



## Research Article

### Apoptotic Gene Expression in Endosulfan Exposed IBD Infected Birds

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#### ABSTRACT

Chlorinated insecticide endosulfan is a common environmental contaminant. Humans, animals, and birds are equally exposed through water and food. The side effects of the endosulfan are comparatively well documented; the toxicological impact of endosulfan in infectious bursal disease (IBD)-infected birds is still unpredictable and unknown. The present study was undertaken to assess whether the endosulfan exposure in IBD infected birds induces significant alterations in apoptotic, heat stress and genes of immunological functions. The chicks were divided into three groups (Group-1: Control, Group-2: IBD infected birds and Group 3: endosulfan intoxicated IBD infected birds). All the chicks from group-2 and -3 were infected with IBD IM+ strain in the laboratory on day 7 and chicks of Group-3 were exposed to 30 ppm of endosulfan-mixed feed for up to 28 days. After the exposure period of 21 days, chicks were sacrificed. Heparinized blood and the liver were collected aseptically and processed under standard protocol of RNeasy<sup>®</sup> Minikit of Qiagen for the gene expression study with single step real-time PCR and DNA content analysis using Propidium Iodide dye using FACS (Fluorescence-activated cell sorting) technique. The FACS analysis showed increased percentage of apoptotic cells in Group-3 birds from that of Group-2 and Group-1 birds. The mRNA level expression of apoptogenic genes (Caspases-3 and NF- $\kappa$ B) was found positively regulated in the Group-3 and Group-2 birds as compared to Group-1. However the expression of Caspase-3 and NF- $\kappa$ B was significantly higher in Group-3 from Group-2 birds. In Group-2 and Group-3 the genes of cell protection machinery such as hemeoxygenase 1, Cyclin-D1, HSP-70, and antiapoptotic mitochondrial pathway genes expression BCL-X1, BCL-2, MCL-1, apoptotic pathway genes CARD, RIPIC, FAS-L, Caspase-10 were negatively regulated as compared to Group-1 birds, but their expression was more profound in Group-2 birds. The results of the study imply that the experimental exposure of birds with Endosulphan follows Caspase-3, NF- $\kappa$ B and mitochondrial pathways of apoptosis in the liver of IBDV infected birds. This is the preliminary report describing the mechanism of apoptosis in liver following exposure to endosulfan at environmentally realistic concentrations in IBD infected birds. The study suggests that in presence of toxicants in feed/water, the pathogenic disease may produce differential and rather more pathogenic consequences.

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#### INTRODUCTION

Endosulfan is a member of the cyclodiene group of organochlorine pesticides and is used worldwide in agriculture. The cyclodiene insecticides are among the most toxic and environmentally persistent insecticides known so far. Endosulfan is a highly toxic pesticide, and it belongs to toxicity class I in the Environmental Protection Agency (EPA) (ExToxNet, 2000). Endosulfan is also a known environmental endocrine disrupter (Rose et al., 1999). Organochlorine insecticides are now harshly regulated in the United States but are still in production and are used

extensively in developing countries because of their effectiveness and inexpensiveness. Along with picrotoxin-binding, cyclodienes inhibit Na<sup>+</sup>, K<sup>+</sup>-ATPase, but more importantly inhibit Ca<sup>2+</sup>, Mg<sup>2+</sup>-ATPase. This leads to an accumulation of intracellular free calcium ions, which promotes the release of neurotransmitters and subsequent depolarization of adjacent neurons.

Endosulfan is stable in the environment and, because of its lipophilicity, can be stored in fat tissue and released later during sickness or starvation. Endosulfan has genotoxic effects on HeoG2 cells (Yuquan et al., 2000) and has been

found to inhibit testicular function in pubertal rats (Chitra et al., 1999). Along with these negative effects, endosulfan is immunotoxic, inhibiting the metabolic activity of peripheral blood lymphocytes and splenocytes in chickens (Aggarwal et al., 2008), induces oxidative stress (Sohn et al., 2004, Ayub et al., 2003, El-Shenawy, 2010, Pradhana et al., 2012) and apoptosis (Aggarwal et al., 2003, Kannan et al., 2000).

