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# Differential gene expression profile of mice lung following chronic dietary exposure to chlorpyrifos and/or cypermethrin

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**Abstract**
Chlorpyrifos is an organophosphate and the cypermethrin is Type 2 pyrethroid insecticide that are frequently used for indoor and outdoor pest control. The combination of both chlorpyrifos and cypermethrin is commonly used to control pest and prolonged exposures of this combination results in chronic or persistent neurologic and teratogenic effects along with abortions and other reproductive failure. We have earlier reported that delay exposure to cypermethrin and chlorpyriphos results in lung injury, however, the molecular mechanism remains to be elucidated. The present study was designed to study differential transcriptional profiling to identify the candidate genes associated with lung injury after exposure to chlorpyriphos and/or cypermethrin in a mouse model. Swiss male albino mice (N=24) ageing 6-8 week were divided into one control group and three treatment groups (n=8each). Treatment group-I, II and III were given orally chlorpyriphos (2.76 mg/kg), cypermethrin (2 mg/kg) and combination dissolved in corn oil for 90 days. Lung samples were collected and subjected to microarray analysis. The data revealed that 365 genes were dysregulated following exposure to chlorpyrifos, cypermethrin and their mixture. Treatment with chlorpyriphos upregulated 194 genes and downregulated 171 genes whereas cypermethrin treatment caused upregulation of 171 genes and downregulation of 152 genes while their combination upregulated 176 genes and downregulated 146 genes (minimum cut off of 12.0 log fold change). *Atp*5*g*2, *Cox*6*a*1, *Tpt*1, *Hint*1, *Fau*, *Rps*27, *Fth*1, *Rplp*0 *Sftpd and Sftpa* were among the top ten dysregulated genes in all the treatments groups. Protein-protein interaction analysis showed that Atp5g2, Cox6a1, Tpt1, Hint1, Fau, Rps27, Fth1 and Rplp0 forms a network with each other whereas Sftpd and Sftpa1 are co- expressed. Data taken together suggest that exposure to chlorpyrifos and/or cypermethrin may induce lung damage via dysregulating the expression of *Atp*5*g*2, *Cox*6*a*1, *Tpt*1, *Hint*1, *Fau*, *Rps*27, *Fth*1, *Rplp*0, *Sftpd and Sftpa*.. The findings of current study have significance as farm workers get frequent exposures to the cocktail of these pesticides in field situations.

**Keywords:** Chlorpyrifos, cypermethrin, lung injury

## Introduction
Indian agriculture sector is one of the largest agricultural producer around the globe thus, it is a vital asset for the economic development [1]. However, the country suffers from approximately 45% of production loss amounting to Rs 6,000 crores each year because of pest infestation. A wide range of pesticide are used to oppose pest and to enhance the agriculture yield [2]. The unrestrained pesticide usage is increasing in developing and developed countries and can be attributed to demand of crop production from the limited agriculture land [3, 4]. The pesticide used against pest infestation are synthetic, persistent and ubiquitous in nature [5]. Extensive use of pesticides in agriculture has posed havoc to the mankind and environment due to their high rate of bioaccumulation and toxicity [6]. Dietary exposure is among the most common route of pesticide exposure [7]. Cypermethrin and chlorpyrifos are popular pesticide that belong to organophosphate and synthetic pyrethroids [8]. Cypermethrin, synthetic pyrethroid, is an insecticide and one of the most common environmental pollutants due to its extensive use against pest infestation at agriculture and domestic level [9]. Chlorpyrifos is the most commonly utilized insecticide in domestic vegetables [10] and found to be is easily absorbed by organ surface such as intestine and lung [11].
Lung is the first organ to come in contact with after inhalation of pesticides [12]. We have earlier reviewed [13, 14] and reported lung injury following exposures to imidacloprid [15, 16], fipronil [17, 18, 19], lindane [20], indoxacarb[21,22], chlorpyrifos [23], 2,4-D [24, 25] and ethion[26, 27, 28]. The pulmonary impairment due to organophosphate causes alveolar congestion [29],

