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Evaluation and physiological correlation of plasma proteomic fingerprints for deltamethrin-induced hepatotoxicity in Wistar rats

Deepika Arora [a,e], Mohammed Haris Siddiqui [e], Pradeep Kumar Sharma [b], Sheelendra Pratap Singh [c], Anurag Tripathi [d], Payal Mandal [d], Uma Shankar Singh [f], Pradhyumna Kumar Singh [g], Yogeshwer Shukla [a,b,*]

[a] Proteomics Laboratory, Food, Drug & Chemical Toxicology Group, CSIR-Indian Institute of Toxicology Research, 31, Vishvigyan Bhawan, Mahatma Gandhi Marg, Lucknow 226001, (U.P.), India
[b] Environmental Carcinogenesis Laboratory, Food, Drug & Chemical Toxicology Group, CSIR-Indian Institute of Toxicology Research, 31, Vishvigyan Bhawan, Mahatma Gandhi Marg, Lucknow 226001, (U.P.), India
[c] Analytical Toxicology Division, Regulatory Toxicology Group, CSIR-Indian Institute of Toxicology Research, 31, Vishvigyan Bhawan, Mahatma Gandhi Marg, Lucknow 226001, (U.P.), India
[d] Food Toxicology Laboratory, Food, Drug & Chemical Toxicology Group, CSIR-Indian Institute of Toxicology Research, 31, Vishvigyan Bhawan, Mahatma Gandhi Marg, Lucknow 226001, (U.P.), India
[e] Department of Bioengineering, Faculty of Engineering, Integral University, Lucknow 226026, (U.P.) India
[f] Department of Pathology, King George's Medical University, Lucknow 226001, (U.P.), India
[g] Plant Molecular Biology & Genetic Engineering, CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, (U.P.), India

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ABSTRACT

Aims: Uprising reports towards deltamethrin (DLM)-induced toxicity in non-target species including mammals have raised a worldwide concern. Moreover, in the absence of any identified marker, the prediction of DLM elicited early toxic manifestations in non-targets remains elusive.
Main methods: Comprehensive approach of proteome profiling along with conventional toxico-physiological correlation analysis was performed to classify novel protein based markers in the plasma of DLM exposed Wistar rats. Animals were exposed orally to DLM (low dose: 2.56 mg/kg b.wt. and high dose: 5.12 mg/kg b.wt.) up to seven consecutive days.
Key findings: The UPLC-MS/MS analysis revealed a dose-dependent dissemination of DLM and its primary metabolite (3-Phenoxy benzoic acid) in rat plasma. Through 2-DE-MS/MS plasma profiling and subsequent verification at the transcriptional level, we found that 6 liver emanated acute phase proteins (Apolipoprotein-AIV, Apolipoprotein E, Haptoglobin, Hemopexin, Vitamin D Binding protein, and Fibrinogen gamma chain) were significantly ($p < 0.05$) modulated in DLM treated groups in a dose-dependent manner. Accordingly, DLM exposure resulted in adverse effects on body growth (body weight & relative organ weight), serum profile, liver function and histology, inflammatory changes (enhanced TNF-α, TGF-β and IL6 level), and oxidative stress. Moreover, these toxic manifestations were suppressed upon *N*-acetyl cysteine (NAC) supplementation in DLM treated animals. Thus, DLM-induced inflammatory response and subsequent oxidative injury to liver grounds the altered expression of identified acute phase proteins.
Significance: In conclusion, we proposed these six liver emanated plasma proteins as novel candidate markers to assess the early DLM-induced hepatotoxicity in non-target species with a minimal invasive mean.
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1. Introduction

Indiscriminate use of pesticides has been documented to induce the adverse biological effects in vitro, in vivo and in clinical settings [30]. Such adverse health impact ranges from emaciating immunity, hormonal imbalance, degenerative disorders and various types of cancers [1,2]. Deltamethrin (DLM), a type II synthetic pyrethroid (SP), is globally used in household and agricultural insect and pest management programmes [10,37]. Some reports on DLM exposure have been ascertained to be associated with metabolic disorders, haematological perturbations, immunotoxic, genotoxic and neurotoxic effects in non-target species, including humans [32,37].

