Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(6): 1108-1112
Received: 13-09-2018
Accepted: 15-10-2018

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Attenuating effects of ascorbic acid on cypermethrin induced histological and biochemical changes in developing brain of *Gallus domesticus*

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Abstract
Cypermethrin [cyano-(3-phenoxyphenyl) methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate), second generation, type-II, pyrethroid insecticide having cyano group which has broad spectrum uses in agricultural sector. Present study aimed to investigate biochemical and histological changes induced by cypermethrin and protective effect of vitamin C in embryonic brain of developing chick of *Gallus domestius*. Fertilized eggs were divided into four groups having 30 eggs each. Group A: served as control, Group B: as vehicle which was administered with distilled water by immersion method, Group C: treatment group; was treated with 25 mg/ml of cypermethrin via immersion method and Group D: recovery group; supplemented with 25 mg/ml of cypermethrin along with 100 mg of vitamin C by air sac method. At embryonic day 0 (ED 0), all the groups treated with requisite doses for 60 minutes. Eggs were recovered and cerebellum was excised and further processed for biochemical and histological changes on embryonic day 16 (ED 16). The results of present study showed cypermethrin induced alterations in the general histology of cerebellum region of developing brain. Cypermethrin adversely affect different layers of cerebellum. Vacuolization was observed in the neurons of outer molecular layer, the middle layer of Purkinje cells showed devastating effects both on the nucleus and cytoplasm. Sign of pyknosis was observed in the nucleus of Purkinje cells whereas cytoplasm was found to be vacuolated. Innermost granular layer showed patchy loss of neurons. The results of light microscopy studies were further strengthened by biochemical studies. Several biochemical alterations were recorded in cypermethrin exposed developing brain. Cypermethrin treatment cause a decrease in protein content by 29.4% as compared to control group. The brain antioxidant marker enzymes such SOD, catalase and GSH were found to be decreased in treatment group as compared to control group and recovery group. The decreasing percentage of SOD, catalase, GSH was 35.75%, 39.16% and 37.43% respectively as compared to vitamin C protected group where estimated decreasing percentage was only 24.6%, 20.75%, 18% respectively. The MDA level was also increased to 115% as compared to vitamin C supplemented group where MDA level was increase to just 58.9%. Acetylcholinesterase, is a key marker enzyme which help in depicting toxicity level of xenobiotics. In the present study, the level of acetylcholinesterase enzyme was decreased to 62.15% as compared to control and vitamin C recovery group where estimated activity of this enzyme only decreased by 39.06% as compared to control group.

Keywords: Cypermethrin, *Gallus domesticus*, neurotoxicity, vitamin C

Introduction
Insecticide is one of the important classes of pesticides that deals with wide range of insects which directly or indirectly harm the cultivated crops. These chemicals are efficient against huge array of harmful pest but due to same physiological baseline, they may accidently trigger beneficial insects, mammals and non-targeted species. On the basis of their chemical structure, insecticides can be classified as organochlorines, organophosphates, carbamates, synthetic Pyrethroids etc. Among all, synthetic pyrethroid gained first preference because of rapid biodegradability [1]. Low bioaccumulation rate and low toxicity to non-target organisms [2]. Pyrethroids are ester derivatives of pyrethrin, procured from the flowers of *Chrysanthemum cinerariaefolium*. These are used in varied formulations like pet shampoos, lice and termite treatments, household insecticide sprays, aerosol bombs, agricultural, public health and domestic applications.

Pyrethroid can be classified as Type-I, pyrethroids, devoid of cyano groups which includes permethrin, allethrin, whereas Type-II, pyrethroid having cyano groups, consist of cypermethrin, cyphenothrin, fenvalarate etc. Cypermethrin [cyano-(3-phenoxyphenyl) methyl] 3-(2, 2-dichloroethenyl)-2, 2-dimethylcyclopropane-1-carboxylate) is a commercially used synthetic pyrethroid which possesses alpha-cyano group at the α-carbon position of the alcohol moiety. It is widely used to spray the walls of poultry houses or studs/livestock farms, [3] in agricultural sector for pest repelling and against diseases causing vectors [4].

