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Fipronil induced modulations in biochemical and histopathological aspects of male Wistar albino rats: A subchronic study

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ABSTRACT

Growing demand of pesticides and their indiscriminate application has led to environmental contamination globally. The present investigation was aimed to elucidate the toxic potentials of fipronil (FPN) on liver biochemistry and histology of male Wistar albino rats. Rats were orally exposed to sub lethal dose of FPN (24.2 mg/kg body weight) for 1 (E1), 45 (E2) and 90 days (E3). The results suggested significant variations (P≤0.05) in catalase, superoxide dismutase and glutathione S-transferase activities under E2 and E3 groups unlike E1, when compared with control group (C). Significantly elevated levels of malondialdehyde in rat liver noticed under E2 and E3 groups indicated oxidative damage to hepatocytes. The tissue damage confirmed through histopathological examination revealed findings like degenerated portal vein and necrosis in liver of FPN exposed rats. The findings suggest oxidative stress potential of FPN resulting from long term exposures (E2 and E3) unlike for single dose administration (E1). The modulations in histoarchitecture nevertheless indicates the possibilities of structural damage to liver under all exposure durations.Based on the outcome it is inferred that FPN is toxic to rats under prolonged exposure. It is therefore suggested that necessary precautions should be taken whenever FPN is used or disposed in areas with close mammalian proximity.

Keywords: *Fipronil, Hepatotoxicity, Oxidative damage, Wistar rats and Xenobiotic.*

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INTRODUCTION

Large scale anthropogenic activities and indiscriminate use of synthetic organic chemicals has contributed to environmental contamination globally (Rhind, 2009; David and Kartheek, 2014). The wide range existence of insect pests has prompted the agricultural community to use broad spectrum insecticides, resulting in increased crop yield and reduced post-harvest losses. However, toxicity due to occupational exposure and accidental poisoning toxicity studies has been always debated in scientific community (Eisenstein, 2015). Based on the previous reports it is convincing that negligence during handling and application of pesticides could result in the greatest possibilities of pesticide exposure to field applicators and agricultural practitioners (Rauh*et al.,* 2012; Kessler *et al.,* 2015; Lerro *et al.,* 2015).

Pesticides belonging to different class are known to control insect pests through impairing their cellular integrity and vacillating their biochemical mechanisms *in vivo* (David and Kartheek, 2016; Alavanja *et al.,* 2013; Mnif *et al.,* 2011). This mechanism is generally carried out by imparting critical conditions like oxidative stress resulting from elevated levels of reactive oxygen species (ROS) within biological systems (Costa *et al.,* 2008). Assessment of pesticides for their potential to induce oxidative stress in the exposed organism is one of the crucial methods for indicating pesticide toxicity (Arnal *et al.,* 2011).

ROS which is the responsible factor for imparting oxidative stress is never the less considered to be an inevitable and obligatory composite of mitochondrial oxidative phosphorylation in eukaryotes (Ray *et al.,* 2012). Although its role in signalling pathways is highly critical, its occurrence in additional amounts is thought to result in catastrophical damage to cellular proteins (David and Kartheek, 2015), with the processes often being irreversible (Ahn and Baker, 2016). Its availability in appropriate threshold is therefore necessary to regulate and maintain the health and survival ability of the organism (David and Kartheek, 2015; Schieber and Chandel, 2014). The mammalian liver is of exceptional concern for studying oxidative stress, as it is recognized to be the site of detoxification of almost all xenobiotic components (Jaeschke*et al.,* 2002). As a result of which it is known to be largely affected through the breakdown of abundantly available poly unsaturated fatty acids and could interdependently result in production of ROS production (Cichoż-Lach, and Michalak, 2014).

