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Concomitant Effect of Chlorpyrifos and Intranasal Endotoxin Administration on Apoptosis Related Protein Expression in Lung of Mice

AA Chougule, RS Brar*, HS Banga, ND Singh, A Goyal and PD Gadhave

Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India

Abstract

Pesticides and bacterial endotoxin have been implicated in variety of respiratory dysfunctions. In present study, mice were administered chlorpyrifos at 3 mg/Kg BW/day for 30 days (by oral gavage) and/or challenged with bacterial endotoxin at 80 µg/mice by intranasal route to investigate expression of apoptosis related proteins such as p53, Bax and caspase-3 in lung. The results of immunohistochemistry of lung showed significant increase in number of p53 expressing cells in chlorpyrifos treated mice as compared to control while endotoxin and chlorpyrifos+endotoxin treated mice showed increased number of p53 expressing cells as compared to control and chlorpyrifos treated mice. Chlorpyrifos or endotoxin administration resulted in increased expression of Bax and Caspase-3 while chlorpyrifos treated and endotoxin challenged mice showed synergistic effect on expression of these proteins. From these results, it is concluded that chlorpyrifos and/or endotoxin increased critical apoptosis related proteins such as p53, Bax and Caspase-3 in lung of mice, which may play pivotal role in pathophysiology of pulmonary dysfunctions resulting from exposure to pesticides and endotoxin.

Keywords: Apoptosis; Chlorpyrifos; Endotoxin; Mice; Pesticide

Introduction

Pollution due to pesticides attracts wide spread public concern as, low-dose exposure to these are increasingly linked to human health hazards such as respiratory dysfunction(s), immunosupression, hormonal disruption, diminished intelligence, reproductive abnormalities and increasing cancer incidences [1,2]. Chlorpyrifos, an organophosphate is one of the most widely used insecticides in the world. Route of chlorpyrifos exposure include ingestion, inhalation and dermal exposure, however, dietary exposures appear to be the main source of nonoccupational exposures to chlorpyrifos [3]. Neurotoxicity is the primary effect observed in chlorpyrifos toxicity [4]; however pulmonotoxic [5], cardiotoxic, hepatotoxic [6], immunotoxic [7] also hemotoxic [8] potentials of this compound have also been reported. Epidemiological studies linked exposure to chlorpyrifos to the onset of respiratory dysfunction(s) [2,9]. Experimental studies showed chlorpyrifos induced apoptosis in T lymphocytes [10], retina [11] and placental cells [12].

Lipopolysaccharide (LPS) is a principal pro-inflammatory component of the Gram-negative bacterial cell wall. Agricultural environments and other occupational settings like small food storing and processing settings, grain mills and textile mills showed high concentration of endotoxin [13,14]. Occupational exposure to organic dust was found to deliver 30 to 60 µg of endotoxin to the lung; over in eight hours work shift [15]. It has been implicated in number of occupational lung diseases like acute airway obstruction, hypersensitivity pneumonitis, chronic bronchitis and decreased lung function in humans, most of which are associated with organic dust exposure [16-18]. Earlier studies of Kitamura et al. [19] suggested that excessive apoptosis might be reason for acute lung injury resulting from LPS exposure.

Apoptosis is physiological cell death which is critical process in the development and the homeostasis of multicellular organism [20]. The process of apoptosis can be triggered by external stimuli, such as soluble cell death ligands released during inflammatory responses, or intrinsic stimuli induced by alteration of cellular function and metabolism [21]. Apoptosis has been implicated in the physiopathology of several diseases and supposed to be involved in pathological cell death [22]. During the process of apoptosis, there may be selective transcription of certain critical genes required for apoptosis, including Bax, Bcl2, p53 and caspase-3 [23].

Therefore, present study contemplates to investigate expression of apoptosis related proteins such as p53, Bax and caspase-3 in pathophysiology of pulmonary dysfunctions putatively induced by chlorpyrifos and bacterial endotoxin.

