Effect of Chlorpyrifos and Lipopolysaccharide on Primary Cultured Astrocytes

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Abstract. This study aimed to investigate the effect of chlorpyrifos (CPF) and lipopolysaccharide (LPS) on primary cultured astrocytes of the neonatal rat cerebral cortex. Neurodevelopmental disorders (NDDs), including obstacles of learning, emotion, memory, and so on, in patients induce a severe decline in social adaptation, resulting in a heavy burden on the family and society. However, the exact etiology and pathogenesis of NDDs are unclear. Adverse environmental factors can seriously impact the development of the nervous system. Methods: Trypsin digestion was used to isolate and purify astrocytes, and identify cells using the immunofluorescence method. The activities of astrocytes were evaluated using a cell counting kit 8 assay. Immunofluorescence and Western blot were used to measure the changes in the expression level of glial fibrillary acidic protein (GFAP) and high-mobility group box 1 (HMGB1). Results: The expression level of GFAP increased in 12 h after astrocytes were exposed to 0.1 and 1\(\mu\)g/mL LPS. The results indicated that 1\(\mu\)g/mL LPS could cause a significant activation of astrocytes. The expression level of GFAP and HMGB1 increased after astrocytes were exposed to 25 and 50\(\mu\)M CPF combined with 1 \(\mu\)g/mL LPS (P<0.05), and decreased after exposure to 100\(\mu\)M CPF combined with 1 \(\mu\)g/mL LPS (P<0.05). CPF and LPS can not only cause astrocyte activation but also increase the expression of GFAP and HMGB1. The effect of CPF combined with LPS is more obvious than CPF or LPS alone on astrocytes, indicating that they exert a synergistic effect.

Introduction

The etiology of neurodevelopmental disorders (NDDs) is unclear. The incidence of NDDs is not low. According to statistics, 4 million newborns are born each year in the United States, of which 3\%–8\% have neurobehavioral disorders [1, 2]; about 60,500 newborns have mental disorders, autism, or cerebral palsy [3]. Studies have shown that the prevalence of mental retardation in children in China was 0.9\%, accounting for 56.7\% of children with disabilities [4]. NDDs in patients lead to a heavy burden on the family and society [3]. However, their etiology and pathogenesis are unclear. Therefore, NDDs require a high degree of attention, and it is important to study the factors that may contribute to the prevention, diagnosis, and treatment of these disorders.

Many researchers have mainly focused on the early life as the origin of disorders in adults. Recent studies suggest that the complex interactions of genes and environment result in NDDs [5]. Pesticides are common environmental chemicals, and people are usually exposed to low
doses of pesticides in a long term. However, no obvious symptoms of pesticide poisoning are observed. The harmful effects of pesticides are increasingly recognized with in-depth studies on their low doses [6,7]. Previous studies suggested that the subtoxic doses of chlorpyrifos (CPF) induced brain damage [6,8]. Infection is a common cause of illness during the development process. Although current studies focus on single factors, the growth process is often influenced by the combined action of a variety of environmental factors [9]. Recently, double-hit models are used to study chronic/tonic pain [10] and stress dysregulation [11]. This in vitro study was designed to investigate the combined effect of CPF and lipopolysaccharide (LPS) on astrocytes, using glial fibrillary acidic protein (GFAP) and high-mobility group box 1 (HMGB1) as indexes to provide new strategies to combat NDDs.

Methods

Cell culture: Primary astrocyte cultures were prepared from the cerebral cortices of 1- to 3-day-old Sprague–Dawley rats. Furthermore, isolated cells were maintained in DMEM/F12 (HyClone, United States), supplemented with 10% of fetal bovine serum in an atmosphere of 5% CO2/humidified air (95%) at 37°C. After cell enrichment, astrocytes were detached from the culture flask with 0.1% trypsin and 0.04% EDTA in the Hank’s balanced salt solution (Sigma–Aldrich, United States). Notably, this was repeated three times. Astrocytes were plated onto poly-L-lysine-coated glass coverslips and supplemented with the culture medium. Experiments were performed 2–3 days after cell plating.

Intervention method: The DMEM/F12 culture mediums with different CPF and LPS concentrations were used to cultivate the prepared astrocytes for 12, 24, and 48 h. Then, the cells were washed with phosphate-buffered saline (PBS) and used for the next processing.

Immunofluorescence: The immunofluorescence assay was conducted on the cells using the primary antibody against GFAP and its corresponding secondary fluorescent antibody, and also the blue fluorescent nuclear stain DAPI (4’,6-diamidino-2-phenylindole).

