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Ameliorative effect of omega-3-Fatty acid on Cypermethrin induced developmental neurotoxicity in chick embryo

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Abstract
Pyrethroids are a class of insecticides that are used for crop protection worldwide. Cypermethrin, a class II pyrethroid, is used to control insects in the household and agricultural fields. It crosses the blood-brain barrier and induces neurotoxicity and motor deficits. Cypermethrin prolongs the opening of sodium channel, thereby causing depolarisation leading to hyper-excitation of the central nervous system. It also modulates chloride, voltage-gated calcium and potassium channels, alters the activity of glutamate and acetylcholine receptors and adenosine tri-phosphatases and induces DNA damage and oxidative stress in the neuronal cells. The present study aimed to evaluate the Cypermethrin induced developmental neurotoxicity in chick embryo. We have also investigated the ameliorative effect of omega-3-Fatty acid on Cypermethrin induced toxicity. Sixty eggs (0 day) of species *Gallus domesticus* were used and divided into six groups -control, vehicle, CYP (50 ppm), CYP (100ppm), CYP (50ppm) + DHA (200µg/egg) and CYP (100ppm) + DHA (200µg/egg) were kept in an incubator at 38±0.5°C with a relative humidity of 60-70% and proper ventilation. Control group have untreated eggs. The vehicle group was supplied with distilled water while the eggs in remaining four groups were administered by air sac method. 16-day old embryos were recovered from all the groups for assessing morphometric, biochemical parameters and histopathology. Results showed that group treated with higher dose of cypermethrin (100ppm) showed a marked decrease in morphometric parameters. Administration of docosahexanoic acid (200µg) in combination with cypermethrin ameliorated the neurological changes produced by Cypermethrin alone in the chick embryo.

Keywords: Cypermethrin (CYP), Omega-3-fatty acid, docosahexanoic acid (DHA), *Gallus domesticus*, morphometric parameters.

Introduction

Pesticides are the chemical compounds that are used to kill pests, including insects, rodents, fungi and unwanted plants (weeds). Pesticides are also used in public health to kill vectors of disease, such as mosquitoes and in agriculture to kill pest that damage crops (Alaa-Eldin *et al.*, 2017 and WHO). There are numerous occupational and domestic uses raise many questions about their deleterious health effects. Because each pesticide has different physiological and toxicological properties (Hocine *et al.*, 2016). The ideal concentration of the pesticides is about 1-5% to control the target pests and remaining amount of the pesticides drift into the environment. According to the WHO data every year about 220 million tons of pesticides used worldwide (WHO., 2002; Landrigan *et al*., 2004 and Greene and Pohanish., 2005). From the total quantity, more than 500,000 tons of unused and absolute pesticides are threatening the environment as well as public health in many countries all over the world which is estimated by the Food and Agriculture Organization (FAO) (Series F.P.D., 2000). This unused amount of pesticide residues has been found in dairy, meat products, food products, soft drinks and water (Alaa-Eldin *et al.*, 2017). Excessive use of pesticides leads to accumulation in the tissues through the contaminate food products and results in major serious toxicities in the non-targeting tissues (Assayed *et al*., 2010). The amount of pesticides in the environment causes morbidity and mortality in developing countries. There are about 3.5-4 million cases of pesticides toxicity resulting 2.2 million causalities occurs annually (Abdou *et al*., 2012).

Materials and method:

Cypermethrin, $C_{22}H_{19}Cl_2NO_3$ ((RS)-alpha-cyano-3- phenoxybenzyl-(1RS, 3RS, 1RS, 3SR)-3-(2,2-dichlorovinyl) - 2,2-dimethyl-cyclopropane carboxylate) having CAS no.52315-07-8, 99% purity was procured from Pesticide India Ltd. New Delhi. All the other chemicals used for research work were of analytical grade and purchased from Rankem (RFCL ltd.) Mumbai and Hi-Media Laboratories Pvt. Ltd.

Experimental Design

Test Animals

Eggs (0 day) of chick (*Gallus domesticus*) were procured from the Central Poultry Development Organization (Northern Region), Ministry of Agriculture & Farmers Welfare, Government of India, Industrial Area, Phase-I, Chandigarh. They were divided into 6 groups (10 eggs each) and were stored into the incubator at 37.5 ± 2˚C.

Pesticide and Ameliorant Dose

Cypermethrin of analytical grade (99.8 % v/v) obtained from Insecticide India Pvt. Ltd. Two doses of cypermethrin were selected for the study, 50 ppm and 100 ppm ($1/13^{th}$ and $1/26^{th}$). The LD_{50} of Cypermethrin for chick eggs was observed around 675 ppm (Anwar., 2003; Anwar., 2003 and Anwar., 2010). DHA (Docosahexaenoic Acid) was taken as the standard drug to ameliorate the neurotoxicity produced by the two different doses of CYP (50 ppm & 100 ppm). The standard dose of DHA was 200 µl/egg and selected on the basis of literature survey (Berman *et al.*, 2013 and Arteaga *et al.*, 2017& Kishore *et al.*, 2009).

Dosing Schedule

Sixty eggs (0 day) of species *Gallus domesticus* were used in this study and were classified into six groups (10 eggs each group) as follows:

Group I: Incubated without any dosing served as control group.
Group II: Received 0.05ml of vehicle and served as control vehicle group.
Group III: Received 0.05ml the pesticide CYP 50ppm per egg on "0 day" incubation through Air sac method.
Group IV: Received 0.05ml the pesticide CYP 100ppm per egg on "0 day" incubation through Air sac method.
Group V: Received 0.05ml the pesticide CYP 50ppm and DHA 200µg/egg on "0 day" incubation through Air sac method.
Group VI: Received 0.05ml the pesticide CYP 100ppm and DHA 200µg/egg on "0 day" incubation through Air sac method.