Materials and Methods

Experimental animals

The experiment was conducted after approval by Institutional Animal Ethics Committee (IAEC), GADVASU, Ludhiana. Forty Swiss albino mice (8-10 week) were housed in laboratory animal cages with room temperature of around 18-22°C and 12:12 h light-dark cycle. The mice were provided feed (Godrej Agrovet Limited, Khanna, Punjab, India) and drinking water *ad libitum*.

Chemicals

The chlorpyrifos technical grade (Minimum 96% pure) was received from Ravi Organics Pvt. Ltd. Muzaffarnagar (UP), India. Chlorpyrifos solution was prepared by dissolving known amount of chlorpyrifos in groundnut oil. The solution was kept in refrigerator and was brought to room temperature before use. Lipopolysaccharide from *Escherichia*

***Corresponding author:** Dr. RS Brar, Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab-141004, India, E-mail: rsbraratemail.com

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coli (0127:B8) was purchased from Sigma Aldrich Chemicals Pvt. Ltd. The solution of lipopolysaccharide was prepared by dissolving 2 mg of lipopolysaccharide in 2 ml of pyrogen free 0.9 % normal saline. The xylazine-ketamine combination anesthetic solution was prepared by mixing 0.5 ml (10 mg) xylazine (Xylazin®) and 2 ml (100 mg) ketamine (Aneket®) in 7.5 ml of isotonic normal saline. Primary antibodies used for immunohistochemistry were purchased from Santa Cruz Biotechnology Inc., USA and secondary antibody was purchased from Vector Laboratories, Burlington, Ontario, through local distributors.

Experimental procedure

Mice were randomly divided into four groups (10 each). Group C was administered groundnut oil for 30 days and challenged with normal saline solution at 80 µl/mice by intranasal route on day 30. Group CPF was administered chlorpyrifos at 3 mg/Kg BW for 30 days and challenged with normal saline solution at 80 µl/mice by intranasal route on day 30. Group LPS was administered groundnut oil for 30 days and challenged with LPS at 80 µg/mice by intranasal route on day 30. Group CPF+LPS were administered chlorpyrifos at 3 mg/Kg BW for 30 days and challenged with LPS at 80 µg/mice by intranasal route on day 30. Mice from all groups were anaesthetized by intraperitoneal administration of anesthetic at 10 mg xylazine+100 mg ketamine/ kg BW after 16 hours of LPS/ saline administration and humanely sacrificed.

Immunohistochemistry

The necropsy of sacrificed mice was performed and tissue samples of lungs were collected in 10% neutral buffered formalin for fixation. Tissues were washed overnight in running tap water, dehydrated in ascending grades of alcohol and cleared in benzene. The 4-5 µm thick tissue sections were obtained on Poly-L-Lysine coated clean glass slides. Following dewaxing, antigen retrieval was performed by citrate buffer by using EZ-Retriever™ System (BioGenex Laboratories Inc., San Ramon, California, USA). After unmasking antigen, tissue sections were washed thrice with PBS (pH 7.2-7.4) for 5 minutes each time. The endogenous peroxidase was quenched with a solution of 3% H_2O_2 in methanol for 15 minutes at room temperature in a humid chamber, followed by three washings with PBS (pH 7.2-7.4) for 5 minutes each time. After blocking nonspecific sites with 1% bovine serum albumin in PBS buffer for 30 minutes, the sections were treated with primary antibody for overnight in a humid chamber at 4°C. The sections were then washed three times in PBS for 5 minutes each, followed by incubation in biotinylated secondary antibody for 30 minutes at room temperature in a humidified chamber followed by three washes with PBS for 5 minutes each. Then tissue sections were incubated with Vectastain ABC reagent for 30 minutes in a humid chamber followed by thrice washing with PBS for 5 minutes each. The reaction was visualized using a color development kit (Vector Laboratories, Ontario, Canada). The sections were also counterstained with hematoxylin. Immunohistochemical controls included incubation with normal goat IgG or omission of primary antibodies. The numbers of cells showing immunoreactivity to p53 were counted per 40X field and statistically analyzed by one way ANOVA. The details of antibodies used for the immunohistochemical studies are given in Table 1.