It is effective against insects by modulating the levels of γ-aminobutyric acid (GABA) and sodium ion channels. It prolonged the activation state of sodium ion channels resulting in hyper-excitation of the nervous system [5]. Beside the beneficial effects, long term or high dosage exposure of cypermethrin reported to cause teratogenic, reproductive toxicity and genotoxicity in some of the non-targeted species [6]. Studies reported that this agrochemical may accumulate primarily in the central and peripheral nervous system, thus triggers several neural disorders [7].

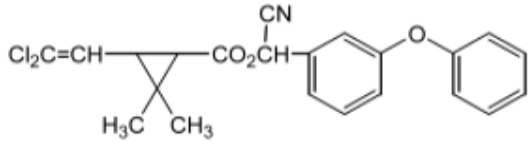
Poultry culture is emerging globally, as one of largest production industry for fulfilling rising demand of food. To elevate the productivity, agrochemicals and pesticides including pyrethroid are more in use in the feeds and management strategies [8]. However, the increased pyrethroid exposure induce several devastating alterations in adults as well as in developing embryos. Studies reported that developing embryos are more prone to pesticidal effects as compared to adults due to lack of well-developed immune and antioxidant enzyme system. For evaluating pesticide induced teratogenic changes, Chick embryo is known to be best animal model as it has easy accessibility, small size, known embryonic development pattern [9, 10].

Ascorbic acid (vitamin C) is water-soluble vitamin that forms important antioxidative components of fruits/vegetables. It has pivot role in pruning pesticide induced hepatotoxicity, nephrotoxicity, neurotoxicity and reproductive toxicity [11, 12]. Previous findings suggested efficient antioxidant potential of vitamin C in scavenging reactive oxygen species (ROS) level [13, 14].

Materials and Methods

Chemicals

Cypermethrin, $C_{22}H_{19}Cl_2NO_3$ having CAS no. 52315-07-8, molecular mass: 416.3 g/mol with 99% purity was obtained from Pesticide India Ltd, New Delhi. Requisite dose of (25mg/ml) cypermethrin was made in 3L distilled water. Ascorbic acid was procured from Hi-media, India. All other chemicals used for the study were of analytical grade.



Chemical structure of Cypermethrin

Test animals

Fertilized eggs of *Gallus domesticus* of BV 300 breed were obtained from Bulbul Hatchery Near Kurukshetra (29.9°N, 76.8°E), India.

Experimental design

Fertilized eggs were brought to Lab., washed with distilled water and disinfected with 70% alcohol. Eggs were divided into four groups consisting of 30 eggs in each group. These eggs were subsequently, exposed to distilled water, cypermethrin and vitamin C for vehicle, treatment and recovery group respectively. Immersion technique depicts exposure of eggs as in normal farm condition [15]. The grouping of eggs was as follows:

Group A: served as control

Group B: vehicle control; eggs were treated with distilled water

Group C: treated group; eggs were tested for aqueous emulsions of 25 mg/L of cypermethrin

Group D: recovery group; eggs were administered with aqueous emulsions 25 mg/L of cypermethrin along with 100 mg/egg vitamin C

The eggs from vehicle and treated group were immersed in 3L of distilled water along with requisite dose of cypermethrin in treated group for 60 minutes whereas eggs from recovery group was supplemented with vitamin C by air sac method, on embryonic day 0 (ED 0). Thereafter, all the eggs were incubated for 16 days in an incubator at 38±0.5°C and 60-70% of relative humidity with proper ventilation. To avoid sticking of embryo to the egg shell membranes, eggs were kept rotating twice a day. On 4th day of incubation, all the eggs were examined via candling to check the survivability of embryos. Unfertilized and underdeveloped eggs were considered as dead embryo thus, discarded. On 16th day of incubation (ED 16) embryos from all the groups, were recovered and processed for biochemical and histological analysis of chick embryo brain.

