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Dietary Long-Term Exposures to Fipronil Alter the Expression of Catalase in Lung and Serum

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Abstract

Fipronil is a broad-spectrum insecticide that belongs to the phenylpyrazole chemical family. We have earlier reported that long term dietary exposures to fipronil cause lung inflammation. Endotoxins are frequently prevalent among agricultural settings and have also been associated with lung damage. However, the mechanism of fipronil induced lung damage with or without endotoxin remains to be elucidated. The present investigations included male Swiss albino mice (N=36) aging 6-8 weeks to estimate catalase expression in lung and serum following exposure to fipronil with or without LPS. Animals were divided into two treatments and one control group (N=12, each). Treatment groups were orally administered high (1/10th of LD50) and low (1/20th of LD50) dose of fipronil dissolved in corn oil for 90 days followed by LPS (80 µl/animal) or NSS challenge via intranasal route. Low dose of fipronil along with LPS resulted 3.75 folds increase in the mRNA expression of catalase in the lung. High and low dose of fipronil alone or in combination with LPS showed strong immunopositive reactivity for catalase in the airways and bronchial epithelial cells. There was a significant increase in the number of immunopositive cells for catalase following exposure to individual high or low dose of fipronil or in combination with LPS as compared to control and LPS group. High dose of fipronil significantly ($p<0.05$) increased the protein concentration of catalase as compared to control group suggesting dose dependent dysregulation of catalase in serum. The data taken together suggest that exposures to fipronil with or without LPS altered the pulmonary expression of key genes associated with oxidative stress.

Keywords: Fipronil; Lung; Catalase; Oxidative Stress

Introduction

Pesticides are used for destroying harmful insects or other organisms and among these fipronil is used on a very large scale [1]. It has been reported to cause various toxic effects on target and non-target organisms including human by acting as antagonism of Gamma-Amino Butyric Acid/GABA [2]. Endotoxin is a Lipo Poly Saccharide (LPS) molecule derived from Gram-negative bacteria and endotoxin exposure has been related with pulmonary dysfunction [3]. Our previous reports indicate that endotoxin interact with various pesticides to alter the pulmonary responses during pesticide induced lung damage [4-9]. Further long term exposure to fipronil with or without endotoxin cause lung damage and dysregulate the key genes of Wnt pathway in mice [8].

Pesticides increase the production of Reactive Oxygen Species (ROS) resulting tissue damage and oxidative stress [9]. Catalase is a common antioxidant enzyme that uses either iron or manganese as a cofactor and catalyzes the degradation or reduction of H_2O_2 to water and molecular oxygen [10]. It is present almost in all living tissues such as liver, kidney and erythrocytes [11] and plays an essential role in cell defence against oxidative stress in these organs [12]. Catalase is also expressed in the lung during the later stages of development and is constitutively expressed in the airway, alveolar epithelial cells and macrophages to play an important role in the endogenous antioxidant defence system [12]. Targeting of catalase directly to the mitochondria in lung epithelial cells protects the cells from H_2O_2-induced apoptosis [13] and deficiency in catalase activity in the lungs predisposes the lung to worsening lung inflammation and subsequent fibrosis [14]. Overexpression of catalase prevent ROS induced damage including initiation of apoptosis and suppress age-related DNA oxidation in skeletal muscle [15].

However, there is no report on the expression of catalase during fipronil and/or LPS induced lung damage. Hence, the present study was conducted and we report the first data on the mRNA expression of catalase in the lung and its serum concentration following exposure to fipronil alone or in combination with LPS.

Material and Methods

Experimental design

Healthy male Swiss albino mice aging 6-7 weeks (N=36) were randomly divided into three groups *viz* two treatments and one control group (n=12 each group). Treatment group I and II were orally administered fipronil @ 1/10th and 1/20th of LD50, respectively for 90 days. Fipronil was dissolved in corn oil. Control group was administered with corn oil only. At the end of experiment half animals from all groups (N=6) were anaesthetized with xylazine ketamine combination anesthesia and challenged intranasally with LPS @ 80 µl/animal. The remaining animals of each group were administered same amount of Normal Saline Solution (NSS) intranasally. The animals were sacrifices after 9 hr of LPS or NSS challenge and lung samples were collected in

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RNA Later solution and paraformaldehyde for quantitative real time PCR analysis and immunohistochemistry, respectively. Blood samples were also collected to estimate serum concentration of catalase in all the groups.