hemorrhage, neutrophil infiltration [30], emphysematous changes and cellular aggregation in vascular walls or air spaces [310, 32]. Cypermethrin causes oxidative stress [33] and developmental neurotoxicity [33], cellular infiltration, necrotic changes, thickening of alveolar septa and inflammation of lung tissues. The synergistic effect of chlorpyrifos and cypermethrin has shown to cause lung damage in mice [35].
However, there is no transcriptomic data available on the lung following long term dietary exposure to chlorpyrifos and cypermethrin individually or in combination. Hence, the current study was aimed to investigate transcriptomic profile of the mice lung in order to identify the differentially expressed genes exposed to chlorpyrifos and/or cypermethrin.

## Methods

### In vivo experiments

The experiment has been conducted as per approved protocol of institutional animal ethics committee (CPCSEA), Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. A total 24 albino Swiss male mice ageing 6-8 weeks were purchased from the disease free small animal colony maintained by Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar Haryana. Mice were acclimatized for a period of one week. Mice were weighed and divided randomly into 4 groups (n=6) in polypropylene cages at institutional small animal house under controlled condition with 12h light and dark cycle. Animals were fed synthetic pelleted mice feed obtained from Ashiward feed industries, Chandigarh and water *ad libitum*.

### Experimental design

Animals were randomly divided into three treatment groups that received either cypermethrin (cypermethrin @2mg/kg) or chlorpyriphos @2.76mg/kg or their mixture orally dissolved in corn oil for 90 days. The control group received corn oil for 90 days. Immediately after the experiment, all animals were euthanized with xylazine and ketamine combination (Xylazine @ 0.5 ml; 20 mg/ml mixed with Ketamine @ 2ml; 50 mg/ml) intraperitoneally.

### Tissue collection

Lung tissue was collected in 2ml tube Rnase free centrifuge tube containing 1ml RNA later (Ambion, Austin, TX,USA) and stored at -80ºC and were subjected to RNA isolation for transcriptional analysis.

### Microarray gene expression and analysis

About 50mg of lung tissue was used from each animal to extract RNA using Trizol method (Ambion, Life Technologies, USA). The quality of the isolated RNA was determined in Agilent 2100 Bioanalyzer as per manufacturer's protocol using the Agilent RNA 6000 Nano Kit. The RNA samples with an RNA integrity > 7 were selected for microarray hybridization. Low input quick Amp WT labeling Kit was used to label 100ng of total RNA as per manufacturer's protocol. RNA samples of three mice from each group were pooled into two biological replicates and one color microarray based exon analysis were performed in duplicates using two mouse microarray slides (8x60K: Agilent—028005). The quality check of labelled cRNA was performed by NanoDrop. The signal intensities were extracted after generation of the microarray scan images using Feature extraction software version 10.7.3. The obtained microarray data were analyzed to identify the differentially expressed genes (DEGs) with cut off of 12 log fold change using DAVID online Bioinformatic tool(https://david.ncifcrf.gov/tools.jsp).

### Pathway analysis and functional Annotation

David bioinformatic tool was used to perform the analysis of the DEGs with cut off of 12. Gene ontology (GO) enrichment analysis of the top 10 Differently expressed genes were then conducted to investigate the biological processes enriched in the different experimental groups. Four categories of gene ontology i.e, biological process, cellular component, molecular function and protein class were analyzed separately by panther online tool. The heat maps of the top 10 dysregulated genes were generated in Prism 9.0.