Results and Discussion

In present study, the expression of three apoptosis related proteins such as p53, Bax and Caspase-3 were investigated by immunohistochemistry method.

P53

In present study, control group mice showed p53 immunoreactivity in few bronchial epithelial and septal cells (Figure 1). Administration of chlorpyrifos to mice resulted in significantly increased number of p53 immunoreactive bronchial epithelial cells as compared to control ($p<0.05$) and weak intranuclear and intracytoplasmic expression of p53 in bronchial and septal epithelium (Figure 2). Wu et al. reported increased expression of p53 in brain of rats treated with insecticide deltamethrin [24]. Chen et al. [25] reported increased mRNA expression p53 in lung epithelial cells exposed to herbicide paraquat. Reports of Uzun et al. [5] showed chlorpyrifos induced oxidative stress in lungs of rats. Increased reactive oxygen species and subsequent DNA damage might be a reason for expression of p53 in lung of mice, which in consonance with the observations in the present study.

In present study, intranasal LPS challenge to mice resulted in significant increase in number of p53 immunoreactive bronchial epithelial cells and cells of alveolar septa as compared to control and chlorpyrifos treated mice ($p<0.05$) and there was weak intranuclear and cytoplasmic immunoreactivity of p53 in these cells (Figure 3). These results are in unison with finding of Vernooy et al. [26] who reported DNA fragmentation and apoptosis in bronchial epithelial cells after intratracheal instillation of LPS in mice. Animals treated with both chlorpyrifos and LPS showed significant increase in number of p53 immunoreactive cells ($p<0.05$) as compared to control and chlorpyrifos treated mice. The bronchial and septal epithelia cells showed moderate expression of p53 (Figure 4). These results showed synergistic effect of chlorpyrifos and LPS on expression of p53 in lung of mice.

Antibody used	Manufacturer	Dilution	Secondary antibody
Rabbit anti Bax	Santa Cruz Biotechnology Inc., USA (sc-526)	1:50	Biotinylated universal antibody raised in horse (Vectastain PK 6200; Vector Laboratories, Burlington, Ontario)
Rabbit anti P53	Santa Cruz Biotechnology Inc., USA (sc-6243)	1:50	
Rabbit anti Caspase-3	Santa Cruz Biotechnology Inc., USA (sc-7148)	1:50	

Table 1: Details of antibodies used for the immunohistochemical studies.

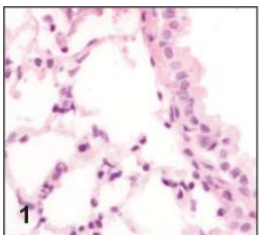

Figure 1: Section of lung from group C showing no p53 immunoreactivity. IHC. 40X.

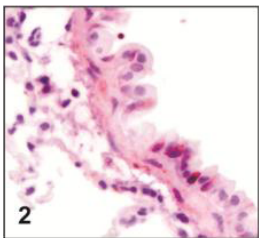

Figure 2: Section of lung from group CPF showing weak p53 immunoreactivity. IHC. 40X.

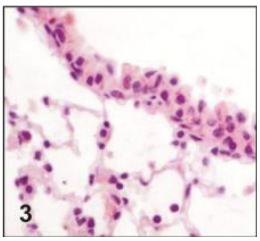

Figure 3: Section of lung from group LPS showing weak p53 immunoreactivity. IHC. 40X.

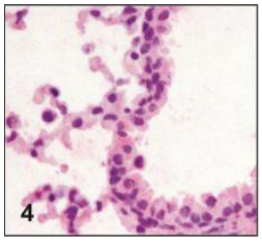

Figure 4: Section of lung from group CPF+LPS showing weak to moderate p53 immunoreactivity. IHC. 40X.