Quantitative Real Time PCR (qPCR)

Right lung from each animal stored in RNA later solution at -80°C was used for detection of expression of catalase mRNA by qPCR. About 50 mg of frozen lung from all the samples was homogenized using Qiagen Tissue Ruptor II (Cat No: Invitrogen 9002755). Total RNA was extracted by using Trizol (Ambion, Life Technologies, USA) method and the quality as well quantity of the resulting RNA was assessed by spectrophotometrically by Nanodrop (Thermo Fisher) and also by visualizing via agarose gel electrophoresis. The concentration of total RNA varied between 100-700 ng/ml in different samples. The amount of total RNA used for cDNA synthesis was adjusted to 100 ng/µl for each sample. Total RNA was reversed transcribed into cDNA using a Revert aid cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instruction. qPCR was performed using Syber green chemistry. Primer sequences used for amplification of MYCN gene was F 5'- GCGTCCAGTGCGCTGTAGA -3' and R 5'- TCAGGGTGGACGTCAGTGAA -3' [16]. Further, β-actin (F5'-CTGTCCCTGTATGCCTCTG -3' and R5'-ATGTCACGCACGATTTCC -3 was used as an endogenous control. Transcript levels were normalized by comparison with β-actin.

Immunohistochemistry

Processed paraffin blocks were used to obtain 5µm thick paraffin sections using a rotary microtome. Paraffin sections were subjected to immunohistochemical staining as described earlier [17] to localize immunopositive catalase cells in the lungs of various groups. The sections were processed and incubated with primary antibody against catalase (Goat polyclonal antibody anti catalase; Elabscience, Dilution 1:25) for one hour followed by incubation with secondary antibody (anti goat, dilution 1:400) for 30 minutes. Colour development was done with a commercial kit (SK4100; Vector Laboratories, USA) followed by counter staining with haematoxylin.

Enzyme-Linked Immuno Sorbent Assay (ELISA)

Sandwich ELISA was conducted to estimate serum concentrations of catalase in different groups by using mouse catalase kit (Immunotag, USA range: 0.2 ng/ml-70 ng/ml and sensitivity 0.093 ng/ml). Standards of different concentrations were prepared such as Standard 1 (2.5ng/ml), Standard 2 (5.0ng/ml), Standard 3 (10.0ng/ml), Standard 4 (20.0ng/ml) and Standard 5 (40.0ng/ml) as per the manufacturer's instructions. About 40 µl of serum samples from all the groups were diluted (1:10) in phosphate buffer saline and were added to the flat bottom polystyrene pre-coated plates. A control consisting of Standard Diluent and Blank (PBS) were also incorporated in the plates. About 50 µl standard solution were added to the wells designated as standard wells. Then 10 µl antibody against the catalase was added to the corresponding sample wells. Standard solution already contains biotinylated antibody. So, antibodies were not added separately to the standard wells. Next, 50 µl streptavidin-HRP conjugate was added to the sample wells and standard wells (not blank) and mixed well. The plates were covered with sealer and incubated for 60 minutes at 37°C. After incubation, the plates were washed five times with 300 µl wash buffer at room temperature for 5 minutes each to remove any unbound antigen. Finally, 50 µl substrate solution A followed by 50 µl substrate solution B was added to each well and the plates were incubated undisturbed at 37°C in the dark for 10 minutes till color was developed. The color reaction was stopped by adding 50µl stop solution into each well. The Absorbances (OD) were measured at a wavelength of 450 nm on ELISA reader. The Absorbance (OD) of unknown sample were inter plotted against the exponential curve to determine serum concentration of catalase in different groups of mice.

Statistical analysis

Data were presented as mean (± SE) and analysed by single analyses of variance (ANOVA) using GraphPad Prism 7 software followed by group comparisons with post-hoc tests. The significance was accepted at $P<0.05$.