FPN belongs to a new class of phenylpyrazole group (Tingle *et al.,* 2003) and is the relatively new yet, most widely used insecticide, having acknowledged for addressing issues related to insect resistance and public health hazards that are commonly encountered with conventional group of pesticide families (Bonmatin *et al.,* 2015). Majority of pesticides are known for disrupting biochemical and even possess tissue damaging propensities (Raj *et al.,* 2013). The literature support for outcome of biochemical and histopathological disruption remains well established for organophosphates (Subramaneyaana *et al.,* 2012), organochlorines (; Lakroun *et al.,* 2015) and pyrethroids (David and Kartheek, 2016; Fetoui *et al.,* 2010). However, similar toxicity effects have been rarely addressed for FPNso far, which aggravated the need to gauge their impact on mammalian class (Murillo *et al.,* 2011). Furthermore, the studies suggesting the impeding effects on functioning of

hepatic and renal tissues in a consolidated pattern are found to be very limited (Ncir *et al.,* 2015). Hence in the current effort an attempt has been made to investigate the toxic potentials of FPN on male Wistar albino rats by evaluating biochemical and histopathological modulations following exposure to sub chronic intervals.

MATERIALS AND METHODS

Experimental design

Male Wistar albino rats (7-8 weeks) were obtained from animal house facility, Department of PG studies and Research in Zoology, Karnatak University, Dharwad, Karnataka, India. Rats were housed in the polypropylene cages with *ad libitum* access to standard pellet feed and drinking water. The room was maintained under a 12/12 h light– dark cycle, an ambient temperature of 23-30 °C and a relative humidity of 45 (±15)%. The rats were divided into four groups namely, Control (C), 1 day (E1), 45 day (E2) and 90 day exposure (E3). Each group was maintained in triplicate (n=6).

Experimental toxicant

A test doseof24.2 mg/Kg body weight (BW)($1/4^{th}$ of LD_{50}) was chosen based on the previous reports of acute toxicity, environmental availability, half-life and its bioaccumulation tendency(USEPA, 1996; Gunasekara *et al.,* 2007; Chopra *et al.,* 2011; Shui *et al.,* 2012).

Catalase activity (CAT)

Catalase (EC 1.11.1.6) activity was determined by measuring the decrease of hydrogen peroxide concentration at 240 nm according to method described by Luck (1963). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0) and 10 mM H_2O_2 which was added freshly. The enzyme activity was expressed as U/mg protein.

Superoxide oxide dismutase activity (SOD*)*

Superoxide dismutase (EC 1.15.1.1) activity was measured by methodology as described by Kakkar *et al.,* (1984). The reaction mixture consisted of 0.052 M sodium pyrophosphate buffer (pH 8.4), 186 µM phenazine methosulphate (PMS), 30 µM nitrobluetetrazolium chloride (NBT) and 780 µM NADH. Activity is reported in units of SOD per milligram of protein. The activity was calculated wherein, one unit of the enzyme concentration required to inhibit 50% of the optical density of chromagen formed in one unit at 560 nm under the assay condition. The enzyme activity was expressed as U/mg protein.

Glutathione S-transferase activity (GST)

The glutathione *S*-transferase (EC 2.5.1.18) activity was assayed by the method of Habig *et al.,* (1974). The increase in absorbance was noted at 340 nm using 1-chloro-2, 4-dinitrobenzene (CDNB). The reaction mixture consisted 0.3 mM phosphate buffer (pH 6.5), 30 mM 1-chloro-2,4-dinitrobenzene and double distilled water. After pre-incubating the reaction mixture at 37 °C for 5 min, the reaction was started by the addition of homogenate and glutathione as substrate. The absorbance was recorded for 5 min at 340 nm. The enzyme activity was calculated and expressed as U/mg protein.

Lipid peroxidation (LPO)

Lipid peroxidation levels were assessed according to the method described by Buege and Aust (1978) and was estimated by thiobarbutyric acid reactive substance (TBARS) assay performed by optically measuring malondialdehyde (MDA) in reaction with thiobarbituric acid. To the liver homogenate, 10% trichloro acetic and 0.67% thiobarbituric acid were added to adjust to a final volume of 1.0 ml. The reaction mixture was placed in a micro centrifuge tube and incubated for 15 min at 95 °C. After cooling, it was centrifuged at 5000×g for 15 min, and optical density was measured by spectrophotometer at 530 nm. TBARS levels were expressed as nmol MDA/ mg protein.

