Ascorbic acid modulates non enzymatic antioxidants in swiss albino mice targeted to imidacloprid: Brain v/s Liver

Mohammad Waseem, Shruti Jain, Suhel Parvez and Mohd. Akram

Abstract
Pesticides may provoke oxidative stress leading to produce free radicals and alternate antioxidant or oxygen free radical scavenging enzyme system. Imidacloprid (Imd) is a systemic insecticide and act as a toxic substance frequently utilized into combat insects, rodents and plants pests and other creatures that can pose agricultural problems. Oxidative stress has been proven to be involved in Imd-induced toxicity including neurotoxicity and hepatotoxicity. The aim of current study was to explore the dose dependent damage caused by Imd on liver and brain under in vitro conditions. The study was also planned to assess neuroprotective and hepatoprotective actions of Ascorbic acid (AsA) on Imd induced oxidative stress in liver and brain of swiss albino mice as an in vivo model. Oxidative stress machinery were evaluated against hepatotoxicity and neurotoxicity induced by Imd. AsA modulated altered non-enzymatic machinery in liver and brain of swiss albino mice. AsA was found to be more effective in ameliorating mice against Imd-induced oxidative stress by enhancing antioxidant defense mechanisms.

Keywords: Hepatotoxicity, neurotoxicity, imidacloprid, ascorbic acid, antioxidant

Introduction
Pesticides are ubiquitously employed for regulation of pests in several fields to protect public health, subsistence of crops, food and other materials essential to mankind and animals. Some of the advantages of pesticides are that they are fast-acting, work against a specific pest, control large infestations, easy to obtain and apply, increase crop production by reducing crop losses, and their usage against pests may significantly improve human health. Some of the disadvantages of pesticides are that they may damage and/or accumulate within the environment, may kill non-target species, may be dangerous to users/pets/native species, and can drift from their original point of application. Imd is relatively a novel pesticide which belongs to the class of the neonicotinoid compounds. It is indexed to regulate insect pests on nursery and agricultural crops, structural pests and parasites on companion animals (USEPA, 1994). It has been investigated that neonicotinoids may be related to nicotine in their structure and site of action at the platform of nicotinic acetylcholine receptor [1]. Recent investigations have indicated that toxic manifestations stimulated by pesticides may be linked with the elevated generation of reactive oxygen species (ROS), which confers the clarification for multiple varieties of toxic responses. It has been studied that the production of ROS is caused by a mechanism in which xenobiotics, toxicants and pathological consequences may induce oxidative stress and produce several tissue damage including liver, kidney and brain [2-4]. Many investigators have found that oxidative stress may occur by production of ROS that reverse the antioxidant potential in the target cell, leading to the damage and/or dysfunction of macromolecules such as nucleic acids, lipids and proteins causing modulation in the target cell function and resulting to cell death [5]. The cells have been known with its several mechanisms to modulate oxidative stress and repair damaged macromolecules. The key defense is put forwarded by enzymatic and non-enzymatic antioxidants, which have been displayed to scavenge free radicals and ROS. Non-enzymatic antioxidants are able to prevent the unregulated production of free radicals or hinder their reaction with biological sites, also the damage of most free radicals rely on the oxidation of endogenous antioxidants mainly by scavenging and minimizing molecules [6,7].

Natural products are generally either of prebiotic origin or originate from microbes, plants, or animal sources [8]. Vitamins are also natural products. AsA or vitamin C is thought to be an important water soluble antioxidant which is reported to neutralize ROS and reduce the oxidative stress [3, 4, 8, 9].
In recent years, it has been shown that environmental contaminants can alter antioxidant system in fish [11, 12], snails [13] and in rat [14]. Nevertheless, there is a paucity of literature on the effect of Imd on oxidative stress in animals. Therefore, the aim of our study was planned to extend the therapeutic display of AsA to control the hepatotoxic and neurotoxic effects of Imd exposure in liver and brain of Swiss albino mice.

Material and Methods

**Chemicals**

Imd were obtained from Parijat chemicals (New Delhi, India). AsA was procured from Hi- Media Labs (Mumbai, India) respectively. Routine Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and SRL Pvt. Ltd. (Mumbai, India).