IBDV is a double stranded RNA virus belonging to the *Avibirnavirus* genus in the family *Birnaviridae* causes an acute disease known as Gumboro disease. This disease is characterized by high mortality and morbidity. Chickens infected with IBDV at <3 weeks of age usually have little or no clinical signs but they are immunosuppressed. IBDV can induce apoptosis in chicken peripheral blood lymphocytes (Vasconcelos and Lam 1994, Jain et al., 2013), indicating that it may play a crucial role in the depletion of lymphoid cells, in apoptosis and immunosuppression. Previous studies have shown chicken embryo fibroblasts exhibited morphological and biochemical features of apoptosis when infected *in vitro* with IBDV (Tham et al., 1995, Kumar 2009). Infection of chicken embryos and young chickens with IBDV induced contagious disease of young chickens (Vasconcelos and Lam 1995).

There are several types of viruses and pesticides contribute to oxidative stress and apoptosis. Reports also confirm the ability of endosulfan and IBDV to induce apoptosis independently. But no report confirms the toxic effects of endosulfan inducing apoptosis, heat stress and other related genes of apoptotic functions in IBDV infection. The main purpose of the present research was to investigate the side effects of sub acute exposure of endosulfan in environmentally realistic conditions on IBD infected chickens, and secondarily to analyze the potential pathways involved in endosulfan-induced apoptosis in IBDV infected birds.

## MATERIALS AND METHODS

### *Animals*

Day old chicks were procured from Central Avian Research Institute, Izatnagar, India. The chicks were acclimatized for seven days and maintained under controlled conditions of temperature and light (12/12-h light/dark cycle). Food and water were provided *ad-libitum*. After the period of acclimatization the chicks were divided into three groups (Control (Group-1), IBD infected birds (Group-2) and endosulfan intoxicated IBD infected birds (Group-3) comprising seven birds in each group.

### *Preparation of Pesticides*

Endosulfan was purchased from Sigma Aldrich Ltd. and was mixed in feed with a rate of 30 ppm (30 mg/kg feed). The endosulfan-mixed feed were prepared just before use and fed to chicks *ad-libitum*.

### *Treatments*

All the chicks were infected with the IBD IM+ strain by ocular route with  $10^{3.0}$  TCID<sub>50</sub> of virus on day 7. Same day chicks were exposed to 30 ppm of endosulfan-mixed feed for up to 28 days. The dose of the endosulfan in the present study was chosen based on the study of Aggarwal et al., (2003).

### *Sample Collection*

After the exposure period of 21 days, chicks were sacrificed. Heparinized blood, bursa and the liver were collected aseptically and processed under standard protocol of

RNeasy® Minikit of Qiagen for the gene expression study with single step real-time PCR and DNA content analysis using PI dye using FACS (Fluorescence-activated cell sorting) technique.

### *Apoptosis Detection (Flowcytometric Method Using Propidium Iodide Dye)*

The experimental birds were sacrificed and bursa was collected. Bursa was minced finely with scissors, and minced tissue was washed several times in PBS. Bursal cell suspension obtained from control and IBDV infected birds were layered on Histopaque and centrifuged at 1500 r.p.m. for 10 minute. The cells at the interphase was collected, washed twice in Phosphate buffer saline and suspended in 500 µl of propidium iodide hypotonic buffer and incubated at 37 °C for 15 Minuit in the dark. The PI fluorescence was measured through FL-2 filter in Becton Dickenson Flowcytometer (NJ, USA).