Histological Studies

The cerebellum recovered from different experimental groups was processed for histopathological studies by method of Pearse (1968) [16]. Tissues were cut into 1-2mm and fixed in Bouin's fixative for 48 hours. Post fixed sections were washed for 2-3 hours under running tap water to remove excess fixative. Tissues were dehydrated through ascending series of alcoholic grades. Embedding of dehydrated tissues was done in paraffin wax (melting point 60-62^0 C). Tissues of 5 µm thickness were sliced off. Dewaxing of slides was carried in xylene for 15-20 min. which were further processed by series of alcoholic grades and stained with haematoxylin and eosin. Sections were observed and photographed using Olympus digital camera.

Preparation of homogenate

Cerebellum of brain was excised from all the groups. The explant was washed with normal saline solution, weighed and homogenized (10%) in ice cold phosphate buffer saline (pH 7.4) for different biochemical assays.

Biochemical analysis

Total protein content of cerebellum was estimated according to the method described by Lowry *et al*. (1951) [17]. The Liebermann and Burchardt reaction were used for estimating total cholesterol content of the tissue [18]. Antioxidant level of enzymatic: Catalase (CAT; EC 1.11.1.6), Glutathione (GSH; EC 1.11.1.9), Superoxide dismutase (SOD; EC 1.15.1.1) and non-enzymatic i.e. Malondialdehyde (MDA) was measured for estimating oxidative stress status. CAT was estimated using method described by Aebi (1984) [19]. GSH content was determined by method of Ellman (1959) [20]. SOD assay was performed by method of Marklund and Marklund (1974) [21]. MDA level was measure of thiobarbituric acid (TBA) reaction, using the method of Ohkawa *et al*. (1979) [22].

Stastical Analysis

All data were expressed as Mean±SEM. Comparison between groups were performed by One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using the Graph pad Prism (version 5.03, San Diego, CA, USA) to establish significant differences (*p<0.05, **p<0.01, ***p<0.001 vs control group) among groups.

Results

Histological studies

Histopathological studies showed that cypermethrin induced changes in normal architecture of embryonic cerebellum of *Gallus domesticus* as compared to control group. Present study revealed that control group showing normal intact cerebellum with proper demarcation in between different layers of cerebellum i.e. outer molecular layer, middle layer of purkinje cells and innermost layer of granular cortex. Purkinje cells were well developed with healthy dendrites (Fig. 1 A). Vehicle group exhibit normal arrangements of granular and molecular layer except some sign of vacuolization in both the layers (Fig. 1 B). The middle layer of purkinje cells showed several devastating effects such as pyknotic nuclei and dilatation of sub-arachnoid space. The shape and size of Purkinje cells were appeared distorted and irregular. Dendrites exhibit degenerative changes. The innermost granular cortical layer showed varying degree of vacuolization with areas of patchy cell loss (Fig. 1 C). as compared to control group. Vitamin C supplemented group showed signs of recovery. Granular and molecular layer showed normal morphology except some vacuolization at one or two places. Purkinje cells restored their normal shape and appeared healthy with dendritic processes (Fig. 1 D). Most of the Purkinje cells were found to be flask shaped with regular nucleus and cytoplasm.