**Animals**

Male Swiss albino mice weighing between 18±2 g were acquired from the Central Animal House of Jamia Hamdard (Hamdard University), New Delhi, India. The mice were acclimatized for a week prior to the initiation of the experiments. Animals were kept at ambient temperature 22 ± 2°C with relative humidity at 65 ± 10 % and at a photoperiod of 12 h light/dark cycle. Standard pellet rodent diet and water were provided to the animals ad libitum. All the experiments were performed according to the standard guidelines of Institutional Animal Ethics Committee.

**Experimental Design**

**In vitro Model**

The hepatotoxicity and neurotoxicity of Imd was appraised under *in vitro* conditions. The liver and brain samples were incubated with designed doses (20, 50 and 100 µM) for 1 h at 37 °C under temperature controlled water bath. The doses established for Imd was based on an *in vitro* study with non-hepatic cells [15]. The exposure regime were planned in such a way that the end point of all the groups took place at the same time.

**In vivo Model**

To assess the neuronal and hepatic toxicity, Imd was investigated under *in vivo* condition. Mice serving as control had received corn oil only. After 24 h the toxicity of Imd was calculated according to Weill (1952) and was expressed as LD<sub>50</sub> value and its confidence limits.

The dose established for Imd and AsA was based on an *in vivo* study with hepatic cells [16]. The effect of Imd on the oxidative stress biomarkers in the liver and brain of male mice and the role of AsA were investigated by dividing the animals into four groups, each including six animals and were treated orally as follows:

**Group 1**: Corn oil (5 ml/kg) and served as control.

**Group 2**: AsA (200 mg/kg b.wt.).

**Group 3**: AsA (200 mg/kg bw), 30 min prior to the administration of Imd (1/10 LD<sub>50</sub>) and considered as pretreatment group.

**Group 4**: Imd alone in a dose equivalent to 1/10 LD<sub>50</sub>. The effect of Imd and ameliorative role of AsA on oxidative stress biomarkers were evaluated in liver and brain of Post Mitochondrial Supernatent (PMS).

**Sample preparation**

PMS was prepared by differential centrifugation method with minor modifications [17]. The liver was homogenized 1:10 w/v in 0.1 M sodium phosphate (pH 7.4) buffer with a Potter–Elvehjem homogenizer. Homogenate was centrifuged at 12,000 rpm for 20 min with the temperature at 4°C, using a REMI C-24 centrifuge (Remi Sales and Engineering, Ltd., Mumbai, India), and the supernatant obtained, after only pelleting the nuclei during subcellular fraction. The PMS was immediately used for biochemical assessments.

**Non-Enzymatic Antioxidants**

**Determination of Reduced Glutathione (GSH)**

GSH content was estimated according to the method of Tabassum et al. [18]. In this method, 5,5-dithiobis-2-nitrobenzoic acid (DTNB) is reduced by –SH groups to form 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitromercaptobenzoic acid anion released has an intense yellow color and can be used to measure –SH groups at 412 nm. The PMS was precipitated with 1 ml of sulphosalicylic acid (4%). The brain and liver samples were kept at 4 °C for one hour and then centrifuged at 1,200 g for 15 min at 4 °C. The assay mixture contained 0.4 ml of filtered aliquot, 2.2 ml of sodium phosphate buffer (0.1 M sodium phosphate buffer, pH 7.4) and 0.4 ml DTNB in a total volume of 3 ml. The absorbance of reaction product was read at 412 nm on a double beam spectrophotometer. The GSH content is expressed as µmoles GSH/g tissue.

**Protein determination**

The protein concentration in all samples was estimated by the method of Lowry et al., [19], using 0.03 % bovine serum albumin (BSA) as standard.

**Statistical analysis**

Results were expressed as mean ± Standard deviation (SD). All data were analyzed using analysis of variance (ANOVA) followed by Tukey’s test. Values of p < 0.05 were considered as significant. All the statistical analyses were performed using graph pad prism 5 software (Graph Pad Software, Inc. San Diego, CA, USA).