## Results and Discussion

To determine whether or not exposure to chlorpyrifos and/or cypermethrin cause any change in the expression profile of pulmonary genes in mice model, the transcriptomic analysis was performed for samples from all the groups using microarray technique. The genes that displayed a 12 folds dysregulation were considered. A total of 365 genes were dysregulated following exposure to chlorpyrifos, cypermethrin and their mixture. Treatment with chlorpyriphos upregulated 194 genes and downregulated 171 genes whereas cypermethrin treatment caused upregulation of 171 genes and downregulation of 152 genes while their combination upregulated 176 genes and downregulated 146 genes (minimum cut off of 12 log fold change) (Fig 1A). The gene overlap study between differentially expressed genes (DEG) in all the groups showed 241 commonly expressed genes whereas chlorpyrifos, cypermethrin and their mixture showed 67, 32 and 13 DEG's respectively (Fig 1B). We have earlier reported that fipronil exposure induces the lung damage and alters the pulmonary transcriptomic profile in mice [36].
Further, we selected top 10 DEG's for the identification of candidate genes responsible for lung damage. The gene expression and heat map along with their functional significance of these genes from microarray as has been presented in Figure 2. Protein-protein interaction (PPI) network was also generated for these genes using STRING online program on the basis of co-expression, co-occurrence and molecular function (combined score >0.4; K means clustering=3) (Fig 3). The PPI clustered *Atp*5*g*2, *Cox*6*a*1, *Tpt*1, *Hint*1, *Fau*, *Rps*27, *Fth*1 and *Rplp*0 while strong coexpression and co-occurance was depicted for *Sftpd and Sftpa1*. Microarray data revealed dysregulation of mRNA expression of *Atp5g2, Cox6a1, Tpt1, Hint1, Fau, Rps27, Fth1, Rplp0, Sftpd,* and *Sftpa1* in all treatments groups.
Gene ontology study revealed that *Cox*6a1, which is a mitochondrial complex IV (Fig 3A- C) and *Atp5g2* that play vital role in mitochondrial functioning. There was downregulation of *Atp5g2* (-14.22, -13.75 and -13.73) and Cox6a1 (-14.91, -14.65 and -14.80) following the exposure to chlorpyrifos, cypermethrin and their combination, respectively (Table 1). *Cox*6a1 and *Atp5g2* are associated with mitochondrial dysfunction and are under expressed in thyroid cancer [37] and clear cell renal cell carcinoma [38], respectively. Pulmonary surfactant-associated protein D (*Sftpd*) and Pulmonary surfactant-associated protein A (*Sftpa1*) contributes to the lung defence against inhaled organisms and toxins beside modulating leukocyte action in immune system. Exposure to chlorpyrifos, cypermethrin and their combination downregulated the pulmonary expression of

*Sftpd* (-14.30, 14.15 and -14.46) and *Sftpa1* (-14.10, 14.26 and -14.46) (Table 1). The expression of these pulmonary surfactant-associated proteins is inversely correlated with lung cancer progression [39, 40]. Further, exposures simultaneously downregulated the *Tpt1* (-14.79, -14.52 and -14.40) which have a novel role as the negative regulator of autophagy [41].

There was downregulation of *FTH1* (-14.83, -14.52 and -14.64) and *Hint1* (-14.6, -14.39 and -14.47) following the exposure to chlorpyrifos, cypermethrin and their combination, respectively (Table 1). Under expression of *FTH1* has close association to tumorigenesis in breast cancer and colorectal carcinoma, suggesting a tumor-suppressive role [42]. Similarly *H*int1 is a tumor suppressor gene that inhibits oncogenic factors and enhances the expression of p53 and Bax with the down expression of Bcl2, an apoptotic inhibitor to regulate apoptotic pathways [43]. Previously available data suggest decline in hint1 expression may lead to cancer [44].