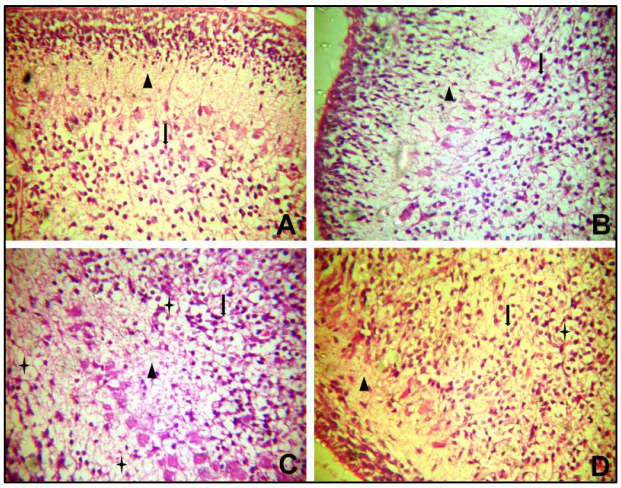


Fig 1: (A) (Control) showing normal cerebellum having mature granular layer and molecular layer (arrow), well developed purkinje cells with dendrites (arrow head). (B) (Vehicle) showing healthy cerebellum having vacuolization at some places in granular layer (arrow head) and molecular layer (arrow). (C) (CYP Treated) group showing significant increase in vacuolization of granular layer (arrow) and molecular layer (asterisk), degeneration of dendrites (arrow head). (D) (CYP + Vit. C) group showing reduced vacuolization and healthy purkinje cells (arrow head). H&E (×400)

Biochemical Analysis

The results of present study reported that cypermethrin induced biochemical alterations in developing brain of 16th day old chick embryo. The antioxidant enzyme level was severely affected by the exposure of cypermethrin thus resulting in oxidative stress in brain. The decreasing percentage of brain antioxidant enzymes i.e. SOD, catalase, GPx was found to be 35.75%, 39.16% and 37.43% respectively in cypermethrin treated group as compared to control group. Cypermethrin exposure enhanced lipid peroxidation level of brain. Around 115% increase in MDA level was reported in treatment group as compared to the control group. The activity level of marker enzyme of brain i.e. acetylcholinesterase was found to be negatively affected by cypermethrin. The decreasing percentage of acetylcholinesterase was 62.15% which was much lower than the control group. Decrease in level of the total protein was observed in treatment group (Table 1).

Table 1: Showing biochemical changes in different groups

Parameters	Control	Vehicle	CYP	CYP + Vit C
SOD (U/mg protein)	7.225±0.299	7.007±0.128	4.642±0.085***	5.447±0.198**
Catalase (U/mg protein)	1.325±0.023	1.213±0.051	0.8067±0.027***	1.05±0.041***
GSH (µM/mg protein)	0.08917±0.001	0.08683±0.001	0.05133±0.001***	0.06671±0.001*
MDA (nmole/g tissue)	197.8±1.352	192.3±1.606	425.2±6.705***	313.7±3.383***
Total Protein (g/dl)	87.12±0.32	86.81±0.72	61.43±0.17***	76.35±0.28***
AChE (µmol/min/mg protein)	2.465±0.1537	2.223±0.07632	0.9333±0.02028***	1.502±0.1271***

All data were represented as Mean±SE. Comparison between groups was done by One-way analysis of variance (ANOVA) followed by Turkey's multiple comparison. *p<0.05 **p<0.01, ***p<0.001 vs control group. Abbreviations: SOD-Superoxide dismutase, GSH-Glutathione, MDA-Malondialdehyde, AChE- Acetyl cholinesterase

Discussion

Chick is proven to be an excellent model for cypermethrin induced teratogenic disorders in embryonic brain of *Gallus domesticus*. Present studies showed that cypermethrin, a pyrethroid insecticide have the potential of inducing histopathological and biochemical changes in developing chick embryo. Present work revealed that cypermethrin intoxication cause varying degree of vacuolization in different layers of the cerebellum including molecular, Purkinje and granular layers. Vacuolization in outer molecular layer was observed, Innermost granular layer showed loss of neurons. Purkinje cells showed pyknosis in the nucleus along with degenerative dendritic processes as compared to control group. Vitamin C supplemented group along with cypermethrin showed normal purkinje cells with euchromatin nucleus and prominent nucleolus. The cytoplasm of purkinje

cells has increased number of double layered mitochondria except some with dilated cisternae of Golgi. The granular layer was intact with healthy neurons. Present study support findings of Manna *et al*. (2005) [23] who explained deltamethrin induced mild to moderate histological alterations in lungs, liver (congestion and fatty changes), stomach, kidney, testes and cerebellum of rats. Cypermethrin treatment induced cerebral edema, hemorrhage, loss of lobar architecture, coagulation necrosis, congestion of blood vessels, fibrosis, necrosis in chick brain [24] The oral exposure to cypermethrin introduced significant histopathological alterations in the brain of rats [25] Several studies explained that pyrethroid treatment leads to histo morphoic distortions in cerebellar and cerebral tissues of albino rat [25, 26].