Result and Discussion

Pulmonary expression of catalase mRNA

In the present study, the pulmonary mRNA expression of catalase was evaluated by using real time PCR in the lung samples of all the groups following long term exposures to fipronil with or without LPS. Catalase plays a central role in the antioxidant system of the lungs due to its ability to convert H_2O_2 to oxygen and water. Catalase is the most abundant antioxidant gene among the 22 oxidative and metabolic stress-related genes and is expressed in bronchiolar epithelium in normal adult lung [18]. In the present observations, LPS or both individual doses of fipronil did not alter the mRNA expression of catalase (Figure 1). There was downregulation of catalase activity during bleomycin-induced inflammation and fibrosis in the lung of mice [19]. Catalase handles the intracellular accumulation of H_2O_2 and its toxic derivatives, however hydrogen peroxide reversely downregulates catalase expression [20]. Low levels of catalase expression are correlated with high H_2O_2 production and activation of signaling pathways to cause proliferation, migration and invasion of cancer cells [21]. Decreased catalase elevates H_2O_2 which activate signal transduction pathways for the formation of squamous cell carcinomas [22]. Similarly [23] observed that the immortalization and transformation of mouse liver cells with SV40 (Simian virus 40) decreased catalase expression resulting oncogenesis via increasing the levels of ROS in transformed cells as cancer cells require higher ROS amounts than healthy cells [24].

In the present investigations, low dose of fipronil along with LPS resulted 3.75 folds increase in the mRNA expression of catalase in the lung (Figure 1). Catalase expression increases after initial smoke exposure in lungs of mice [24] reported that catalase overexpression (7-fold) in MCF-7 cells impaired the proliferation and migration capacities of MCF-7 cells and suggested that changes in the expression of catalase

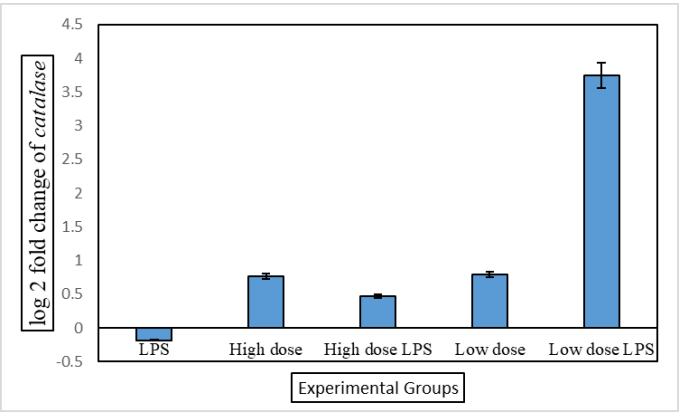

Figure 1: Fold change of catalase mRNA expression in LPS, high dose of fipronil alone or in combination with LPS, low dose of fipronil alone or in combination with LPS.

may be a mechanism of resistance of cancer cells towards redox-based chemotherapeutic drugs. Similarly, [22] observed that catalase plays an important role as an antioxidant to maintain the redox state during the progression step of carcinogenesis.

In the present study, the lung sections incubated without primary antibody did not show any colour development in control group (Figure 2). There was moderate catalase immunopositive reactivity in the cells of airways and bronchium following exposure to LPS (Figure 3). Further, LPS did not alter the number of immunopositive cells (Table 1). High and low dose of fipronil alone or in combination with LPS showed strong immunopositive reactivity for catalase in the airways and bronchial epithelial cells (Figure 2). There was a significant increase in the number of immunopositive cells for catalase following exposure to individual high or low dose of fipronil or in combination with LPS as compared to control and LPS group (Table 1). Catalase is expressed in later stages of pulmonary development and is constitutively expressed in airways and alveolar epithelial cells and macrophages [19].

Catalase expression in serum

In the present study, individual LPS or low dose of fipronil did not alter the serum concentration of catalase (Table 1). However high dose of fipronil significantly ($p<0.05$) increased the protein concentration of catalase as compared to control group suggesting dose dependent dysregulation of catalase in serum (Table 1). Increased serum catalase activity alter immune function, viral replication and/or repair processes [25]. Catalase has both beneficial and deleterious effects as addition of catalase protected cultured endothelial cells against H_20_2 induced damage, while the same concentrations did not effect the neutrophil bactericidal activity or mononuclear cell cytotoxicity in vitro [26]. Catalase expression increases following inhibition of the PI3K/Akt signaling pathway in vascular smooth muscle of rat and human MCF-7 cancer cells [25-29].