Xenobiotic including insecticides and endotoxin induced production of reactive oxygen species, which resulted in generation of oxidative stress [27-29]. Oxidative stress causes depletion of mitochondrial energy, induction of proteolytic enzymes, DNA fragmentation and apoptosis [11,30] at intermediate concentrations but induce necrotic cell death at higher concentrations [31]. The normal p53 acts as suppressor of cell growth but in the cells, which have irreparably damaged DNA, p53 promote apoptosis or allow the cells to proliferate [32]. Over expression of p53 induce apoptosis in various cells types [33]. In present study, p53 expressed in both in nucleus as well as in cytoplasm. Studies with other compound showed that cytoplasmic localization of p53 can trigger apoptosis through activation of Bax [34].

Bax

Control group showed very weak cytoplasmic immunoreactivity to Bax (Figure 5) in the bronchial epithelium. Administration of chlorpyrifos resulted in weak to moderate cytoplasmic expression of Bax in bronchial epithelium (Figure 6) and in few cells of alveolar septa of lungs as compared to control. In chlorpyrifos treated mice, cytoplasmic immunoreactivity for Bax in bronchial epithelium was higher as compared to control. Kashyap et al. [35] reported increased levels of protein and mRNA expression of Bax, p53 in PC12 cells treated with organophosphate insecticide monocrotophos. Similarly, Chen et al. [25] reported increased mRNA expression of Bax in lung epithelial cells exposed to herbicide paraquat. Wu et al. [24] reported increased expression of Bax in brain of rats treated with deltamethrin.

Intranasal administration of LPS to mice showed weak to moderate cytoplasmic immunoreactivity for Bax in bronchial epithelium (Figure 7) and in few cells of alveolar septa and expression higher as compared to control and chlorpyrifos treated groups. Reason for increased expression of Bax might be endotoxin produced oxidative and subsequent activation of Bax and release of cytochrome C [36].

Mice treated with both chlorpyrifos and LPS showed moderate to intense cytoplasmic immunoreactivity for Bax in bronchial epithelium (Figure 8), few cells of alveolar septa and in alveolar macrophages. The expression was higher as compared to other groups. Number of cells showing the reaction were also higher.

The members of Bcl-2 family protein are important regulators of apoptosis and have key role in the pathogenesis of the pulmonary inflammation, fibrosis and apoptosis [37]. Among the Bcl-2 family proteins, Bcl-2 was identified as a repressor gene of apoptosis [38] while Bax was identified as a promoter of cell death whose pro-apoptotic function was directly antagonized by Bcl-2 through formation of Bax/Bcl-2 heterodimers [39]. These Bcl-2 family proteins are localized in

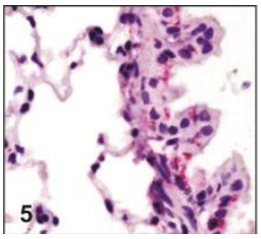

Figure 5: Section of lung from group C showing very weak Bax immunoreactivity. IHC. 40X.

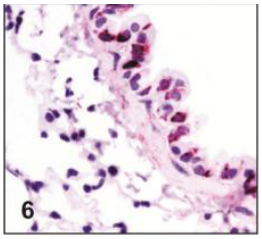

Figure 6: Section of lung from group CPF showing weak Bax immunoreactivity. IHC. 40X.

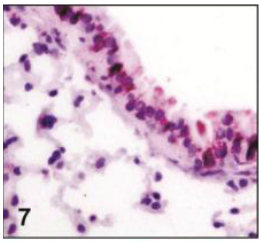

Figure 7: Section of lung from group LPS showing moderate to intense Bax immunoreactivity. IHC. 40X.

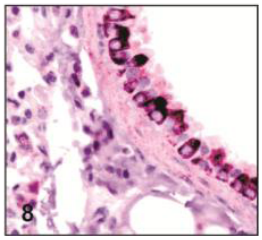

Figure 8: Section of lung from group CPF+LPS showing intense Bax immunoreactivity. IHC. 40X.

the mitochondrial membrane and regulate apoptosis by controlling release of cytochrome C into the cytosol [33]. The cells in which Bax is over expressed, the susceptibility of cells to apoptotic stimuli is enhanced and Bcl-2 over expression show reduced susceptibility of cells to apoptosis. Thus, Bax/Bcl-2 ratio determines the susceptibility of a cell to apoptosis [39,40].