Histological analysis

For the histopathological examination, the method was followed as described by Humason(1972). The liver was isolated and immediately fixed in Bouin's fluid for 24 to 48 hoursand was further processed in a series of graded alcohol and embedded in paraffin. The embedded liver in paraffin was cut into 5 µm sections by using semi-automated microtome (LeicaRM 2255), and sections were stained primarily with haematoxylin and counter-stained with eosin (H&E) for light microscopic examination (Lille, 1969). The sections were observed under ×200 magnification. The microscopic view was photographed by using an Olympus phase-contrast microscope (Olympus BX51, Tokyo, Japan) with attached photography machinery (ProgResC3, Jenoptic-Germany). The photographed images were further observed for differences, and the findings were recorded.

Statistical analysis

The antioxidant activities are reported as the mean ± standard error of the mean (SEM) obtained from triplicates. The data were subjected to one-way analysis of variance (ANOVA) and further subjected to Tukey's test for post hoc analysis by defining the significance level at $P \leq 0.05$.

Ethical committee

All rats were housed for a duration of 1 week for acclimatization before initiation of any experiment. The maintenance of experimental rats and all the procedures implemented are in accordance with standard guidelines issued by CPCSEA followed with approval of the Institutional Animal Ethics Committee (IAEC) of the institute.

RESULTS

Antioxidant status

The activity of antioxidant enzymes *viz* CAT, SOD and GST in the liver of rats showed significant variations under group E2 and E3unlike E1 ($P<0.05$) following exposure to 24.2mg/Kg BW/day of FPN. The liver CAT was found to be elevated (+3.84%)at E1. However, the decrease of -22.09 and -51.58% were noted for E2 and E3 respectively. Determination of SOD activity indicated a slight increase (+2.70) at E1 and drastic decline (-34.93%) was noticed for E3 followed by E2 group (-20.07%). Decline was also noticed for GST, which showed maximum deterioration in enzymatic activity at E2 (-35.62%), followed by E3 (-29.99%) and finally E1 (-5.47%). The LPO studied as an indicative factor of oxidative damage suggested elevated MDA levels, which were significantly ($p<0.05$) higher in liver of FPN exposed rats. The pattern of oxidative damage suggested that the damage was dependent on the duration of exposure. While E3indicated the highest MDA level (+99.50 %);E2 showed comparatively lesser damage with percent change of+71.64 %. This was followed by E1which confirmed comparatively Lesser, yet considerable increase in MDA faction (+7.96 %) suggesting minimum damage to hepatocytes for one time dosage.

Histopathology

The histopathological investigation revealed findings in liver histoarchitecture of rats under E1, E2 and E3 groups unlike that of C group. The sameare presented in Figure 5. The important findings noticed in liver of exposed rats include mild degeneration of portal vein (DPV), degeneration of bile duct (DBD), pyknotic nuclei (PN), slight degeneration of hepatocytes (DH) and dilation of blood sinusoids (DS) at E1. The intensity of damage noticed in E2 was comparativelyhigher than that of E1 and comprised of similar findings with higher prominence. These findings included increased sinusoidal dilation (DS), nuclear degeneration of hepatocytes (ND), pyknotic nuclei (PN),

degenerated hepatocytes (DH), degenerated portal vein (DPV) and mild cytoplasmic degeneration (CD). Among all the exposure groups, the highest damage due to FPN ingestion was witnessed in E3and findings like necrosis (N), degenerated hepatocytes (DH),increased cytoplasmic degeneration (CD), increased number of pyknotic nuclei (PN), completely degenerated portal vein (DPV), lymphocytic infiltration (L) around the inner periphery of damaged portal veincomplex and appearance of necrotized cells (NC) were clearly visible (Figure 5).

DISCUSSION

In addition to traditional pesticides, a large number of other xenobiotics have also been identified for their potential to generate excessive amounts of free oxy radicals in biological systems (Henkler *et al.,* 2010). This potential to generate ROS is nevertheless found to be dependent on exposure duration of toxicants, as reported in previous investigations (David and Kartheek, 2016). The role of ROS in the pathophysiological mechanisms of some diseases have been previously studied by Agarwal *et al.,* (2006) and are thought to be related to different levels of toxicant exposure. The common enzymes scavenging such ROS which mainly include CAT (Hu and Tirelli, 2012), SOD (Afonso *et al.,* 2007) and GST (Townsend and Tew, 2003) are known to be affected under pesticide stress.