### *Real-Time Polymerase Chain Reaction (RT-PCR)*

The aseptically collected liver were homogenized for single cell suspension and total RNA was isolated using RNeasy® Minikit of Qiagen under standard protocol for the gene expression study with single step real-time PCR. The primer set for NF-κB, caspase-8, FAS-L, BCL-XL, BCL-2, cyclin-D1, Heme oxygenase (decycling)-1 (HMOX1), heat shock protein-70 (HSP-70), and glyceraldehyde phosphate dehydrogenase (GADPH) (House Keeping Gene) were the published sequences (Kumar et al., 2010). Other primer sequences used were sense: 5'-CACGGACGCATTGGTCTCAT-3', anti-sense: 5'-CAGGTCTCAACTCGGAAGAAG-3' for MCL-1; sense: 5'-GTGTGGCAGCAGAAGCAATG-3', anti-sense : 5'-TTAGCACCATCCCTGAGACAAC-3' for Ripic, Receptor-Interacting Serine-Threonine Kinase-2, (RIPK-2); sense: 5'-CAGCGAGAAGGACATCAAC-3', anti-sense: 5'-AAGGAGCCACAGGACAAG-3' for Caspase Recruitment Domain Family, Member-10 (CARD); sense: 5'-TATTCTACTGCTCCAGGCTACTAC-3', anti-sense: 5'-ACACGAGTTAAAATCTGCATGAGT-3' for Apoptosis-Related Cysteine Peptidase (caspase-3) and Sense : 5'-CAGCGTCCTGTCTATGTTG-3'; anti-sense: 5'-AAGAGGCATATCCATCAATCG-3' for Apoptotic Cysteine Protease (Caspase-10). We used a relative quantification method ( $\Delta\Delta Ct$  method) to calculate the gene expression values as described (User Bulletin No. 2, Applied Biosystems). In brief, the amplification plot is the plot of fluorescence versus PCR number. The threshold cycle value (Ct) is the fractional PCR cycle number at which the fluorescent signal reached the detection threshold. Therefore, the input mRNA copy number and Ct are inversely related. Ct value was automatically converted to fold change RQ value. The Fold change (RQ) =  $2^{-\Delta\Delta Ct}$ , where  $\Delta\Delta Ct = (\Delta Ct_{\text{trt}} - \Delta Ct_{\text{control}}) = -[(Ct_{\text{Target}} - Ct_{\text{GADPH}})_{\text{trt}} - (Ct_{\text{Target}} - Ct_{\text{GADPH}})_{\text{control}}]$ . The RQ values from each gene were then used to compare the apoptotic gene expression across all time intervals. Statistical analysis of data was performed using SPSS 10 software. Various parameters were expressed as mean±SE. Means of various parameters in IBDV Im+ virus treated group with control group by using one way ANOVA with Tukey's B multiple comparisons as post hoc test (Snedecor and Cochran, 1989). A p value of p<0.05 was considered as statistically significant.

## RESULTS AND DISCUSSION

The FACS analysis using Propidium Iodide dye showed increase in the percentage of apoptotic cells in bursal cells harvested from endosulfan fed IBDV infected birds from that of IBD alone infected and control birds. In endosulfan intoxicated IBDV infected birds the proportion of cells with a lower DNA content increased considerably, than the IBDV alone infected and control birds, indicating the profound induction of apoptosis.

Previous studies revealed that endosulfan induces oxidative stress (Dorval et al., 2003 Sohn et al., 2004, Ayub et al., 2003, Dorts et al., 2009, Ballesteros et al., 2009, El-Shenawy 2010). Previous studies have indicated that certain chemical exposures can result in the alteration of secondary messengers, such as free radicals or ROS, and these alterations have been linked to the induction of apoptosis in immune cells (Corcoran et al., 1994). Free radicals influence gene expression, regulate cellular responses to cytokines, as well as proliferative events of a cell. All these events have also been implicated as possible triggering mechanisms of apoptosis. Oxidative stress may trigger the mitochondrial pathway. Increased permeability of the mitochondrial membrane during apoptosis triggers the release of cytochrome *c* from mitochondria in the apoptotic process. The release of cytochrome *c* into the cytoplasm is a critical step, which is followed by the activation of different caspases like caspase-9 and caspase-3 (Shen et al., 2004). In the present study there is significant down regulation of mitochondrial pathway genes like MCL-1 and BCL-XL was observed in both the endosulfan intoxicated IBDV infected birds (0.36±0.23 and 0.33±0.008 respectively) (Figure 1) and IBDV alone infected birds and (0.11±0.0012 and 0.008±0.0012 respectively) in comparison to control birds. Down regulation in of mitochondrial pathway genes were significantly more profound in the IBDV alone infected birds. This finding suggests though the roles of intrinsic

