



Pulmonary expression of MYCN mRNA following exposure to 2,4-D with or without endotoxin challenge

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ABSTRACT

The present study aimed to observe the expression of MYCN in lungs of mice following chronic exposure of 2,4-D with or without lipopolysaccharide (LPS). 2,4-D was administered orally dissolved in corn oil at high and low dose (1/10th and 1/20th of LD50) for 90 days. After 90 days of exposure, animals from each group were challenged with LPS/normal saline solution at 80 µg/animal. The lung tissues were processed for microarray and real time studies. LPS resulted decrease (–0.173 fold) in m-RNA expression level of MYCN as compared to control, while High dose of 2,4-D alone and in combination with LPS resulted 0.949-fold change and 1.656-fold change increase in expression of MYCN m-RNA, respectively, as compared to control. Similarly, Low dose of 2,4-D alone or in combination with LPS also altered MYCN expression. The microarray data when validated by Real Time PCR was found to be in concordance with the Real Time PCR data. The data taken together suggest that, high and low exposure of 2,4-D alone or in combination with LPS alters expression of MYCN at m-RNA level.

Key words: 2,4-D, Mice, Microarray, MYCN, Quantitative real time PCR

To achieve sustainable production, we either use genetically improved high yielding variety of crops which enhance the soil fertility via chemical fertilization and control the pests via the use of synthetic pesticides (Oerke and Dehne 2004). Among these pesticides, 2,4-dichlorophenoxyacetic acid is one of the most widely used herbicide around the world (Aydın *et al.* 2005). This 2,4-D herbicide belongs to the phenoxy or phenoxyacetic acid herbicide family (Sarikaya and Selvi 2005). It acts similar to natural auxin (indole-3-yl-acetic acid) and plays important role in the plant cell division, differentiation and elongation (Song 2014).

However, herbicides exposure to different environmental conditions causes their degradation process via biological or photolytic mechanism (Sklivagou *et al.* 2010). The frequent use of 2,4-D possess environmental contamination and damage to non-target species (Kalipci *et al.* 2011) as it interferes with some chemical reactions, enzymes activity, cell functions and DNA structure causing mutation, free radical formation and apoptosis (Song 2014).

MYCN is a family of proto-oncogene protein and regulator genes that plays key roles in the cell proliferation, cell cycle, differentiation and apoptosis (Okuyama *et al.* 2010). However, aberrant MYCN gene expression has been

identified in almost all human tumors (Mai and Mushinski 2003). MYCN gene activation is frequently associated with the tumors progression, poor patient prognosis and malignant properties such as increased mobility, invasive and metastatic capacities (Dang 2012). However, there is no data on the pulmonary expression of MYCN following exposure to 2,4-D.

Endotoxin is a lipopolysaccharide (LPS) molecule, present ubiquitously in the environment (Heinrich *et al.* 2003). Its exposure is associated with increased risks of respiratory health and decrement in lung function (Liebers *et al.* 2006). We have earlier reported that endotoxin interaction with pesticides alters the pulmonary responses (Pandit *et al.* 2016, Kaur *et al.* 2016, Tewari *et al.* 2017, Sethi *et al.* 2018, Verma *et al.* 2019, Pandit *et al.* 2019). Keeping all this in view, the present study was planned to understand the effect of chronic exposure of 2,4-D alone or in combination with LPS on the expression of MYCN mRNA in lung homogenates in a mouse model.

MATERIALS AND METHODS

Chemicals: Technical 2,4-Dichlorophenoxyacetic acid, Plant Culture Tested (Catalogue no. PCT0825) was obtained from Himedia, Nashik. Corn oil (Catalogue no. C8267) and lipopolysaccharide (LPS) from *Escherichia coli* (CAS no L3129) were obtained from Sigma-Aldrich, Bengaluru, India. The others chemicals included Trizol reagent (Life Technologies), c-DNA first strand synthesis kit (Thermo Scientific, USA).