Both the pesticide (CYP) as well as Ameliorant (DHA) were administered through Air Sac method (by puncher the upper shell of the egg on the vegetal portion and inject the both using tuberculin syringe). On 16^{th} day chick embryos were isolated and homogenize the organs like brain, liver and heart by crushing organs separated in homogenate tube by mixing with 10 volumes of the phosphate buffer of tissue weight and centrifuged at 5000rpm for 5min. to separate the supernatant and discard the cell debris. The supernatant was again centrifuge at same rpm for 5 min. and discard the supernatant and add 1-2 ml of phosphate buffer to the tube and dissolve the sedimented pallets and same procedure was followed for 2 time and final supernatant was used for biochemical estimation; AChE (Ellman *et al.*, 1960), SOD (Marklund and Marklund., 1974), CAT (Aebi., 1984), GPx (Rotruck *et al.*, 1973), FRAP (Benzie and Strain., 1999) and MDA (Okhawa *et al.*, 1979).

Stastical Analysis

The data was analyzed by one-way analysis of variance (ANOVA) followed by t-test to determine the level of significance using Graph pad prism 5.0 (Chicago, USA). The value of $p<0.05$ was considered as significant results are expressed as mean.

Result

The present study showed the neuroprotective effects of Docosahexaenoic acid (DHA) on cypermethrin induced developmental neurotoxicity in chick embryo. The developmental toxicity of cypermethrin was evaluated by observing the changes in the different morphometric, biochemical parameters and histopathology in chick embryo. The experimental plans were conducted in such a manner that the groups of fertilized eggs were treated with two different doses of cypermethrin on the "critical period" ('0' day incubation) of embryogenesis of developing chick embryo. The surviving embryos were opened on the day 16 and were examined for morphometric parameters. The brain was taken out from embryos for their biochemical and pathology studies. The morphometric parameters of 16day old chick embryo was found to be decreased in the cypermethrin (50 ppm & 100 ppm) treated group in dose-dependent manner in comparison with control group. The results

concluded that the group treated with lower dose of cypermethrin (50ppm) showed slight deviation in morphometric parameters but the group treated with higher dose of cypermethrin (100ppm) showed a marked decreased in above parameters. The other parameters don't affect to estimation of the toxic effect of cypermethrin. But the toxic effects were significantly ameliorated by the 200µg dose of DHA. The change in the morphometric parameters due to cypermethrin intoxication and its amelioration by DHA were reported (Table 1 and Fig. 1,2). Effect of Cypermethrin on various biochemical parameters such as Superoxide Dismutase (SOD), Catalase (CAT) Glutathione Peroxidase (GPx), Lipid Peroxidation, Acetylcholinesterase Assay, Ferric Reducing/ Antioxidant Power Assay (FRAP ASSAY) was reported **(**Table 2 and Fig. 3-8). Light microscopic studies showed that CYP affects the normal architecture of embryonic brain of *Gallus domesticus.* Deteriorating effects of cypermethrin were observed in CYP treated groups as compared to control group (Fig.9A-F). The group treated with lower dose of CYP (50ppm) and DHA (200µg) treated embryonic brain section showed showing highly improved cerebellar cortex with increased number of small neurons in granular layer, Purkinje cells in Purkinje cell layer and glial cells in molecular layer while the group treated with CYP (100ppm) and DHA (200µg) showed less improved cerebellar cortex with increased number of cells in both Purkinje cell layer, granular layer and molecular layer**.**

Table 1: Effects of Cypermethrin on the morphometric parameters of 16day old chick embryo0

Groups	Morphometric Parameters							
	Body Weight (g)	**Head Diameter (cm)**	**Eye Diameter (cm)**	**Beak Length (cm)**	**Neck Length (cm)**	**Tibia Length (cm)**	**Femurs Length (cm)**	**Crown Rump Length (cm)**
Control	15.51 ±0.12	1.53±0.06	1.03± 0.15	1.03± 0.15	1.63± 0.06	1.2 ± 0.1	1.77± 0.06	10.10 ±0.36
Vehicle	15.06 ± 0.30	1.47± 0.06	0.87± 0.06	0.87± 0.06	1.5 ± 0.1	1.1 ± 0.1	1.60± 0.1	9.67± 0.15
CYP (50ppm)	14.52± 0.17*	1.37± 0.06*	0.83± 0.06*	0.8±1.36*	1.37± 0.06**	0.93± 0.06**	1.53± 0.06**	9.33 ± 0.15**
CYP(100ppm)	13.48± 0.1**	1.27± 0.71**	0.73± 0.06**	0.73± 0.06**	1.23± 0.06**	0.9 ± 0 ***	1.40± 0.1***	9.03 ± 0.21**
CYP (50ppm) +DHA(200µg)	14.76± 0.17*	1.47± 0.06*	0.867± 0.06	0.933± 0.06*	1.533 ± 0.06*	1 ± 0*	1.53± 0.06**	9.50 ± 0.17*
CYP(100ppm) +DHA(200µg)	14.48± 0.22**	1.37± 0.06**	0.83± 0.06**	0.83± 0.06**	1.4 ± 0.1**	0.97± 0.06**	1.43± 0.06**	9.13 ± 0.15**