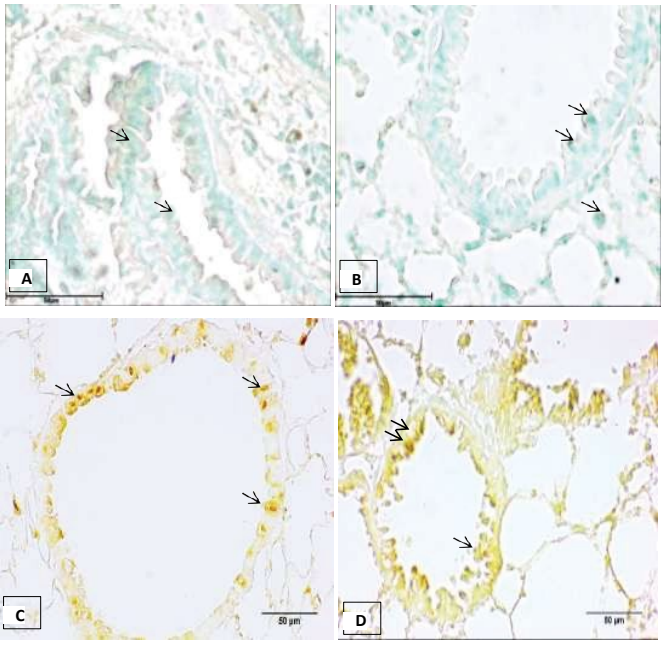

Figure 2: Control section without primary antibody (A-B) resulted in no colour development. Lung sections showed immunopositive reactivity in cells of airways epithelium (arrow) in control group (C-D). Original magnification: 40x.

Table 1: Immunopositive Score and serum concentration of catalase in various groups.

Groups	Number of immunopositive cells	Serum concentration (ng/mL)
Control	199.16 ± 9.19	30.32^{a} ± 0.92
LPS	78.00^{a} ± 6.42	31.45^{a} ± 2.00
High dose	80.66^{c} ± 9.62	78.74^{b} ± 6.68
High dose + LPS	85.50^{b} ± 8.76	29.53^{a} ± 1.32
Low dose	99.33^{b} ± 12.63	29.81^{a} ± 2.56
Low dose + LPS	277.50^{c} ± 14.97	30.36^{a} ± 4.97
Values are Mean ± SE. Means bearing superscript ^{ab} differ significantly at $p \leq 0.05$.		

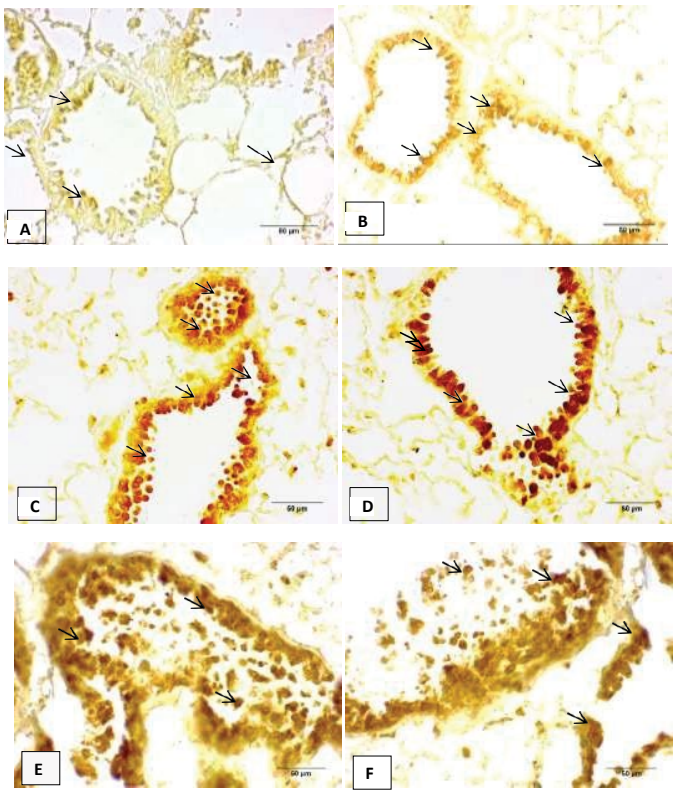

Figure 3: Lung section showing immunopositive Catalase cells in the airway epithelial cells (arrow) in control (A), LPS (B), high dose of fipronil alone (C) or in combination with LPS (D), low dose of fipronil alone (E) or in combination with LPS (F) group. Original magnification: 40x.

Acknowledgement

None

Conflict of Interest

None

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