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Animals: Forty-eight healthy male albino mice aged 6–8 weeks were purchased from the Disease-Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. The animals were given synthetic pelleted diet and water *ad libitum*. These mice were acclimatized for one week prior to the start of the experiment. All the protocols were approved by Institutional Animal Ethics Committee (IAEC), Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana.

Experimental design: Mice (N=48) were randomly divided into three groups, viz. two treatments and one control group (n=16 each group). The treatment groups were administered 2,4-D @1/10th and 1/20th of LD50 orally dissolved in corn oil for 90 days. At the end of experiment eight animals from all groups were anaesthetized with Xylazineketamine combination anesthesia (1/10th of the body weight) and challenged with LPS @ 80 µl/animal intranasally. The remaining eight animals of each group were administered 80 µl of normal saline solution (NSS) intranasally. After anaesthesia, the animal was dissected and lung samples were collected in RNAlater solution for microarray and quantitative real time PCR analysis.

Microarray for MYCN: Microarray analysis was performed using the mouse microarray slide of format 8 × 60 K (ID No: 0307760384 Agilent Technologies). Labeling of RNA samples was done with Low Input Quick Amp WT Labeling Kit as per manufacturer's instructions. Microarray was performed in duplicates, so in each group RNA samples from three mice were pooled into 2 samples. Using Nanodrop, the quality check of the labeled cRNA was done. Hybridization and scanning was performed as per the manufacturer's protocol. After generating the microarray scan images, the feature extraction was done by using Feature Extraction software version 10.7.3 and data generated after feature extraction was further analyzed by using Agilent software Genespring version 14.9 to identify the differentially expressed genes (DEGs) (P<0.05) in mice lung samples.

Quantitative Real Time PCR (qPCR): Right lung from each animal stored in RNAlater solution at –80°C was used for detection of expression of MYC-N mRNA by qPCR. 100 mg of frozen lung tissue was homogenized using QiagenTissueRuptor II (Cat No: Invitrogen 9002755) and total RNA was extracted from all the samples using Trizol (Ambion, Life Technologies, USA) method following manufacturer's instructions. The quality as well as quantity of the resulting RNA was assessed spectrophotometrically by Nanodrop (Thermo Fisher) and also by visualizing the ribosomal RNA bands 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'-CCTCCGGAGAGGATACCTTG-3' and R 5'-TCTCTACGGTGACCACATCG-3' (Wu *et al.* 2017). Further, β-actin (F5'-CTGTCCCTGTATGCCTCTG-3' and R5'-ATGTCACGCACGATTTC-3' (Chen *et al.* 2014) was used as an endogenous control. Amplification of MYCN mRNA required an initial denaturation step at 95°C for 10 min. Temperature cycling consisted of 40 cycles of denaturation at 95°C for 15s, annealing and elongation at 60°C for 1 min. Transcript levels were normalized by comparison with β-actin.

Statistical analysis: Data were presented as mean (±SE) and appropriate transformations were made. The data were analyzed 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.

RESULTS AND DISCUSSION

Microarray analysis for MYCN: Microarray analysis revealed the up regulation of MYCN mRNA following exposure to 2,4-D with or without LPS. There was 0.949, 1.656, 1.138, 1.51 fold increase in the expression of MYCN following exposure to high dose of 2,4-D alone or in combination with LPS and low dose of 2,4-D alone or in combination with LPS, respectively. LPS downregulated (–0.173 folds) the mRNA expression of MYCN. MYCN, is one of the MYC family members that plays an important role in regulating cell growth and division/proliferation and the self-destruction of cells/apoptosis (Fulda *et al.* 2000). MYCN contributes to all facets of metastasis, viz. adhesion, motility, invasion and degradation of surrounding matrices (Huang and Weiss 2013).