Each value was mean ± SEM of three observations, *p<0.05, **p<0.01 and ***p<0.001 vs. cypermethrin treated groups and cypermethrin + DHA treated groups. (ANOVA followed by t-test)

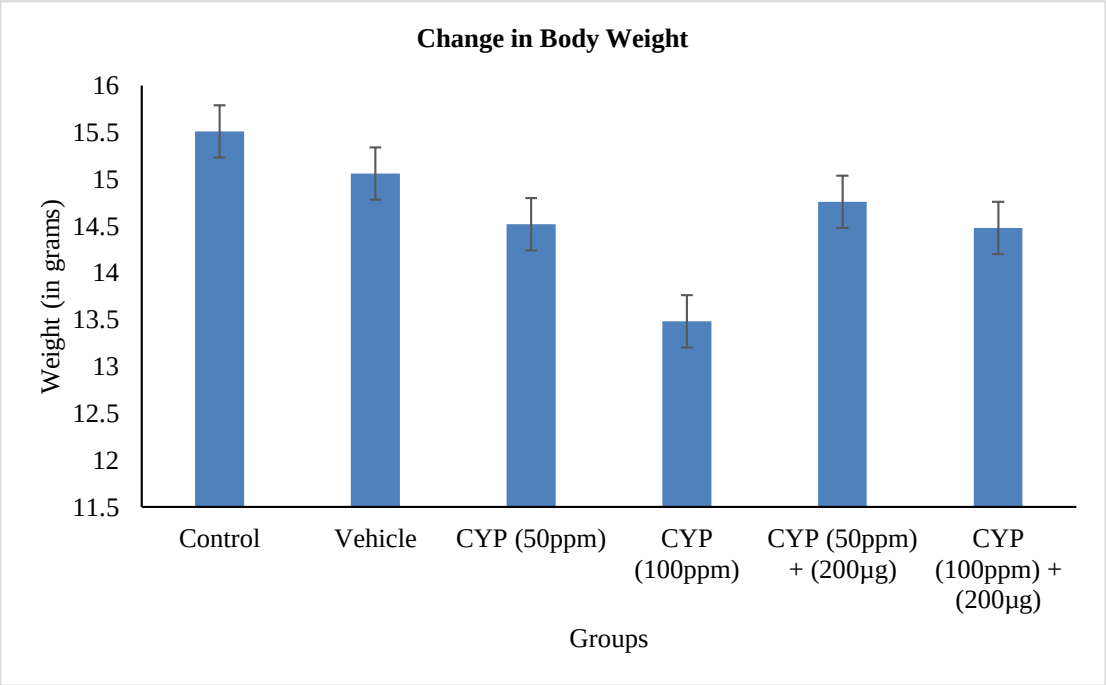

Fig. 1 Changes in the Body weight of 16dayschick embryo of different groups

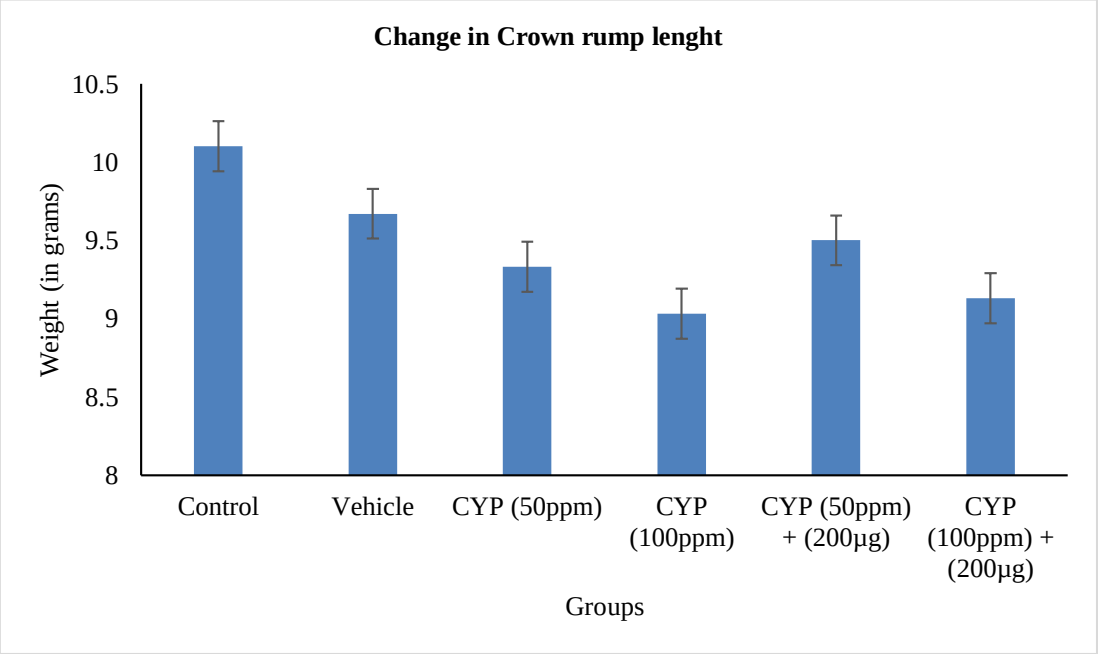

Fig. 2 Changes in the Crown rump length of 16 days chick embryo of different groups

Table 2: Effect of Cypermethrin on the biochemical parameters of 16 days old chick embryo