Owing to its high hydrophobicity, DLM could exert effects on biological membranes and on absorption in body tissues through hydrolytic ester cleavage by cytochrome P450's and oxidative route) it rapidly transformed into sulphate and glucuronide conjugates [23,24]. Moreover, the hydrolytic degradation of the cyano group at the α-position of DLM could generate cellular toxicity due to formation of hydrogen cyanide in the human body [27]. Previously, in order to monitor the risk elicited due to DLM exposure, various cellular perturbations such as enhanced lipid peroxidation, impaired antioxidant status and

* Corresponding author at: Proteomics Laboratory, Food, Drug & Chemical Toxicology Group, CSIR-Indian Institute of Toxicology Research, 31, Vishvigyan Bhawan, Mahatma Gandhi Marg, Lucknow 226001, (U.P.), India.
E-mail addresses: yogeshwer_shukla@hotmail.com, yshukla@iitr.res.in (Y. Shukla).

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metabolite concentrations have been proposed as its contributory factors [3,12,40]. However, due to non-specificity and fewer predictive values, their substantial translation at the clinical level is limited.

Currently, strategies to accelerate the development of early fingerprints of chemical toxicities have shown that with the advent of high throughput "omics" (transcriptomics, proteomics and metabolomics) in toxicological science, the analysis of complex biological responses to chemical exposure is achievable [42,43]. The combinatorial assessment approaches are being efficiently employed to develop the appropriate quantifiable signatures that can give us a better mechanistic understanding of toxicants [38]. A comprehensive proteomics investigation has been heralded as an innovative mean that divulges the alterations occurring at the level of proteome including quantitative changes in protein synthesis and degradation [16]. Furthermore, the proteomic information about the body fluid is highly conserved across species and holds a substantial association among preclinical model and human subjects.

In the present study, we attempted to identify a set of plasma candidate markers in response to acute DLM administration in male Wistar rats. Furthermore, we have correlated our proteome findings with the physiological alterations in rats under similar treatment conditions to find out the possible mechanism for proteomic modulation(s) if any. By using comprehensive 2-DE/MS plasma profiling, we successfully identified six liver emanated proteins, namely Hemopexin, Apolipoprotein A IV, Apolipoprotein E, Haptoglobin, Fibrogen gamma chain and Vitamin D binding protein. A dose-dependent decrease in the expression of these proteins was observed in DLM treated animals. Moreover, the DLM-induced inflammatory changes and consequent oxidative liver injury were found as a probable reason for their reduced expression. Thus, the present study advocates whom the proteomic alterations effectively connote the liver patho-physiology and provide us an alternative mean to assess the acute DLM toxicity.

2. Material and methods

2.1. Reagents

Decis (deltamethrin, 2.8% E.C.) was purchased from Bayers Crop Science Ltd. (Mumbai). IPG strips and 0.5% pH 3–10 IPG buffer were procured from Bio-Rad Laboratories (Hercules CA, USA). General chemicals, 3-Phenoxy Benzoic Acid, Acetonitrile, Methanol, CHAPS, DTT, iodoacetic acid (IAA), acrylamide, Bis, TEMED, ammonium persulphate, sodium orthovandate, sodium fluoride, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), Polystyrene Latex Beads, and Trizma base were purchased from Sigma (St. Louis, MO). Polyvinylene difluoride (PVDF) membrane was procured from Millipore (Billerica, MA, USA). Primary antibodies (TNF-α, IL6, GAPDH, ICAM-1, iNOS) and HRP-conjugated secondary *anti*-mouse, anti-rabbit and anti-goat antibodies were from Cell Signalling Technologies. TGF-β and COX2 antibodies were procured from Abcam (Cambridge, MA, USA).