Ribosomal P complex consists of the acidic ribosomal P (RPLP) proteins *RPLP0*, *RPLP1* and *RPLP2* and recruits translational factors to facilitate protein synthesis. Exposure to chlorpyrifos, cypermethrin and their combination downregulated the pulmonary expression of Rplp0 (-14.46, -12.71 and -14.00) (Table 1). Reduced expression of *Rplp0* causes cell cycle arrest in gastric cancer cells [45, 46]. *FAU* encodes a ubiquitin-like protein called FUBI with ribosomal protein S30 as a carboxy-terminal extension and is a pro-apoptotic regulatory gene. There was downregulation of Fau (-14.72, -14.47 and -14.63) and Rps27 (-14.85, -14.60 and -13.92) following the exposure to chlorpyrifos, cypermethrin and their combination, respectively (Table 1). Its downregulation has been reported in prostate and ovarian tumors and are strongly associated with poor prognosis in breast cancer [47]. *Rps*27, also called metallopanstimulin-1 (MPS-1), play important role in regulation of the mitotic checkpoint, spindle assembly and sister chromatid biorientation [48]. Its role in mitosis suggest that a decline in the expression RPS27 may cause mitotic check point dysfunction and chromosome alignment defect [49]. Data taken together indicate that long term dietary exposures to chlorpyrifos and cypermethrin individually or in combination downregulated the mRNA expression of Atp5g2, Cox6a1, Tpt1, Hint1, Fau, Rps27, Fth1, Rplp0, Sftpd, and Sftpa1 in the lung of mice.

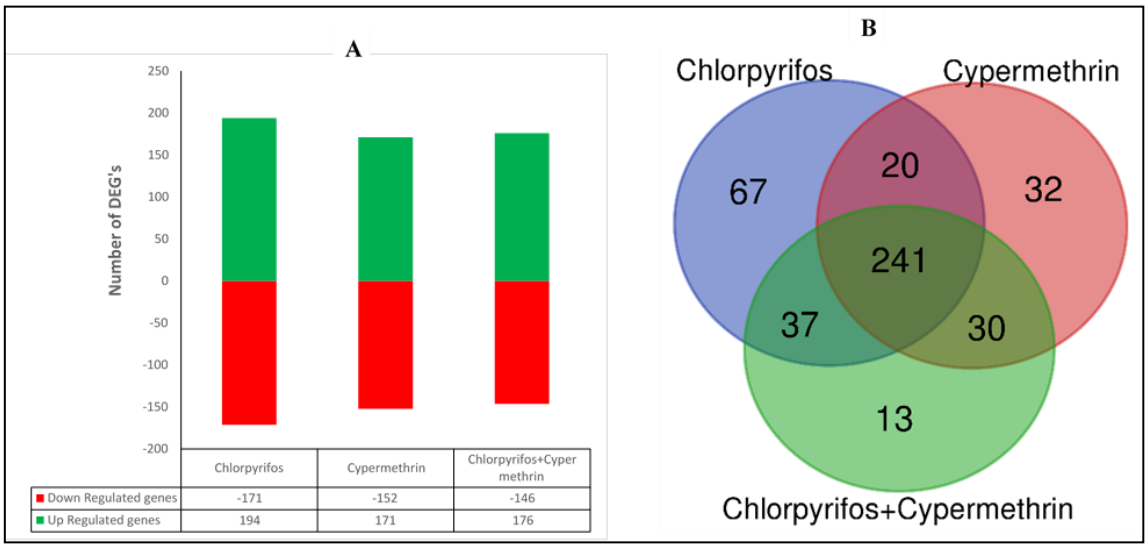


**Fig 1:** Global View (A) Venn diagram (B) of differentially expressed genes ( cut off of 12 log fold change) following exposure to Chlorpyriphos, Cypermethrin and their combination.