Parameter	Groups					
	Control	Vehicle	CYP (50ppm)	CYP (100ppm)	CYP (50ppm) +DHA(200µg)	CYP (100ppm) + DHA (200µg)
SOD	7.037 ± 0.126	6.744 ± 0.165	6.231± 0.252***	5.289 ± 0.162***	6.27 ± 0.0635***	6.124 ± 0.107***
CAT	0.857 ± 0.011	0.823 ± 0.0115	0.725 ± 0.0087**	0.666 ± 0.0135***	0.763 ± 0.0085**	0.737 ± 0.0123***
GPx	4.443 ± 0.300	4.032 ± 0.125	3.767 ± 0.091**	3.449 ± 0.188***	3.849 ± 0.075**	3.597 ± 0.176***
LPO	177 ± 8.598	197 ± 8.626	234.8 ± 18.834***	256.1 ± 10.697***	221.1 ± 7.350***	211.1 ± 4.015**
AChE	0.117 ± 0.007	0.103 ± 0.006	0.095 ± 0.004**	0.088 ± 0.008***	0.101 ± 0.003**	0.096 ± 0.007**
FRAP	16.24 ± 0.479	15.25 ± 0.554	13.99 ± 0.786**	13.1 ± 0.711***	14.24 ± 0.449**	14.38 ± 0.488*

Each value was mean ± SEM of three observations, *p<0.05, **p<0.01 and ***p<0.001 vs. cypermethrin treated groups and cypermethrin + DHA treated groups. (ANOVA followed by t-test)