Cell counting kit 8 assay: Isolated cells were placed in a 96-well plate (three subholes were provided for each group) after intervention and cleaning. Then, 10 μL of the cell counting kit 8 (CCK-8) solution was added to each well, and the OD at 450 nm was measured using a microplate reader.

Western blot: The whole astrocytes were prepared as described earlier. Meanwhile, the protein concentration was determined using a protein assay kit, and the separation method was sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then, the gel was electroblotted onto a nitrocellulose membrane. The membranes were probed with anti-GFAP (1:400 dilution) and anti-HMGB1 (1:1000 dilution). Furthermore, the filters were washed and incubated for 2 h at room temperature.

Result

Cell Identification

GFAP is a specific marker of astrocytes [12]. GFAP was used in this study to identify cultured cells (Fig. 1). The positive cells were counted as the percentage of the total number of cells, that is, the purity of astrocytes. After trypsin digestion and separation, the purity of primary cultured astrocytes from the neonatal rat cerebral cortex was 95.6% ± 1.5%.
The astrocytes were isolated and purified twice, and then identified by immunofluorescence staining. The blue nucleus is located in the red cytoplasm and represents astrocytes. Note: CyTM3-red: GFAP-positive cells; DAPI-blue: nucleus. (A) Only red fluorescence excitation; (B) excited only by DAPI; (C) fusion of (A) and (B) (× 400, scale: 50 μm).

**Effects of Lipopolysaccharide on Astrocyte Morphology**

Compared with the control group, the number and morphology of astrocytes did not change after treatment with 0.01 μg/mL LPS. When concentrations of 0.1 and 1 μg/mL lipopolysaccharide were applied to the astrocytes for 24 h, the cells became denser and the synaptic connections increased (Fig. 2). After treatment with 10 μg/mL LPS for 12 h, the number of cells decreased and the amount of granulosa-like substance increased, which was more obvious with the increase in time.

The astrocytes were exposed to 0.1 and 1 μg/mL LPS. The PBS group was the control group. The morphology of the cells was observed under an inverted microscope 24 h after exposure. The synaptic connections of astrocytes significantly increased after exposure to LPS for 24 h, and the effect of the dose of 1 μg/mL was more obvious than that of 0.1 μg/mL (× 100, scale 200 μm). (A) 24 h after PBS treatment; (B) 24 h after exposure to 0.1 μg/mL lipopolysaccharide (LPS); (C) 24 h after exposure to 1 μg/mL LPS.

**Effects of CPF and LPS on Astrocyte Viability**

The activity of astrocytes decreased at 10 μg/mL LPS (P < 0.05). Also, 1 μg/mL LPS significantly increased astrocyte viability at 24 and 48 h compared with the control group (P < 0.05) (Fig. 3a).

The activity of astrocytes was not significantly changed after treatment with 25 and 50 μM CPF (P > 0.05). The astrocyte viability decreased after treatment with 100, 200, and 400 μM CPF (P < 0.05). The activity of 100μM concentration group decreased less than 50%, indicating that 25, 50, and 100μM were low doses of CPF (P < 0.05) (Fig. 3b).

The cell viability at 25μM CPF and 1 μg/mL LPS at 24 h was significantly higher than that in the control group (P < 0.05). The OD value of 50μM CPF and 1 μg/mL LPS at 24 and 48 h was higher than that of the control group (P < 0.05). The cell viability at 100μM CPF and 1 μg/mL LPS significantly decreased (P < 0.05) (Fig. 3c). All the OD values decreased not more than 50%, suggesting that the aforementioned concentrations could be used for the next steps.
Figure 3. Effect of CPF combined with LPS on the OD values of astrocytes.

a: Different concentrations of LPS (0.01, 0.1, 1, and 10 µg/mL) were used to establish the cell model. PBS (0.1%) was used as the control group for 12, 24, and 48 h. The OD value of the cells was determined using the CCK-8 method. Note: 12 h: 12 h after exposure; 24 h: 24 h after exposure; 48 h: 48 h after exposure. *P < 0.05; x: compared with the same time point PBS; comparison between different time points for the same concentration: a: compared with 12 h, b: compared with 24 h.

b: DMSO (0.1%) was used as the control group. The cells were exposed to CCK-8 for 12, 24, and 48 h. Then, the cells were exposed to different concentrations (25, 50, 100, 200, and 400µM) of CPF to establish the cell model. Compared with the same time DMSO: comparison between different time points for the same concentration: a: compared with 12 h; b: compared with 24 h; comparison between different concentrations at the same time point: c: compared with 50µM; d: compared with 24 h.