Present study showed that cypermethrin exposure triggers biochemical alterations in brain of developing embryo. Total protein content and antioxidant enzyme activity of SOD, catalase, GPx was significantly decreased whereas the level of lipid peroxidation was significantly increased in treated group of developing brain. The decreased level of antioxidant level in respect to cypermethrin induced ROS, showed the scavenging action of these antioxidant marker. The brain acetylcholinesterase activity was found to be decreased in treated group. Present work strongly supports the findings of Anwar (2003) [24] who postulated that cypermethrin induced teratogenic effects which might have caused some genetic mutations, leading to developmental abnormalities. Biochemical alterations were also reported in maternal and fetal compartments. Svartz *et al*. (2016) [27] has been reported that cypermethrin cause stage dependent toxicities in the American toad species. Larvae were more prone to toxicities than embryos. Murkunde *et al*. (2012) [28] studied transplacental genotoxicity effects of cypermethrin on developmental stages of Wistar rat foeti which was found killed on gestation day 20. Studies reported that cypermethrin influence biochemistry and morphology of 11th day old chick embryo (*Gallus domesticus*) in dose dependent manner. It was also reported that cypermethrin cause alterations in biochemical aspects such as increase in amylase activity whereas decline in the activity of AkP. The level of glycogen, free amino acids, total lipids, cholesterol, DNA and RNA contents were found to be significantly affected in cypermethrin intoxicated groups [29]. Recovery group in the present study showed that vitamin C exhibit antioxidant properties thus increased level of antioxidant marker enzymes in brain was estimated. Vitamin C also helps in restoring total protein level and acetylcholinesterase activity of developing brain. Present study is in consistent with work of Assayed (2010) [30] who reported countracting role of L-ascorbic acid against cypermethrin treated female and male. Sharma and Bhardwaj (2018) [31] reported ameliorative effects of vitamin C in reversing permethrin induced alterations in antioxidant system of goat testis. Vitamin C has remedial properties against cypermethrin induced teratogenic deformities in the lungs and thorax. The protective effect of vitamin C against subcutaneous oedema, eye deformities, brain lateral ventricles dilatations, heart and kidneys malformations. Present study supported the findings of Siman and Eriksson (1997) [32] who stated that vitamin C supplementation reduce the elevated concentrations of thiobarbituric acid reactive substances (TBARS) in serum of pregnant diabetic rats.

Conclusion

It is concluded that cypermethrin instigates both the histoarchitectural and biochemical alteration in the developing embryos of *Gallus domesticus*. Cypermethrin cause vacuolization in different layers of cerebellum. Purkinje cells show degenerative changes in nucleus and dendritic processes. Decreased level of antioxidant enzymes like CAT, SOD, GSH and increase in the level of lipid peroxidase (MDA) in cypermethrin exposed group showed the scavenging action of marker enzymes in response to elevated level of ROS. Vitamin C is potential natural product which effectively reverse the deleterious effects of cypermethrin on histology and biochemistry of developing cerebellum of chick. The elevated level of antioxidant markers in vitamin C supplemented groups validate the protective effect of vitamin C against cypermethrin induced neurotoxicity.

Acknowledgements

The authors are thankful to the Kurukshetra University, Kurukshetra for the financial support granted as University Research Scholarship to Mr. Shivkant Sharma and grateful to Department of Zoology for providing laboratory facilities throughout the study.

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