**Results**

**In vitro**

**Effect of Imd on GSH in brain**

Imd exposure with the dose of 50 µM has shown a significant decline (*P < 0.01*) on GSH level in brain of mice as compared to control group (Fig. 1). Imd also caused a significant depletion (*P < 0.001*) on GSH levels with 100 µM exposed groups as compared to control. Minimal dose of Imd (10 µM) has shown no significant change in GSH contents as compared to control in brain samples of mice.

**Effect of Imd on GSH in liver**

Minimal dose of Imd (10 µM) has shown no significant change in GSH contents as compared to control in liver samples of mice. Imd exposure with the dose of 50 µM has displayed a significant depletion (*P < 0.05*) on GSH level as compared to control group (Fig. 1). Imd also caused a significant depletion (*P < 0.001*) on GSH levels with 100 µM exposed groups as compared to control.

**In vivo**

**AsA ameliorated against deviation in GSH levels in Imd treated group in brain**

The dose of Imd (group IV) caused a significant decrease (*P < 0.001*) in GSH (Fig. 3) contents in brain when compared with control (group I). AsA pre-treatment (group III) significantly enhanced the level (*P < 0.05*) of GSH in brain as compared with Imd-treated group (group IV). AsA alone treatment (group II) showed no significant change on GSH levels as compared to control.
AsA prevented Oxa induced depletion of GSH contents in liver

Imd treatment (group IV) led to significant decrease ($P < 0.01$) on GSH contents (Fig. 4) as compared to control (group I). AsA pre-treatment (group III) significantly enhanced the levels ($P < 0.05$) of GSH in liver as compared with Imd-treatment (group IV). AsA alone treatment (group II) on GSH contents caused no significant change as compared to control.

Fig. 1: Effect of different concentrations of Imd (10, 50, 100 μM) on GSH products in brain of mice. Values were expressed as μmoles GSH/g tissue. Each value represented as mean ± SD (n = 6). Significant differences were indicated by **$p < 0.01$ and ***$p < 0.001$ when compared with control.

Fig. 2: Effect of different concentrations of Imd (10, 50, 100 μM) on GSH products in liver of mice. Values were expressed as μmoles GSH/g tissue. Each value represented as mean ± SD (n = 6). Significant differences were indicated by *$p < 0.05$ and **$p < 0.01$ when compared with control.

Fig. 3: Effect of Imd (1/10 LD₅₀) and protective effect of AsA (200 mg/kg/b.wt.) on GSH contents in brain of mice. Each value represents mean ± SD (n = 6). Values were expressed as μmoles of GSH/g tissue. Significant differences were indicated by ***$P < 0.001$ when compared to the control group and $#P < 0.05$ was used to show significance when compared to the Imd administered group.
The widespread use of pesticides in public health and agriculture programs has caused severe environmental pollution and health hazards, including cases of severe acute and chronic human poisoning [20]. It is well known that the oral LD50 value of any pesticide is highly dependent on the pesticide and the organism. Pesticides can induce oxidative stress by generation of free radicals that might cause lipid peroxidation, alternations in membrane fluidity, DNA damage and finally carcinogenic effects [7, 21]. In vertebrates, the liver is a highly metabolically active organ, with a high activity of antioxidants and associated enzymes, so that it is the main organ responsible for detoxification of xenobiotics [11, 22]. Additionally, pesticide induced oxidative stress has also been a focus of toxicological research for the last few decade as a possible mechanism of neurotoxicity [23]. Regarding non-enzymatic antioxidants, GSH are the first line of defense against free radicals. The depletion of brain and liver GSH has been shown to be associated with xenobiotic-induced toxicity [24]. In our results, decrease in GSH concentrations due to Imd exposure, was seen in both liver and brain of mice. This shows induction of oxidative stress and debilitative of the cellular antioxidant defense mechanisms leading to active participation of GSH in cellular defense against ROS [18]. The pool of GSH is critical in maintaining the functional competency of the organelle and for cell survival [17]. In our results, the level of GSH which were also affected by Imd treatment and strengthened towards normal level by AsA pre-treatment. GSH is one of the essential compounds for maintaining cell integrity against ROS, as it non enzymatic free radical scavenger, participates in the detoxification of ROS and reduce H2O2. GSH participates in the enzymatic reduction of membrane hydroperoxy-phospholipids and prevents the formation of secondary alkoyl radicals when organic peroxides are homolyzed [25]. Earlier research has shown that supplementation of AsA markedly shunted the GSH or thiol ratio in pesticide induced oxidative stress [14]. The reduction of GSH content demonstrated in our study may be attributed to the direct conjugation of Imd and its reactive metabolites with thiols groups thereby interfering antioxidants status. It is a conventional fact that thiols protect cells against pesticide-induced LPO [21].