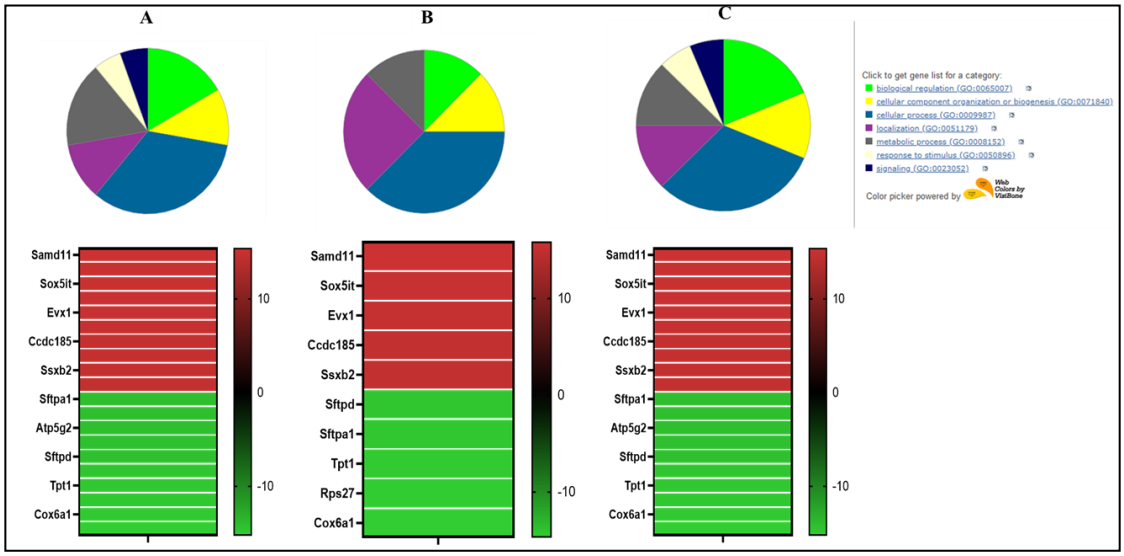


**Fig 2:** (a) Biological significance and Heat Map of Top 10 DEG's following exposure to Chlorpyriphos (A), Cypermethrin (B) and their combination (C).