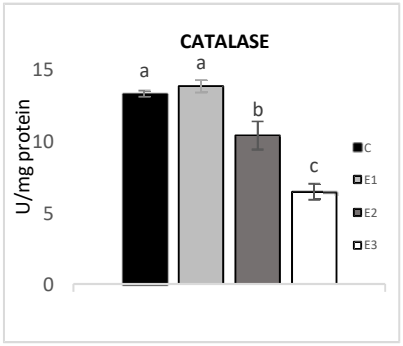

Figure 1: Changes in catalase activity in liver of rats exposed to 24.2 mg/Kg BW/day of FPN

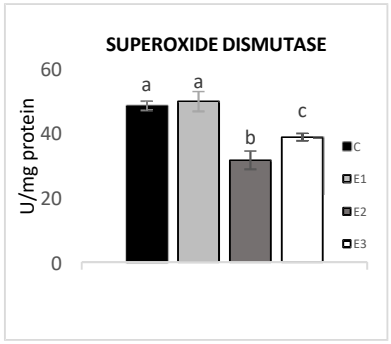

Figure 2: Changes in superoxide dismutase activity in liver of rats exposed to 24.2mg/Kg BW/day of FPN

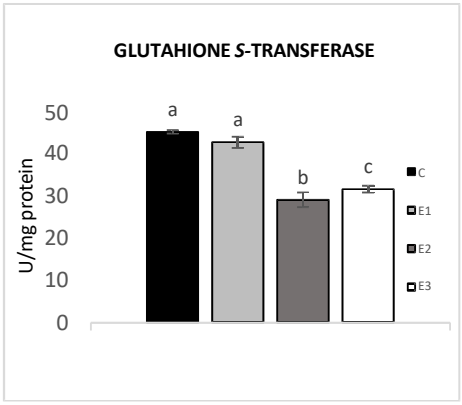

Figure 3: Changes in glutathione *S*-transferase activity in liver of rats exposed to 24.2 mg/Kg BW/day of FPN

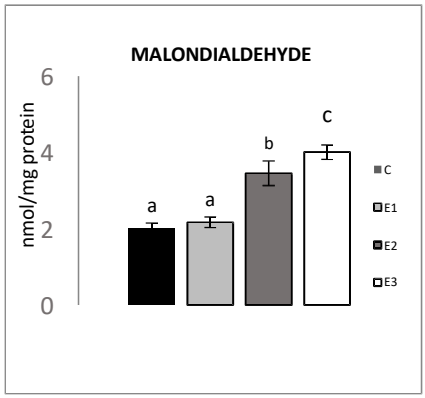

Figure 4: Changes in malondialdehyde levels in liver of rats exposed to 24.2 mg/Kg BW/day of FPN

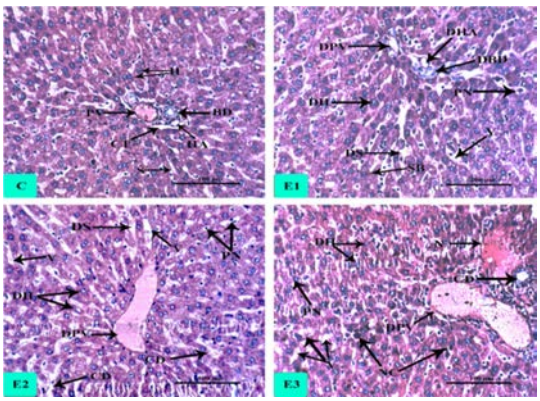

Figure 5: Photomicrograph showing sections of rat liver with changes in tissue architecture in E1, E2 and E3 following exposure to 24.2 mg/Kg BW/day of FPN