Discussion
The widespread use of pesticides in public health and agriculture programs has caused severe environmental pollution and health hazards, including cases of severe acute and chronic human poisoning [20]. It is well known that the oral LD50 value of any pesticide is highly dependent on the pesticide and the organism. Pesticides can induce oxidative stress by generation of free radicals that might cause lipid peroxidation, alternations in membrane fluidity, DNA damage and finally carcinogenic effects [7, 21]. In vertebrates, the liver is a highly metabolically active organ, with a high activity of antioxidants and associated enzymes, so that it is the main organ responsible for detoxification of xenobiotics [11, 22]. Additionally, pesticide induced oxidative stress has also been a focus of toxicological research for the last few decade as a possible mechanism of neurotoxicity [23]. Regarding non-enzymatic antioxidants, GSH are the first line of defense against free radicals. The depletion of brain and liver GSH has been shown to be associated with xenobiotic-induced toxicity [24]. In our results, decrease in GSH concentrations due to Imd exposure, was seen in both liver and brain of mice. This shows induction of oxidative stress and debilitative of the cellular antioxidant defense mechanisms leading to active participation of GSH in cellular defense against ROS [18]. The pool of GSH is critical in maintaining the functional competency of the organelle and for cell survival [17]. In our results, the level of GSH which were also affected by Imd treatment and strengthened towards normal level by AsA pre-treatment. GSH is one of the essential compounds for maintaining cell integrity against ROS, as it non enzymatic free radical scavenger, participates in the detoxification of ROS and reduce H2O2. GSH participates in the enzymatic reduction of membrane hydroperoxy-phospholipids and prevents the formation of secondary alkoyl radicals when organic peroxides are homolyzed [25]. Earlier research has shown that supplementation of AsA markedly shunted the GSH or thiol ratio in pesticide induced oxidative stress [14]. The reduction of GSH content demonstrated in our study may be attributed to the direct conjugation of Imd and its reactive metabolites with thiols groups thereby interfering antioxidants status. It is a conventional fact that thiols protect cells against pesticide-induced LPO [21].

Conclusion
Our findings clearly demonstrate the prophylactic neuroprotective and hepatoprotective efficacy of AsA as it markedly prevented imidacloprid-induced oxidative stress in vivo. Our finding also assumes the importance of AsA is ubiquitously consumed by general human population and is also being employed as a therapeutic agent to alleviate various neurological and hepatic disorders. Thus, sufficient dietary intake of AsA by individuals who regularly come in contact with these pesticides is beneficial in combating the adverse effects of imidacloprid. Future studies should examine the hypothesis with AsA as a antioxidant therapy targeted neurological and hepatic damage induced by imidacloprid may constitute an interesting strategy to mitigate hepatotoxicity and neurotoxicity at sub cellular or molecular levels.

References
7. Zama D, Meraihi Z, Tebibel S, Benayache F, Benayache S, et al. Chlorpyrifos-induced oxidative stress and tissue damage in the liver, kidney, brain and...
fetus in pregnant rats: the protective role of the butanolic extract of Paronychie argentea L. Indian Pharmacol 2007; 39: 145–150.


22. Hinderer RK, Menzer RE. Comparative enzyme activities and cytochrome P-450 levels of some rat tissues with respect to their metabolism of several pesticides. Pest Biochem Physiol 1976;6:148-160.