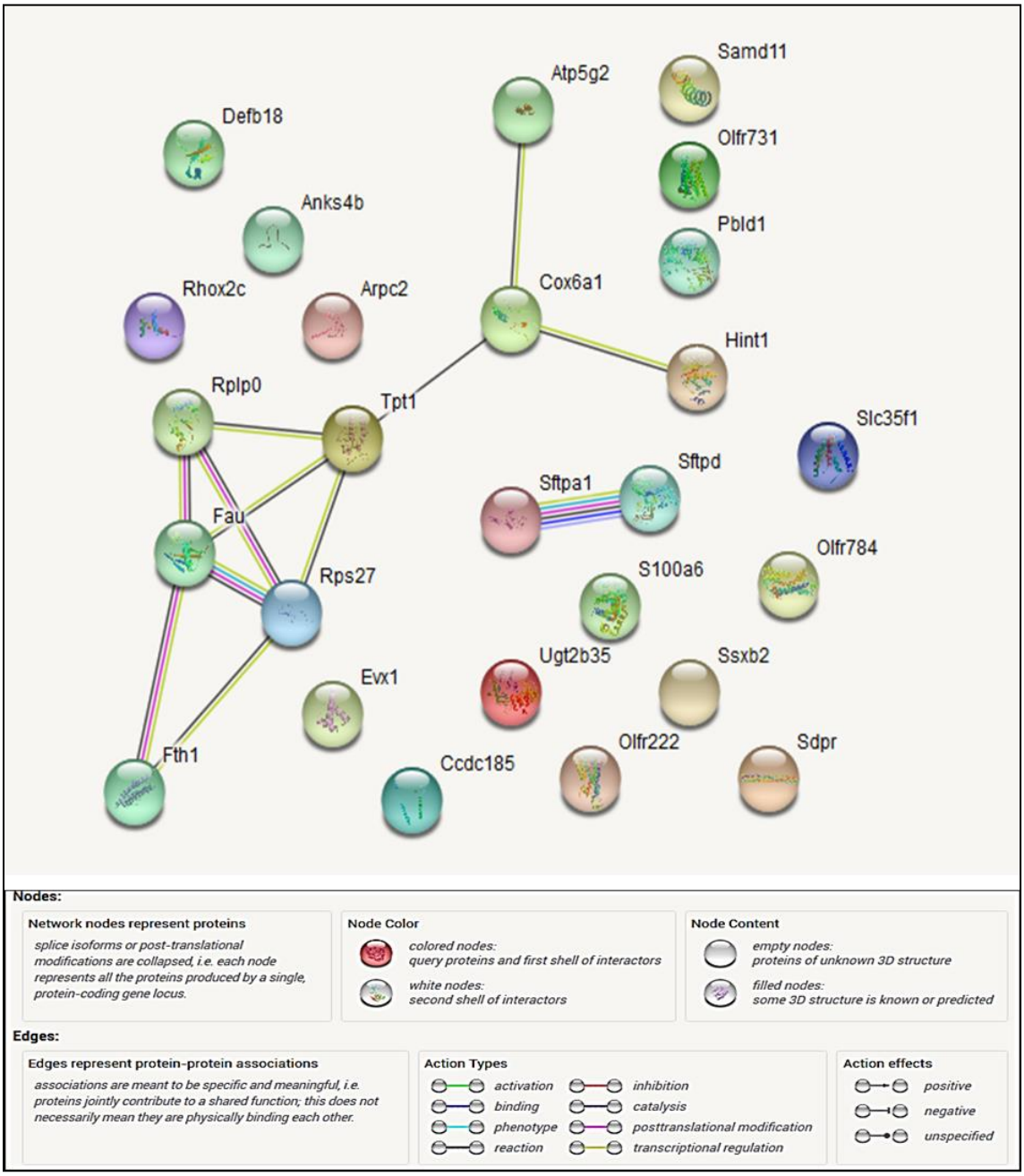


**Fig 3:** PPI network of top 10 DEGs following exposure to Chlorpyriphos, Cypermethrin and their combination.

**Table 1:** Log2 fold change of Top 10 differentially expressed genes in various treatment groups,

| Chlorpyrifos | | Cypermethrin | | Chlorpyrifos+Cypermethrin | |
|---|---|---|---|---|---|
| **Name of genes** | **Log2 Fold change** | **Name of Gene** | **Log2 Fold change** | **Name of gene** | **Log2 Fold change** |
| *Samd11* | 15.35 | *Samd11* | 15.79 | *Samd11* | 15.65 |
| *Sox5it* | 15.00 | *Sox5it* | 15.33 | *Sox5it* | 15.27 |
| *Evx1* | 14.77 | *Evx1* | 15.15 | *Evx1* | 14.82 |
| *Ccdc185* | 14.62 | *Ccdc185* | 14.99 | *Ssxb2* | 14.76 |
| *Ssxb2* | 14.59 | *Ssxb2* | 14.91 | Atp5g2 | -13.73 |
| *Sftpa1* | -14.10 | *Sftpd* | -14.15 | *Sftpa1* | -13.86 |
| *Atp5g2* | -14.22 | *Sftpa1* | -14.26 | *Sftpd* | -14.46 |
| *Sftpd* | -14.30 | *Tpt1* | -14.52 | *Tpt1* | *-14.40* |
| *Tpt1* | -14.79 | *Rps27* | -14.60 | Hint1 | -14.47 |
| *Cox6a1* | -14.91 | *Cox6a1* | -14.65 | *Cox6a1* | -14.80 |

## Conclusion

In this study, we have identified the differentially expressed pulmonary genes following the chronic dietary exposure to chlorpyrifos and/or cypermethrin. PPI showed that *Atp*5*g*2, *Cox*6*a*1, *Tpt*1, *Hint*1, *Fau*, *Rps*27, *Fth*1 and *Rplp*0 forms a network with each other whereas *Sftpd* and *Sftpa1* are co-expressed. Data taken together suggest that exposure to chlorpyrifos and/or cypermethrin may induce lung damage via dysregulating the expression of *Atp*5*g*2, *Cox*6*a*1, *Tpt*1, *Hint*1, *Fau*, *Rps*27, *Fth*1, *Rplp*0, *Sftpd and Sftpa*.. The

findings of current study have significance as farm workers get frequent exposures to the cocktail of these pesticides in field situations.

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