CAT being a tetrameric enzyme is mainly located in the liver peroxisomes and is also found in almost all the aerobic organisms (Usui *et al.,* 2009; Walton and Pizzetelli, 2012). Since peroxisomesare identified to contain rich amounts of CAT (Smith and Aitchison, 2013) their importance in partial if not total contribution towards relieving oxidative stress cannot be ruled out. The significant decline in CAT at E2 and E3 points at the possibilities of liver peroxisomal damage, which could be perhaps due to the continual process of FPN detoxification. Similar kind of findings has been presented by Fetoui *et al.,* (2010) and Crestani *et al.,* (2007) who reported decline in CAT activity due to xenobiotic intervention. The findings at E1 which indicated mild increase in CAT activity could be due to the induced response governed by the survival mechanism in rats upon one time administration of FPN. This part of our study is in terms with the findings of Łukaszewicz-Hussain, (2001) who suggested the increased CAT activity of rat liver upon exposure to Chlorfenvinphos, further attributed the increase to homeostatic mechanism of rat. The continuous administration of FPN resulted in significant inhibition of CAT which was recorded at E2 and E3 and is believed to be due to the failed ability of liver to detoxify and eliminate the regularly up taken FPN residue at subchronic tenures.

SOD is predominantly localized to mitochondrial matrix (Holley *et al.,* 2011) and is directly responsible for dismutation of superoxide anions within biological systems (Halliwell *et al.,* 1999). The property of biological systems to excessively generate free radicals under stressful conditions that are mainly induced by compounds like pesticides (Carvalho, 2012) and heavy metals have been well evidenced (Flora *et al.,* 2012; Karadag and Fırat, 2014).The available reports indicate that the reduction in the activities of SOD in liver of rats might be due to rapid generation of superoxide radicals in more than required amounts (Sheng *et al.,* 2014). It could be therefore presumed that FPN ingestion under current study could have aided in generation of superoxide anion.

Additionally, the existence of mechanism with which CAT and SOD work in tandem is well established and is thought to be responsible for the preliminary neutralization of ROS (Mishra *et al.,* 2016). These enzymes that are usually recognized as the first line of defence against the ROS are thought to safe guard the cellular architecture by scavenging the excess amounts of free oxy radicals that are commonly generated as the consequence of pesticide induced toxicity (McCord, 2008). Thus the diminished levels of CAT and SOD could have prompted in tissue degeneration process (Rosety *et al.,* 2005), and is discussed in detail under histopathological section under present study. One more study suggested that the induction of SOD and CAT activities may be in the order of defence mechanism to escalate the enzymatic concentrations in order to safeguard the cell against oxidative insult resulting from high levels of free oxy radicals (Xiao *et al.,* 2015).

GST enzyme is of cytosolic origin and involved actively in biotransformation and detoxification of xenobiotic compounds (Van der oost *et al.,* 2003; Ezemonye and Tongo, 2010) and is additionally known to provide an active protection against products of LPO like 4-hydroxy-2nonenal (Hayes *et al.,* 2005). Correlating the reports of previous investigations and outcome of present study it could be ascertained that the LPO generated due to FPN could have been briefly attenuated by GST at E1. However, further continuous administration of FPN could have resulted in elevated LPO and therefore causing the decline of GST activity consequently. The alteration in the GST activity directly reflects the metabolic disturbances and cellular damage (Livingstone *et al.,* 2001). LPO is a free radical-mediated chain reaction and once initiated, is thought to be self-perpetuating (Ayala *et al.,* 2014). The length of the chain propagation of LPO, depends upon the activity of chain-breaking antioxidant enzymes (Suraj, 2015).The level of MDA, a marker of LPO was recorded to be exponential higher in liver and respectively at E2 and E3. These findings are in agreement with the reports presented by several authors previously (Pandey *et al.,* 2003; Farombi *et al.,* 2007; Tuzmen *et al.,* 2008).