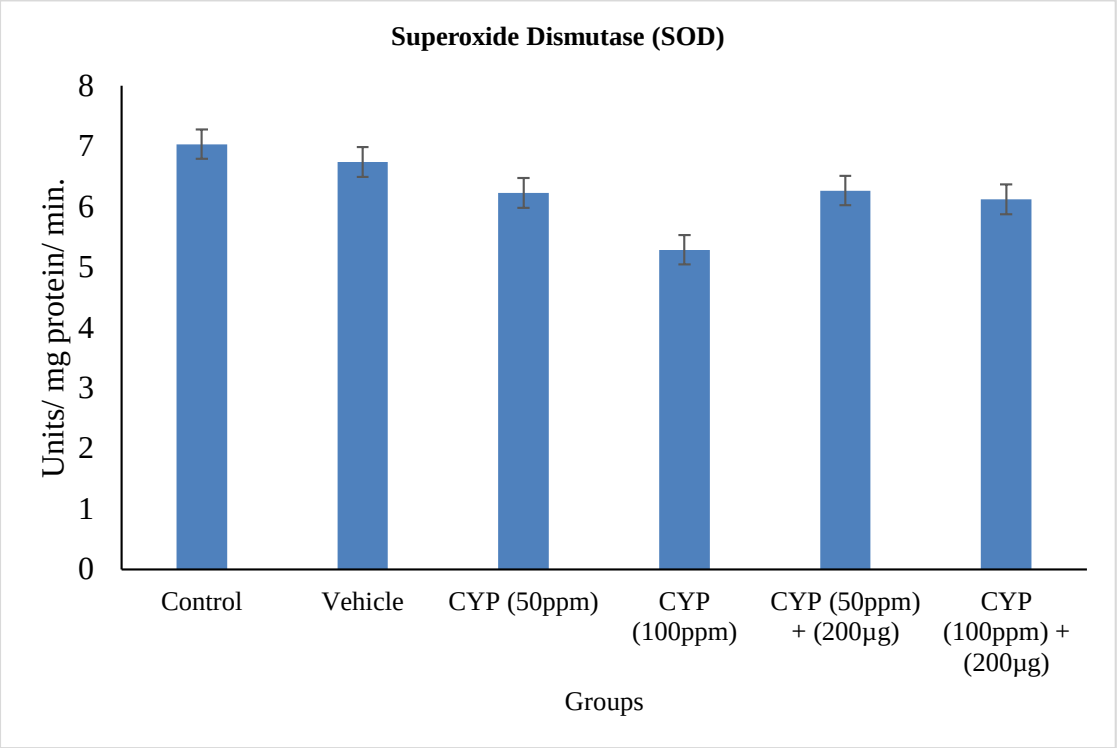

Fig. 3 Changes in the SOD level in 16days chick embryo of different groups

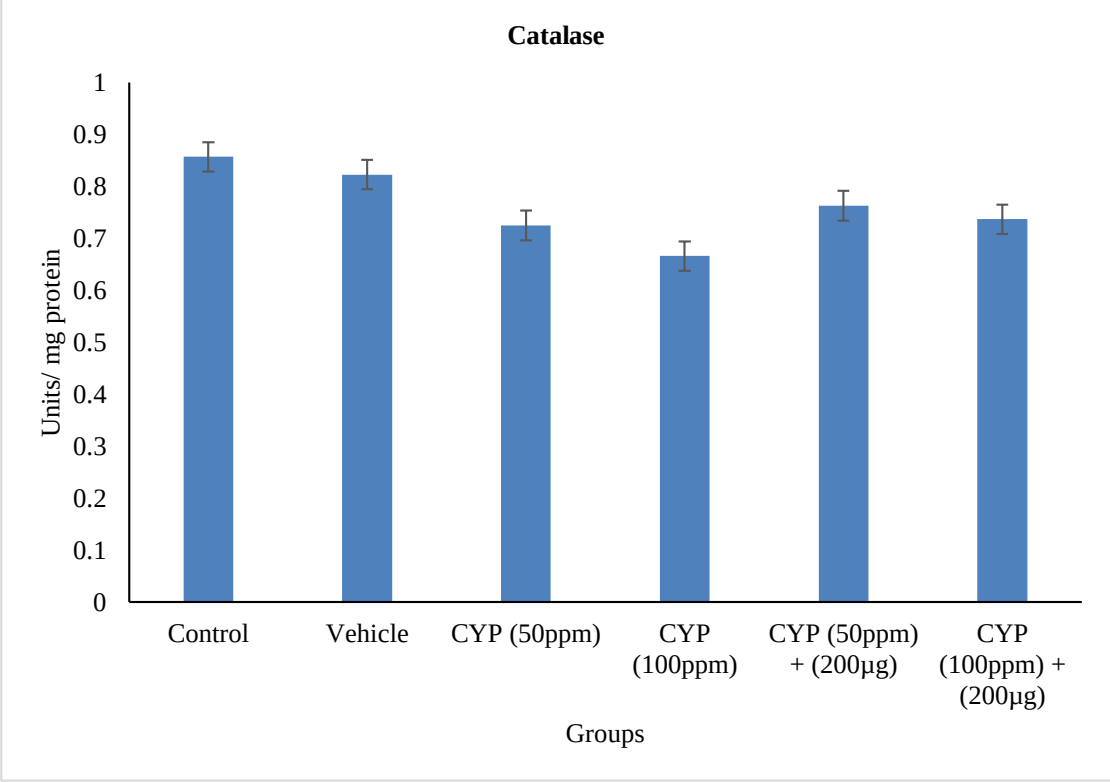

Fig. 4 Changes in the CAT level in 16days chick embryo of different groups

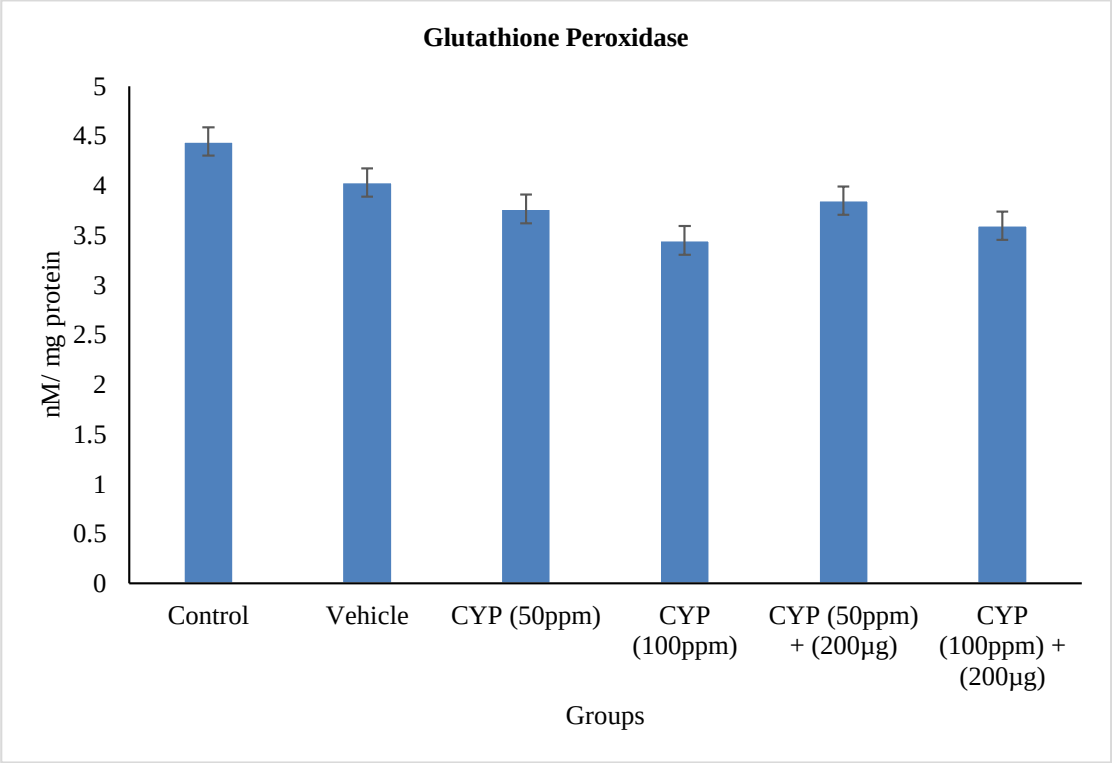

Fig. 5 Changes in the GPx level in 16days chick embryo of different groups

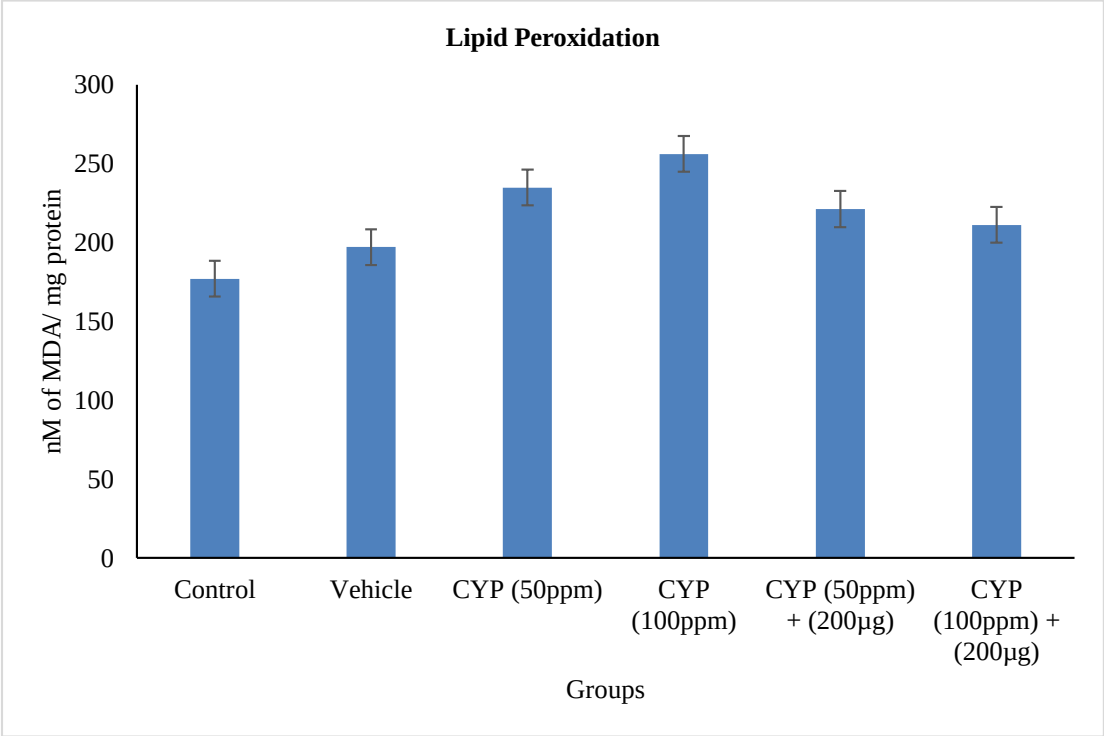

Fig. 6 Changes in the LPO level in 16days chick embryo of different groups

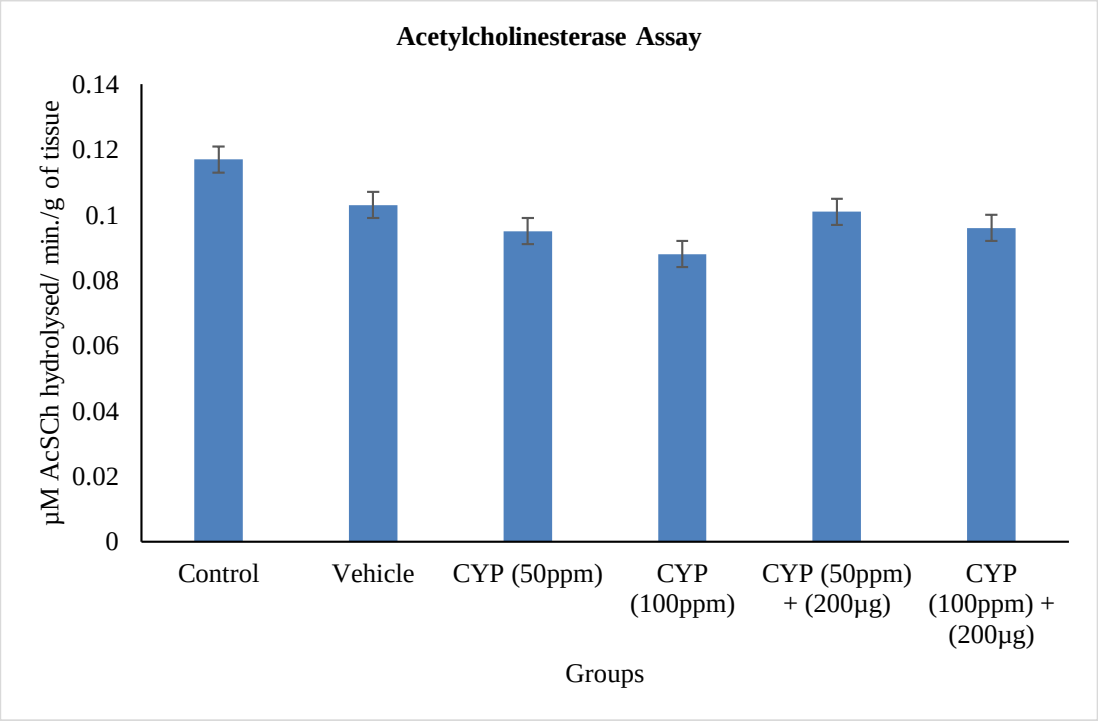

