
<tr>
<td>5</td>
<td>13.3 ± 2.9</td>
</tr>
<tr>
<td>10</td>
<td>13.1 ± 2.9</td>
</tr>
<tr>
<td>20</td>
<td>15.8 ± 3.2</td>
</tr>
</tbody>
</table>

The values represent the number of escape failures recorded on days 2–5 after a 75 min inescapable shock (IS) exposure on day 1. The data are represented as the mean ± S.E.M. An ANOVA model revealed a significant effect for CPF exposure on days 3–5.
The LH paradigm is based on the hypothesis that depression is induced in animals when they are subjected to repeated unavoidable and uncontrollable aversive stimuli [10]. This model has been shown to closely incorporate the disease etiology, and the validity of this paradigm for depression-like behavior has been supported by its reversal through short-term antidepressant treatment [15]. It is also hypothesized that IS depletes brain monoamines; thus, administering drugs that decrease monoaminergic transmission may enhance the escape deficit, while drugs that increase monoaminergic activity may prevent this deficit [10]. In fact, studies on neonatal rats had already shown the significant effects of CPF exposure on neurotransmitters, indicated by a widespread deficiencies in catecholaminergic synaptic functions of neonatal rats [23]. It is predictable that for a narrow window of adolescent exposure, CPF could also elicit deficiencies in monoamine systems, which awaits further assessments.

The FST is the most widely used paradigm for assessing depressive- and antidepressant-like effects [18]. Decreased immobility time in the FST represents antidepressant activity, while increase of this index, as was observed in our study, represents a behavioral despair state that imitates aspects of major depression. To exclude the possibility of false-positives that can result from stimulant compounds or locomotor activating compounds, such as scopolamine [20], we performed an open-field test. For all doses, there was no significant CPF effect on either horizontal (crossings) or vertical movement (rearings).

Furthermore, we observed that repeated exposure to CPF also exerted a major effect on feeding latency in the NSFST. Interestingly, rather than observing an anxiolytic effect, as in our previous study of doses ranging from 10 to 160 mg/kg, we found anxiogenic effects [4]. These conflicting results can probably be explained by the dual effect of CPF exposure: anxiolytic-like behavior is induced by higher doses, and anxiogenic-like behavior is induced by lower doses. The same effect was also observed in the FST of our previous study [4]. The dual effects of CPF exposure need to be further investigated in future studies. Besides, it is important to note that those stress-based behavioral measures used in our present study are believed to reflect relatively comprehensive depression-related symptoms [25]. However, whether CPF facilitates depressive-like symptoms should be further studied in chronic mild stress paradigm.

It is accepted that adolescence is an important period for brain development, particularly in the hippocampus [3,19], a region that is extremely sensitive to stressors and xenobiotics [15]. It is also believed that the immature immune system to be more susceptible to the effects of xenobiotics, by delaying its maturation. If no intervention strategies are employed, life-long defects in immune function [14] and increased risk for major depression may occur. Therefore, it is also of our future interests that to assess the immunotoxicity of CPF on adolescent animals.

5. Conclusion

Our results demonstrated that repeated exposure to CPF elicits depressive-like behavioral alterations in adolescent rats. These effects resemble those of animal models of chronic mild stress. Specifically, repeated CPF exposure elicits a behavioral despair state in the FST, helplessness in the LH test, and anxiety in the NSFST. There were no effects, however, on locomotor activity in the open-field test. These results support the epidemiological findings that pesticide-exposed populations are more susceptible to depression and that adolescents are more susceptible to the neurotoxic effects of pesticides.

Conflicts of interest

The authors do not have any possible conflicts of interest.

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