Histological analysis is found to be a simple, sensitive, effective and critical tool to determine structural changes under tissue level organization (David and Kartheek, 2014; Hussein *et al.,* 2015). The role of oxidative stress in contributing to the pathophysiology of liver damage has been previously reported for other toxicants (Sarin and Choudhary, 2016). These changes under cellular context are known to occur due to exposure to pesticides (Muthuviveganandavel *et al.,* 2008), pathogens (Colton *et al.,* 2011) and radiations (Moore *et al.,* 2013) thereby reflecting the tissue damaging tendency. These changes like degenerated portal vein complex, cytoplasmic vacuolation, degeneration of hepatocytes and dilation of blood sinusoids, that were witnessed in current study may be as a consequence of obstructed circulatory mechanism leading to ischemia (Nath and Szabo, 2012). The outcome suggests the possible role of FPN in impairing circulatory mechanism as seen with other toxicants (Nag and Wadhwa, 2005). The extensive damage to the liver as indicated by loss structural integrity of hepatocytes and degeneration of cytoplasm in addition to severe vacuolization indicates the toxic insult due to FPN dosage. Similar findings have been reported by (Das *et al.,* 2006) in rats dosed with other toxicants.

Functioning of hepatocytes as detoxification ability with additional function of signalling and consequent formation of bile under molecular levels is one of the crucial component of canalicular ATP-binding cassette transporters (Nicolaou *et al.,* 2012). These are specifically vital for maintaining the functional integrity as in addition to retaining the concentration of their respective substrates low and well within the hepatocytic cellular boundary (Pollheimer *et al.,* 2013). Any disturbance to these transporters is thought to result in the reduced detoxification ability, resulting in the life threatening consequences of exposed animal (Nicolaou *et al.,* 2012). The results from present study direct a possibility of partial ABC transporter damage at E1 which might have led to reduced detoxification ability of hepatocytes. Another finding of lymphocytic infiltration observed in rats under E3 group suggests the possibilities of activation of immune response mechanism by the host animal to overcome the FPN induced trauma. As proposed by (Bautista, 2002), the ROS, which prevailed under declined antioxidant enzyme concentration might have prompted the release of inflammatory chemokines that are thought to be involved in the migration of leukocytes into liver (Oo *et al.,* 2010).

Research on cell death has been strengthened by the understanding that necrosis which can occur in a highly regulated and genetically controlled manner (Vanden Berghe *et al.,* 2014). Hotchkiss *et al.,* (2009) elucidated that the rupture of plasma membrane could result in release of cellular constituents into the extracellular environment. This pathological process could elicit a significant inflammatory response (Oo *et al.,* 2010). Despite the improved understanding of immune response triggered hepatotoxicity, the molecular mechanism of cell death remains to be poorly understood. The current study which indicated occurrence of necrotized cells in E3 could be therefore termed as the result of FPN involvement which could have stimulated the immune response thereby causing the lymphocytes to flow in. Nevertheless, due to the combined status of depleted enzymatic antioxidants and elevated LPO, the tissue damage

could have led to rupture of plasma membrane there by subsequently resulting in necrosis.

Although these findings are reported based on biochemical and histopathological investigations, it would be appropriate to state that the exact mechanism of toxicity could be explained by the outcomes of combined strategies like immuno- histochemistry and elucidating the toxikinetics of the FPN in hepatic tissue under molecular level by considering other additional factors like bioaccumulation and elimination rate (Rubio *et al.,* 2012).

CONCLUSION

The outcome of the current study clearly implicates the role of FPN in dysregulation of enzymatic activities of selected antioxidant enzymes as well as the damage to cellular integrity in liver of rats administered with FPN dose. The study since conducted for subchronic exposure helps in understanding the toxicological outcome of FPN toxicity for long term durations. Even though, one time exposure (E1) could have resulted in compensatory approach of enzymatic levels to in turn rescue and reimburse the imbalance caused due to toxic insult by FPN, the prolonged exposures to the same, suggested the overridden physiology and inefficient homeostatic mechanism of exposed rats under E2 and E3 groups. This outcome also suggests the existence of compensatory mechanism in rat observed at E1, which is however viewed as a nutshell under current investigation. Further direct analysis under molecular evaluation techniques may explain the principles behind the cascadic changes in biochemical dynamics of liver and its possible correlation with histoarchitechtural disturbances. The study could contribute in the course of regulatory surveillance as well as for the preliminary approach of cases with possible FPN poisoning.

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Conflict of Interest: The authors hereby declare no conflict of interest.

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