Fig. 7 Changes in the AChE level of 16days chick embryo of different groups

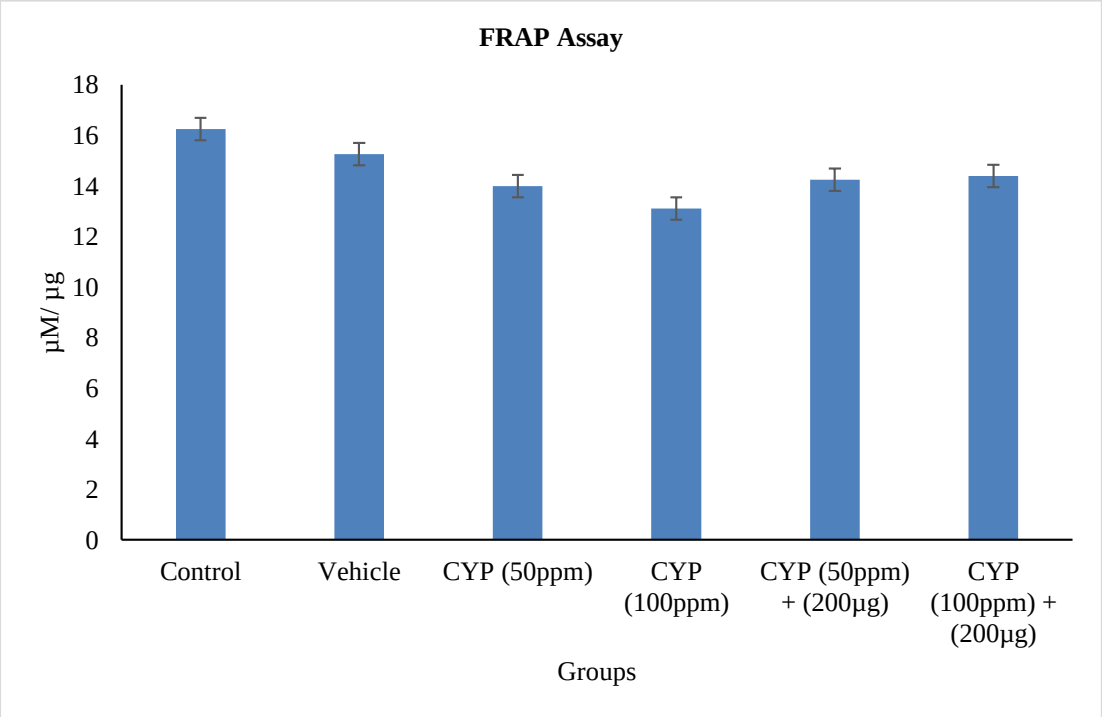

Fig. 8 Changes in the FRAP level of 16 days chick embryo of different groups

Histopathology

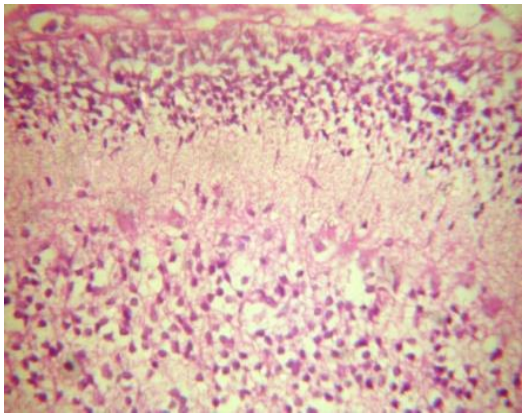

Fig. 9 A (Control) showing healthy histoarchitecture of brain having three well-defined layers, the granular layer, the Purkinje cell layer and the molecular layer.

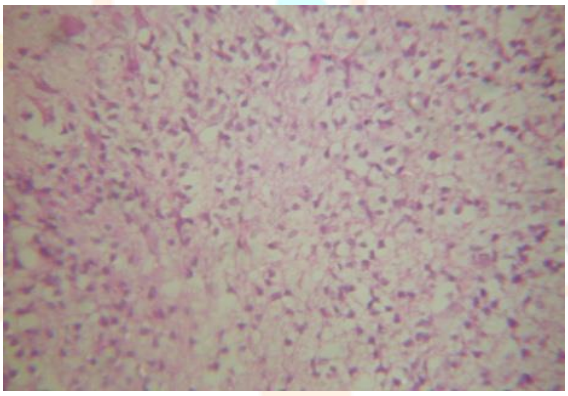