pathway genes in apoptosis of hepatocytes in IBDV infected endosulfan intoxicated birds are there but it is of less magnitude in endosulfan intoxicated IBDV infected birds. This resulted in several morphological and biochemical changes leading to apoptosis of the cell (Hengartner 2000). Mitochondrial (Intrinsic) pathway was shown to get activated at different time point of virus life cycle in IBDV induced apoptosis (Kumar et al., 2010). Anti-apoptotic genes like BCL-2 and BCL-XL acts as inhibitors of cell death and the suppression of apoptosis. BCL-2 can neutralize one another's function, suggesting that their relative concentration in the given cell may act as a rheostat for the suicide program. BCL-2 is localized on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope and counter balance the oxidative damage done to these compartments and affect their behavior (Hockenberry 1995, Antonsson et al., 1997, Yang and Korsmeyer 1999).

Mitochondrial DNA is also heavily damaged by ROS at the bases. Transcription factors such as NF-κB, p53 and AP-1 are also sensitive to redox changes in mammalian cells, primarily through redox regulation of their DNA binding regions (Morel and Barouki 1999; Ho and Bray 1999; Du et al., 1999; Williams and Smith 1993; Li et al., 2013; Li et al., 2014). NF-κB is a transcription factor that has a crucial role in regulation of cell survival, protecting cells from apoptosis under most circumstances and accelerating apoptosis in others. NF-κB is activated via degradation of the inhibitor of NF-κB (IκB). In the present study, gene expression studies by real-time PCR revealed the mRNA level expression of apoptogenic genes Caspases-3 and NF-κB were found positively regulated in the endosulfan intoxicated IBDV infected birds (1.33±0.22 and 3.99±1.2 respectively) and down regulated in IBD alone infected birds ( 0.93±0.0014 and 0.10±0.0029 respectively) in comparison to control birds.