c: DMSO (0.1%) group, PBS (0.1%) group, and CPF (25, 50, and 100µM) groups. The cells were exposed to CPF (25, 50, and 100 µM) and lipopolysaccharide (LPS) (1 µg/mL). LPS (1 µg/mL) served as the control group, the role of 12, 24, and 48 h after the use of CCK-8 cell OD value. Compared with the same time DMSO and PBS: comparison between different time points for the same concentration: a: compared with 12 h; b: compared with 24 h; comparison between different concentrations at the same time point: c: compared with 25µM; d: compared with 50µM; e: compared with CPF (same concentration) and LPS group and without LPS group; f compared with LPS group. CPF25 represents a 25µM dose of CPF; CPF50 represents a 50µM dose of CPF; CPF100 represents a 100µM dose of CPF.

**CPF- and LPS-activated Astrocytes**

In this study, GFAP antibody was labeled with a red fluorescent dye CyTM3 so that astrocytes were stained red. The results showed that both star-shaped glial cells and synaptic contact increased after exposure to LPS combined with low-dose CPF for 24 h. These findings suggested that astrocytes were active (Fig. 4).
Astrocytes exposed to LPS (1 µg/mL) combined with CPF (50µM) were used to establish the cell model. DMSO group, PBS group, CPF (50µM) group, LPS (1µg/mL) group and the control group. Moreover, GFAP expression was observed by immunofluorescence after 24 h. CyTM3-red: GFAP expression; DAPI-blue: nuclei. (A) After DMSO treatment for 24 h; (B) after PBS treatment for 24 h; (C) after CPF (50µM) treatment for 24 h; (D) after LPS (1 µg/mL) treatment for 24 h; (E) after LPS (1 µg/mL) + CPF (50µM) treatment for 24 h.

**CPF and LPS Influenced the Expression of GFAP**

The expression of GFAP was examined using Western blot after astrocytes were simultaneously exposed to low-dose CPF and LPS. The results showed that the expression of GFAP in astrocytes exposed to CPF (25 and 50µM) and LPS gradually increased compared with the control group (P < 0.05), while the expression in astrocytes exposed to CPF (100µM) and LPS decreased (P < 0.05) (Fig. 5).

![Figure 5. Expression of GFAP in astrocytes exposed to chlorpyrifos (CPF) and lipopolysaccharide (LPS).](image)

Primary cultured astrocytes from neonatal rat cortex were exposed to chlorpyrifos (25, 50, and 10 µM) and LPS (1 µg/mL); CPF (25, 50, and 100 µM) group, LPS (1 µg/mL) group, PBS (0.1%) group, and DMSO (0.1%) control group. GFAP expression was observed using Western blot after 12, 24, and 48 h. (A) Expression of GFAP protein was observed to detect changes at different time points; β-actin served as internal control. (B) A graph of the expression of GFAP protein. *P < 0.05; x, group compared with the same time PBS and DMSO; a: compared with the same time CPF, b: compared with the same time LPS; compared with different time points: c: compared with 12 h, d: compared with 24 h, e: compared with 48 h.
Figure 6. Expression of HMGB1 in astrocytes exposed to chlorpyrifos (CPF) and lipopolysaccharide (LPS).

Primary cultured astrocytes from neonatal rat cortex were exposed to CPF (25, 50, and 100μM) and LPS (1 μg/mL); CPF (25, 50, and 100 μM) group, LPS (1 μg/mL) group, PBS (0.1%) group, and DMSO (0.1%) control group. The expression of HMGB1 was observed using Western blot after 12, 24, and 48 h. (A) Expression of HMGB1 protein was observed to detect changes at different time points; β-actin served as internal control. (B) A graph of the expression of HMGB1 protein *P < 0.05; x, group compared with the same time PBS and DMSO; a: compared with the same time CPF, b: compared with the same time LPS; compared with different time points: c: compared with 12 h, d: compared with 24 h, e: compared with 48 h.

**CPF and LPS Influenced the Expression of HMGB1**

The expression of HMGB1 was examined using Western blot after astrocytes were simultaneously exposed to low-dose CPF and LPS. The results showed that the expression of GFAP in astrocytes exposed to CPF (50μM) and LPS gradually increased compared with the control group (P < 0.05), while the expression in cells exposed to CPF (100μM) and LPS decreased (P < 0.05) (Fig. 6)

**Discussion**

This study aimed to explore the double-hit effect of environmental pollutants and infections on the nervous system, so as to explore the interaction between these two effects. The primary cultured astrocytes were exposed to low-dose CPF and LPS in vitro. The results showed that low doses of CPF and LPS changed the morphology and viability of astrocytes and the expression of GFAP and HMGB1. Moreover, the effect of their combination was more obvious compared with their use alone.