Fig. B(Vehicle) showing healthy brain having Purkinje cells.

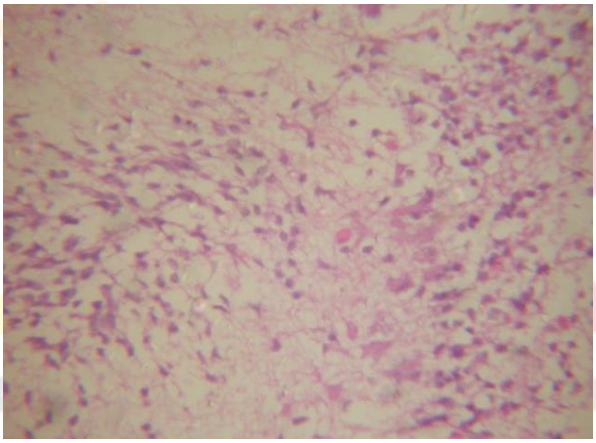

Fig. C (CYP treated 50 ppm) showing slight disrupted cerebellar cortex still keep a series of deeply convoluted folds supported by branching central core with granular layer, Purkinje cell layer, and the molecular layer.

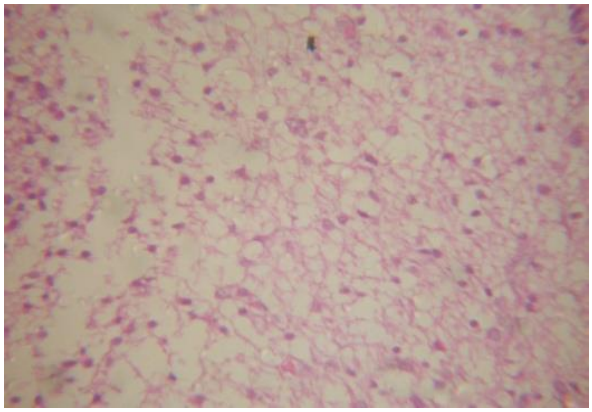

Fig. D (CYP treated 100 ppm) showing full disruption of cortex of cerebellum, decreased number of neurons in both Purkinje cell layer, granular layer.