2.2. Rodent model, maintenance and treatment schedule

Male Wistar rats, (180–200 g bodyweight) were taken from Indian Institute of Toxicology Research (IITR) animal breeding colony and acclimatised for 1 week before the start of the experiments. All the guidelines of Institutional Animal Ethics Committee (ITRC/IAEC/19/15) were followed for the care and use of laboratory animals. The animals were kept under standard laboratory conditions (temperature 23 ± 2 °C, relative humidity 55 ± 5%) and fed with synthetic pellet basal diet (Ashirwad, Chandigarh, India) and drinking water ad libitum. *In vivo* studies were performed to study the distribution kinetics of DLM and to identify protein targets in rat plasma *via* 2-DE proteome analysis. Animals were randomly assigned to 3 different experimental groups (10 animals per group) namely Group I: Control (vehicle), Group II: 1/25th of LD_{50} of DLM (5.12 mg/kg in corn oil) and Group III: 1/50th of $LD_{50}$of DLM (2.56 mg/kg in corn oil). Now onwards the doses of DLM are designated as 1/25th of LD_{50} (high dose) and 1/50th of LD_{50} (low dose) elsewhere in the manuscript. Oral administration (through gavage) of DLM was given to Wistar rats with corn oil (200 μl) for the 7 consecutive days. Vehicle control group received 200 μl of corn oil per animal in the similar manner. Further, proteomic study and other parameters were also carried out in the presence of *N*-acetyl cysteine (NAC). It was given orally (2 g/l in distilled drinking water) right from the first day of treatment and maintained during the experimental period [25,31]. Animals were placed into 3 groups as: Group IV: N-acetyl cysteine (NAC) control, Group V: NAC + 1/50th of LD_{50} and Group VI: NAC + 1/25th of LD_{50}.

At the end of the experimental period, blood samples (*via retro-orbital route*) and peritoneal macrophages were taken accordingly and animals from the different groups were sacrificed. Plasma and serum samples were drawn and stored at −80 °C until further analysis. Spleen, thymus, liver and kidney were collected, cleaned, weighed and processed accordingly for the further experiments.

2.3. Body weight gain

Animal's body weight was measured initially and on 8th day whereas the organs such as liver, spleen, thymus and kidneys were weighed on 8th day immediately after sacrifice. Body weight gain and relative organ weights were calculated as:

Body weight gain (%) = (Final body weights − initial body weights) × 100/initial body weight.

And, Relative weight of organs = mean of weight of organs for each group / body weights × 100.

2.4. Xenobiotic metabolism (UPLC-MS/MS analysis)

Following DLM administration, blood samples were collected on days 1 and 7 by sampling into EDTA containing microfuge tubes at 0.5, 1, 3, 5, 7 and 24 h. Samples were kept at ice until centrifugation. Blood samples were centrifuged within 30 min (15,000 rpm for 5 min at 4 °C) and the obtained plasma samples were stored −80 °C until used for analysis. A simple protein precipitation was used for extraction of DLM, 3-PBA, Centchroman (Internal standard, IS for DLM) and Curcumin (IS for 3-PBA analysis) from rat plasma. For analysis, 100 μl of plasma was precipitated by 200 μl of acetonitrile containing IS, followed by vortex for 2 min and then centrifuged at 12,000 rpm for 10 min. An aliquot of 200 μl of supernatants was separated and 10 μl was injected onto analytical column. Calibration standards of DLM and 3-PBA were prepared by spiking the appropriate aliquots of working standard solutions into pooled blank rat plasma containing sodium fluoride (10 mM) and processed according to the procedures described above. The typical MRM chromatograms of standard DLM and 3PBA are given in Supplementary Fig. 1. Analysis was carried out using UPLC-MS/MS consisting of UPLC (Acquity-Waters, Miliford, 114 USA) and API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada). Chromatographic separation of DLM and 3-PBA was achieved using isocratic and gradient UPLC method respectively at a flow rate of 0.6 ml/min using C18 column. The mobile phase was consisting of methanol and ammonium formate (5 mM) for DLM and acetonitrile and ammonium acetate (5 mM) for 3-PBA. The detection of ions was performed in the multiple reaction monitoring (MRM) modes. The lower limit of quantification for DLM and 3-PBA was 7.81 ng/ml. Pharmacokinetic parameters were determined by non-compartmental approach using WinNonlin (version 5.1, Pharsight Corporation, Mountain View, USA).

2.5. Determination of reactive oxygen species (ROS)

For assessment of ROS, tissue samples were homogenised in 1 ml phosphate buffer (0.1 M) containing 5 mM EDTA and protease inhibitor

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