Development is an interaction between genetic information and environmental factors to produce a specific phenotype with a strong plasticity in the programming process[13]. Adverse environmental factors can change the programming process, resulting in long-term chronic diseases[14]. Some scholars [15] analyzed the relationship between environmental pollution and NDDs and reported the environmentally attributable fraction as 10%. A study, by summarizing the relationship between pesticide residues in the urine of 249 pregnant women
and the Neonatal Behavior Neurological Assessment (NBNA) criteria, concluded that high maternal exposure to pesticides was a major risk factor for neonatal neurobehavioral development [16]. Pesticide intervention in newborn mice led to cognitive dysfunction in adults [17]. Previous studies have also shown that the exposure of newborn rats to CPF can induce dopaminergic neuronal degeneration in adults [6]. Many studies have shown that the infection can lead to premature delivery and brain damage in premature children [18]. People are often passively exposed to pesticides over a long time period. The effects of pesticides on health have attracted people’s attention. Does the applied pesticide conform to "pesticide residue standards"? Is it really safe? A few studies have proposed that the "safe level" of toxic chemical residues is associated with NDDs [19]. Organochlorine pesticides are persistent, and bioaccumulative environmental contaminants have potential neurotoxic effects. The exposure to CPF is closely related to nervous system damage in children [20]; it may cause neurodevelopmental impairment [8], mental retardation [21], ADHD [22], memory loss [23], anxiety, and autism [24].

Previous studies suggested that the subtoxic doses of CPF induced brain inflammation [6,25] via the HMGBl/TLR/NF-kB pathway in the brain amygdala regulated by proinflammatory cytokines. Also, HMGBl could be the target of intervening and treating the neurotoxicity caused by CPF [25].

Infection is a common reason for illness in children. Several studies have indicated that the intrauterine infection caused by fetal inflammatory response syndrome is the most common cause of brain injury in preterm infants[26]. It might be related to cognitive developmental disorders in children [27]. Inflammation is not only a potent inhibitor of neural development but also a high risk factor for cognitive impairment and depression [28]. A prospective study showed that 65% of children with very low birth weight (a total of 6093 cases of newborns) had at least 1 infection. Compared with the normal children, the early childhood infections would significantly increase the risk of NDDs [29].

The proliferation and differentiation of glial cells occur later than those of neurons [30] because the glial cells may be often affected by external factors in the maturity period. Many studies have suggested that the main target of CPF is astrocytes [31]. Astrocytes refer to glial cells. Their main functions include guiding neurons, glutamate and γ-amino butyric acid (GABA) metabolism, buffering of external environmental changes, and contacting neurons through synapses. Also, they can accept a variety of materials through receptor regulation [32]. GFAP is a marker of astrocyte activation [33]. Moreover, a hallmark of gliotic reaction is the upregulation of GFAP, which often precedes the anatomically apparent damages in the brain [34]. CPF combined with LPS, compared with each intervention alone, induced an increase in the expression of GFAP in astrocytes, suggesting that a combination of CPF and LPS can cause astrocyte activation and nervous system damage.

The important feature of HMGBl is that it can be combined with various proteins. Also, it can mediate inflammation and gene transcription [35]. HMGBl induces an inflammatory response via (receptor for advanced glycation end products by glycosylation and Toll-like receptors (TLRs) [36]. In this study, CPF and LPS could cause an increase in the expression of HMGBl in astrocytes, suggesting that CPF and LPS work together to cause nervous system injury. If the expression and secretion of HMGBl are inhibited, can it alleviate the toxic effects of CPF and LPS on the nervous system? Can HMGBl act as a common therapeutic target in these two kinds of injuries? Further research is needed to answer these questions.
This study investigated the effect of CPF and LPS on the primary cultured cortical astrocytes in neonatal Sprague–Dawley rats at different time points and showed that CPF combined with LPS in a certain concentration range could activate astrocytes and increase the expression of GFAP and HMGB1, further mediating nervous system injury.

Summary
This study indicated that CPF and LPS not only cause astrocyte activation but also increase the expression of GFAP and HMGB1. Moreover, the effect of CPF combined with LPS is more obvious than CPF or LPS alone on astrocytes, suggesting that they exert a synergistic effect.

References