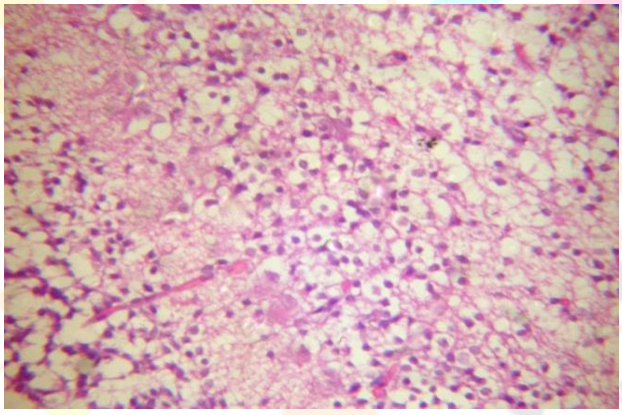

Fig.E. CYP (50ppm) and DHA (200µg) showing highly improved cerebellar cortex with increased number of small neurons in granular layer, Purkinje cells in Purkinje cell layer and glial cells in molecular layer.

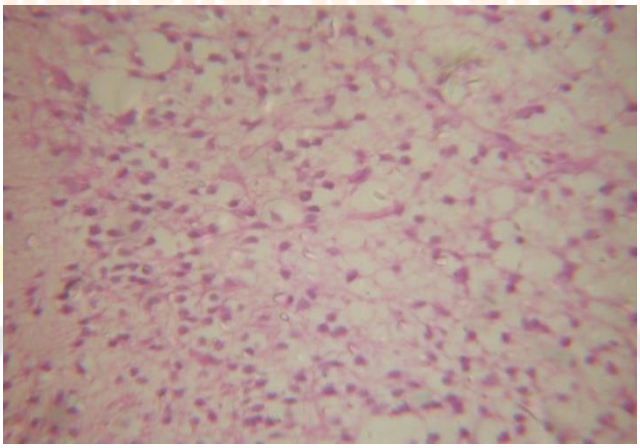

Fig. F. CYP (100ppm) and DHA (200µg) showing less improved cerebellar cortex with increased number of cells in both Purkinje cell layer, granular layer and molecular layer.

Discussion

A large number of environmental as well as manmade chemicals such as drug, toxin, solvents and pesticides exert their toxic effect by interfering with fundamental developmental

mechanisms of an organism and exert them from reaching their proper end points (Uggini *et al.*, 2012). These environmental stressors are also known as teratogens which are responsible for causing teratogenicity in developing embryo or fetus even though these agents have either negligible or no maternal effects. Oxidative stress is reported to be a major mechanism followed by other mechanisms such as mutation, chromosomal abnormalities, mitotic interference, interference with nucleic acid function, nutrient deficiencies, deficient or altered energy supply, change in osmolarity, ultrastructure changes in cell membrane and disruption of retinoid acid signalling pathway by which different types of teratogens exert their developmental effects (Levi, 1987; Paskova *et al.*, 2011). In our study, changes were observed in biochemical constituents of brain of chick embryo at earlier development stages at embryonic day 16 due to administration of cypermethrin. The cypermethrin mediated biochemical changes seems to contribute toward the development of various congenital malformations and pathological changes in the brain of developing embryo. Since the possibilities to conduct experiments during human pregnancy are very limited, therefore many experimental models (chick embryo in present study) are employed comprehensively to examine the mechanisms of teratogenicity as similar pattern of human teratogenesis can also be suspected by various teratogenic agents (Natekar, 2007; Van Gelder *et al.*, 2010). The present study provides the detailed analysis of developmental toxic effects of cypermethrin in developing chick embryo for which there is negligible data available in the literature. This study illustrated that a single dose by air sac method in fertilized eggs of cypermethrin respectively, was showed developmental neurotoxicity in developing chick embryo. The activity of antioxidant may be increased or inhibited under chemical stress depending on the intensity and the duration of the stress applied as well as susceptibility of the exposed species. A decrease in antioxidant levels could be the result of either an increase in ROS that react with the antioxidants or a response to lower production of reactive species. Because of the complex interactions within cells, one test is normally not enough to understand precisely what is going on within the cell. Rather, a broad array of tests is required to determine the different cellular parameters that are occurring within a biological system (Griffin & Bhagooli., 2004).The present study revealed that CYP induce many pathological changes in cerebellum at different doses of CYP in chick embryo including decreased number of purkinje cells, a focal accumulation of the neurocytes cytoplasm of stratum granulosum.

Conclusion

Cypermethrin induced neurotoxicity may be mediated through reduced antioxidant defense mechanism, inhibition of acetylcholinesterase activity. CYP increase the ROS generation in the brain which mediate the change in level of antioxidant enzymes (SOD, CAT and GPx) and also changes the level of oxidative stress markers (LPO and FRAP). Because of change in the biochemical levels the histopathology of cerebellum also affected in dose dependent manner. Administration of DHA (200µg) in combination with cypermethrin ameliorates the neurological changes produced by CYP alone in the 16 days chick embryo. And these findings indicate that the DHA significantly improved the biochemical parameters as well as histopathology of cerebellum.

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