Caspase-3

Control group mice showed negative to weak immunoreactivity for caspase-3 (Figure 9) in lung of mice. Mice administered with chlorpyrifos showed weak immunoreactivity for caspase-3 in bronchial epithelium, cells of alveolar septa and alveolar macrophages as compared to control (Figure 10) and expression was high as compared to control group. Previous studies also reported chlorpyrifos induced caspase-3 mediated apoptosis in human T cells [10]. Kashyap et al. [35] reported increased levels of protein and mRNA expression of caspase-3 in PC12 cells exposed organophosphate insecticide monocrotophos. There was weak immunoreactivity for caspase-3 only in cells of alveolar septa because chlorpyrifos mediated apoptosis is partially mediated by activation of intracellular caspase-3 [10], and is in conformity with the observations of present studies.

Intranasal challenge with LPS resulted in weak to moderate immunoreactivity for caspase-3 in bronchial epithelium, cells of alveolar septa and alveolar macrophages (Figure 11) and expression was higher as compared to control group. These findings are in accordance with Kawasaki et al. [41] and Z'graggen et al. [21]. Z'graggen et al. [21] reported increased caspase-3 in alveolar macrophages, neutrophils, tracheobronchial as well as alveolar epithelial cells after *in vitro* endotoxin stimulation. Apoptotic response induced by bacterial endotoxin may produce acute lung injury [19,41].

Mice administered with chlorpyrifos and subsequently challenged with LPS showed moderate to intense immunoreactivity for caspase-3 in bronchial epithelium and cells of alveolar septa but weak to moderate immunoreactivity in alveolar macrophages (Figure 12). Bronchial epithelium showed higher expression of caspase-3 as compared to other treatment groups.

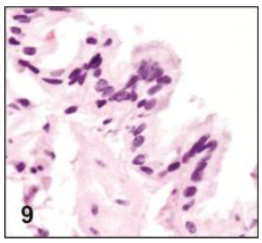

Figure 9: Section of lung from group C showing no caspase-3 immunoreactivity. IHC. 40X.

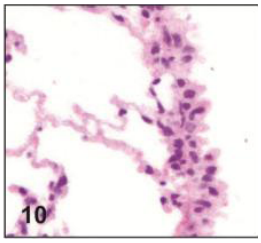

Figure 10: Section of lung from group CPF showing very weak caspase-3 immunoreactivity. IHC. 40X.

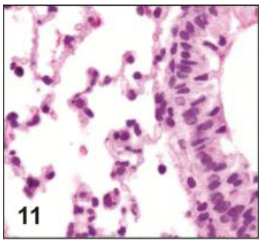

Figure 11: Section of lung from group LPS showing weak caspase-3 immunoreactivity. IHC. 40X.

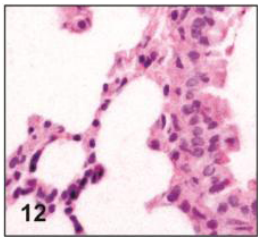

Figure 12: Section of lung from group CPF+LPS showing weak to moderate caspase-3 immunoreactivity. IHC. 40X.

The intrinsic apoptosis pathway triggered by Bcl-2 and the extrinsic pathway of apoptosis initiated by ligation of death activators such as TNF, Fas ligand and TNF-related apoptosis inducing ligand result in activation of caspase-3 [21], which is known to activate endonuclease and induce DNA fragmentation [42].

The reports of previous studies suggested that there might be a distinct process inducing transcription of certain critical genes including Bax, Bcl-2, p53 and caspase-3 required for apoptosis [23]. Combined exposure to chlorpyrifos and LPS produced synergistic effect on expression of apoptosis related proteins such as p53, Bax and Caspase-3 in lung of mice. From the results of present study, it is concluded that apoptotic response exhibited by over expression of p53, Bax and caspase-3 in lungs of mice exposed to chlorpyrifos and endotoxin may have paramount role in pathophysiology of pulmonary dysfunctions, as is evident this over expression of apoptosis related proteins in the present study.

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