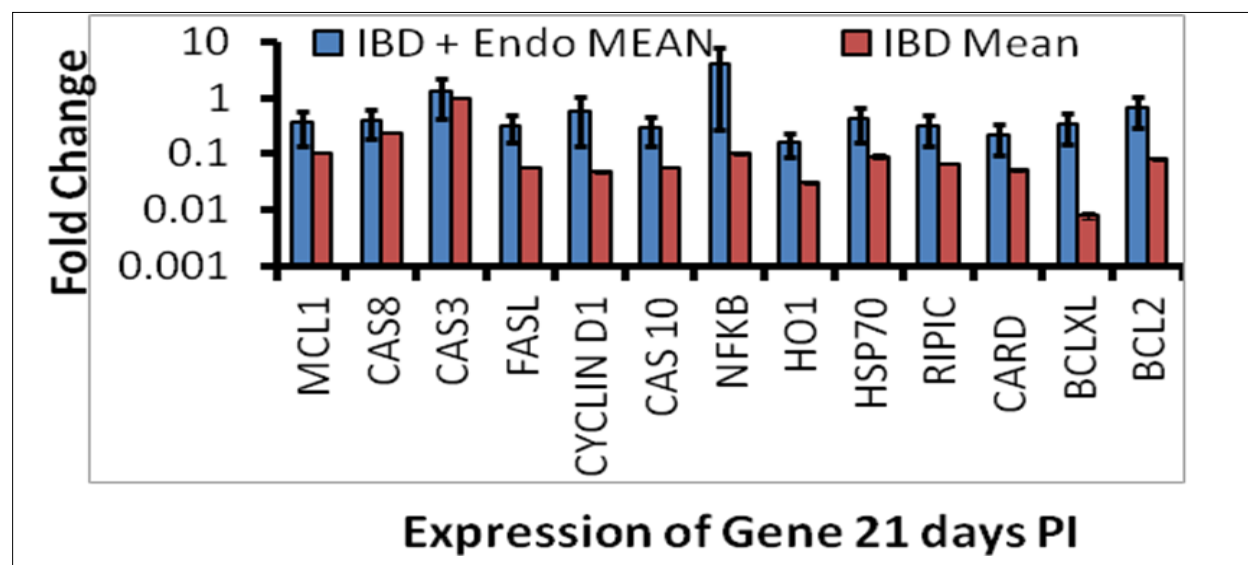


Figure 1: Real-time RT-PCR analysis of mRNA level of genes associated with apoptotic pathways; MCL1; Caspase 8; Caspase 3; Fas l; Cyclin D1; Caspase 10; NFκB; Heme Oxygenase 1 gene; Heat Shock Protein 70 gene; RIPK 2; Caspase Recruitment Domain gene; BCL XL; BCL 2; Data shown are mean±SEM of [log<sub>2</sub> (RQ value)]. RQ value, which was calculated as described in material & methods, is an indicative of mRNA expression level for each gene at different time interval (d pi). a, b, c : means within each gene that are not denoted with common letter are different p < 0.05

The result suggests that the NF- $\kappa$ B pathway and the executioner caspase of extrinsic/intrinsic pathway, caspase-3 plays the pivotal role in endosulfan induced apoptosis in IBDV infected birds. Endosulfan induces uncoupling of oxidative phosphorylation. Oxidative stress occurs under conditions when oxygen radical production is greater than the detoxification capacity of the cell (Kowaltowski and Vercesi 1999, Halliwell and Gutteridge 1989). Oxidative stress occurs when redox homeostasis within the cell is altered. Injury to cells occurs only when the ROS overwhelm the biochemical defenses of the cell (Sies 1986, Horton and Fairhurst 1987, Sies 1985). Reactive oxygen species, (in particular, hydroxyl radicals) can react with all biological macromolecules, i.e. lipids, proteins, nucleic acids and carbohydrates. The initial reaction generates a second radical, which reacts with a second macromolecule and oxidative damage in membrane phospholipids, pose a constant threat to cellular integrity and function. The lipid radicals generated during the early encounter with an oxidant add molecular oxygen to produce lipid dioxy radical (Jaescheke 1995).

It appears that oxidative stress acts as a pleiotropic modulator in both these pathways. Indeed, ROS have been described as second messengers for several growth factors and cytokines. It has been shown that the transcription factors such as NF- $\kappa$ B and AP-1, which is stimulated by ROS, could mediate apoptotic gene expression. Under conditions of mild oxidative stress, cell cycle-related genes are repressed to increase the lengthening of G1-phase. Excess ROS production causes GSH depletion and oxidative stress. Oxidative stress leads to disruption of the mitochondrial transmembrane potential and causes release of cytochrome C and other apoptosis related proteins to cytosol and finally leads to apoptosis. In the present study increased apoptotic DNA content may be due to endosulfan-induced ROS generation and oxidative damage to the cells (Kannan et al., 2000). IBDV induces apoptosis through activation of caspases (Kumar et al., 2009). Genes like hemeoxygenase-1 (0.16 $\pm$ 0.07), Cyclin-D1 (0.58 $\pm$ 0.44) and HSP-70 (0.42 $\pm$  0.26) were negatively regulated as compared to control birds suggest that cells pro-survival machinery is not selected till this stage of intoxication. There was significant down regulation in FAS-L (0.32 $\pm$ 0.16) gene in endosulfan exposed IBD infected birds as compared to control and IBD infected birds revealed that the activation of Caspase-3 and increased mRNA level expression might have followed the intrinsic pathway of apoptosis by activating Caspase-8. Apoptotic pathway genes CARD, RIPIC, FAS-L, Caspase-10 down regulation shows there less significance in combined apoptosis induced by IBDV infection and sub acute endosulfan intoxication.

The gene expression, in general, is modulated by both physiological and environmental stimuli. The results of the study confirm that the experimental exposure of IBDV infected birds with endosulfan affect mitochondrial pathway, Caspase-3 (extrinsic/intrinsic pathway) and NF- $\kappa$ B (oxidative stress-induced) pathways of apoptosis in the liver. The study shows that the infectious disease IBD in conjugation with the environmental pesticides pollutants like endosulfan, may lead to grave prognosis of the disease and may cause more economic losses.

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