Quantitative Real Time PCR (qPCR): The real time PCR (qPCR) showed the up regulation of MYCN gene following exposure to 2,4-D with or without LPS. The increase was 1.14, 1.74, 1.26, 1.46 fold following exposure to high dose of 2,4-D alone or in combination with LPS and low dose of 2,4-D alone or in combination with LPS, respectively (Fig. 1). LPS group showed downregulation of MYCN by –0.25 fold (Fig. 1). The real time data was found to be in concordance with microarray data. Further, MYCN also decreases the expression of anti-proliferative proteins Dickkopf-1 and CDKL5 to disrupt the WNT/β-catenin signalling pathway and arrests cells between G₀/G₁ phase, respectively (Valli *et al.* 2012). MYCN promotes transcription of focal adhesion kinase (FAK), a critical regulator of integrin signalling and generally promotes increased migration and metastasis in tumour cells (Megison *et al.* 2013). Increased and dysregulated expression of MYCN is responsible for the development of multiple tumours, including tumours of the nervous and hematologic systems and neuroendocrine tumours in other organs (Rickman *et al.* 2018). The data taken together suggest that 2,4-D alone or in combination with LPS upregulate the mRNA expression of MYCN in lungs suggesting its role in modulating the lung damage.

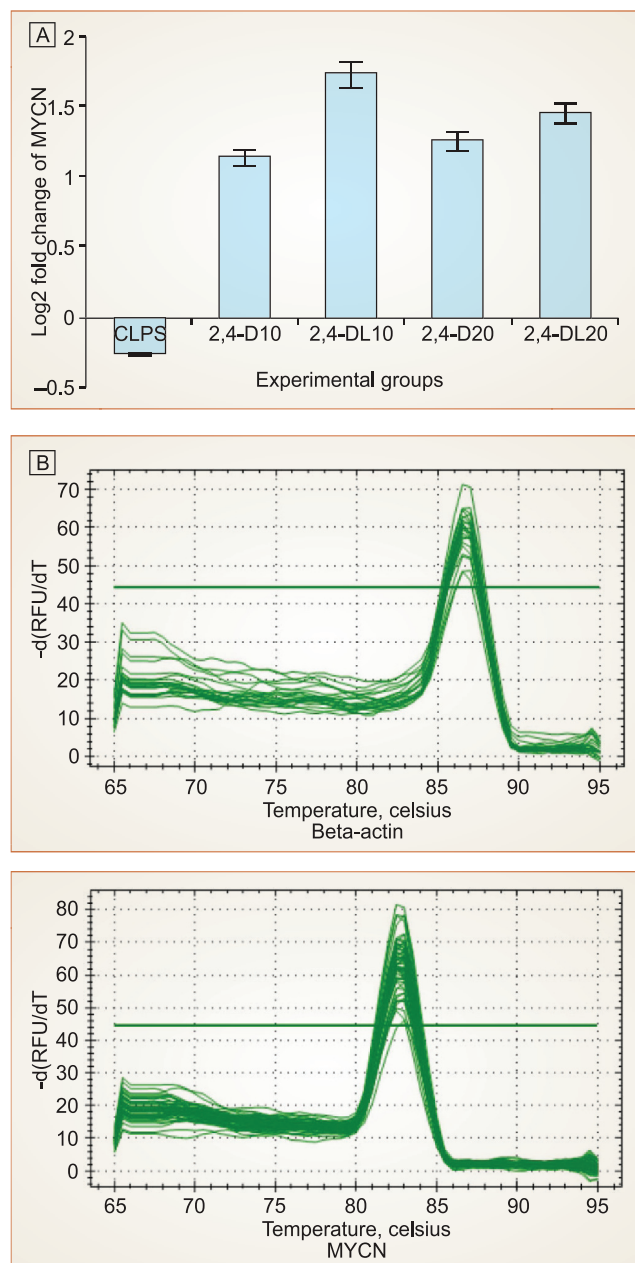


Fig. 1. Log 2 fold change of MYCN mRNA expression in LPS, high dose of 2,4-D alone (D10) or in combination with LPS (DL10), low dose of 2,4-D alone (D20) or in combination with LPS (DL20).

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