Egypt. Acad. J. biolog. Sci., 3 (1): 19- 26 (2011) **F. Toxicology &Pest control**
Email: egyptianacademic@yahoo.com **ISSN: 2090 – 0791**
Received: 28/7/2011 **www.eajbs.eg.net**

Effect of pesticides mixture of dimethoate and methidathion on acetylcholinestrase during embryo development using chick embryo model

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ABSTRACT

Pesticides mixture (dimethoate 30% and methidathion 40%) prepared by the farmers in Yemen and engaged in the cultivation of Qat, which is chewed by people every day including pregnant women. Therefore, the developing embryos in the society are more vulnerable than adults to the chronic cholinergic intoxication. This study aimed to examine the chronic effect of pesticides mixture on the AChE of developmental embryo in an avian model, which does not share the maternal potential confounds. For this, fresh fertile chicken eggs (*Gallus gallus domesticus*) were used. The lethal concentration of pesticides mixture for 50% killing (LD_{50}) values was computed on the basis of probit analysis and was found to be 40 ppm. $1/5^{th}$ LD_{50} and $1/10^{th}$ LD_{50} (8 and 4 ppm) were chosen to be the tested doses. Eggs weighing 54±1 gm were separated in to 3 batches of 10 eggs each batch. One batch of embryos was injected with normal saline and the other batches of embryos injected with pesticides mixture of 4 and 8 ppm each alternative day starting from incubation day 7 for 2 weeks. On day 21 after 12 hours of the last dose an amount of 200 µl blood was collected from the blood vessels surrounding the embryonic membranes and the heamolyzate was used for the assessment of the AChE activity calorimetrically. Result of this study indicated that $1/10^{th}$ of the LD_{50} had only marginal effect on the AChE activity (40.6%). Whereas $1/5^{th}$ of the LD_{50} of pesticides mixture caused significant inhibition of AChE activity (69%) which could not be reversible. So neuro-developmental consequences such as behavioral changes and memory impairment may prolong throughout the life span of the embryo.

Key words: embryo, pesticide mixture, AChE.

INTRODUCTION

Insect pest management is facing the health and ecological challenge worldwide due to the human and environmental hazards caused by majority of the synthetic pesticide chemicals. Although beneficial in protecting the crop against insect pests, these pesticides have posed a grave environmental problem because of their indiscriminate use in fields. The organophosphorus insecticides (OPIs) are one of the most widely used and consequently their propensity to produce developmental neurotoxicity remains a major concern, (Colborn T, 2006, Costa LG, 2006, Landrigan PJ *et al*, 1999 and Weiss B *et al*, 2004). Because pregnant womens are likely to be exposed to OPIs under circumstances that do not elicit outward signs of intoxication (De Peyster *et al*. 1993; Gurunathan *et al*. 1998) and in light of recent findings that such exposures can produce long term cognitive impairment in their children (Rauh *et al*. 2006; Rohlman *et al*. 2005), the mechanisms and consequences of OPIs developmental neurotoxicity remain a major environmental concern. The OPIs are known to affect the nervous system by inhibiting acetylcholinesterase (AChE), the enzyme that modulates the amount of the neurotransmitter acetyl-choline (Fukuto, 1971). The inhibition of

AChE results in the accumulation of acetylcholine that over stimulates cholinergic receptors, which in turn stimulates neurological activity, (Gallo and Lawryk, 1991). Indeed, a number of studies utilizing neural cell lines or micromass cultures have clearly demonstrated direct effects of organophosphates on neurodevelopment, (Bagchi D *et al*. 1995, Bagchi D *et al*. 2006, Crumpton TL *et al*. 2000, Das KP *et al*. 1999, Jameson RR *et al*. 2006, Monnet-Tschudi F *et al*. 2000, Qiao D *et al*. 2001, Qiao D *et al*. 2005, Slotkin TA *et al*. 2007 and Song X *et al*. 1998).

Unfortunately, people are often exposed to different OPIs in different dosages at different or overlapping times. However, the developing embryos remain more vulnerable than adults do to chronic cholinergic intoxication by OPIs. These agents affect the process of neural development itself, leading to permanent deficits in the architecture of the nervous system.

Despite the increasing recognition of the need to evaluate developmental neurotoxicity in safety assessment (Claudio L *et al*. 2000, Eriksson P. 1997 & Tilson HA. 1995), only very few of the mixture commercial chemicals in current use have been examined with respect to neurodevelopmental effects (Grandjean P & Landrigan PJ, 2006).The use of mixture of OPIs raises the possibility of antagonistic, additive, or synergistic neurotoxicity in exposed organisms. Few studies have begun to characterize the toxicological effects of pesticides mixture exposure (Richardson *et al*., 2001; Schuler *et al*., 2005; Moser *et al*., 2006). On the other hand mixing organophosphate insecticides, dimethoate and methidathion (PM), becomes very common among the farmers in Yemen during growing Qat (Katha Edolis Forsk). This plant (Qat) is used to be chewed daily by people in the society including pregnant women and result in an increase in the prevalence of mixed neurodevelopmental toxicity. Recent evidence on direct roles of acetylcholinesterase (AChE) on neuronal development provides additional needs for investigating the developmental toxicity of OPIs. Therefore, the effect of the PM on the AChE of the developing chick embryo was studied by an in vivo approach. Chick embryo model has being preferably used in recent work for studies of developmental neurotoxicity of organophosphates as well as other neurotoxicants (Yanai J *et al*., 2004 & 2008). Studies in mammalian models incorporate both direct and indirect neurodevelopmental effects. The indirect effects are mediated through maternal physiology and maternal/neonatal interactions. With chick embryo, we can administer PM directly to the medium surrounding the embryo without maternal mediation, eliminating the variables related to maternal physiology. In this study the effects of PM examined in an avian model, which does not share these potential confounds. From literature review lot of studies concerned the acute effect of organophosphorous insecticides on the AChE during embryo development, but this study concerned the chronic effect of PM on the AChE of developmental embryo.

MATERIALS AND METHODS

Fresh fertile chicken eggs (*Gallus gallus domesticus*) were used. The eggs were cleaned and placed in a commercial incubator at 37 ± 1 C° and 60 – 65 % RH, and embryonic survival was monitored via candling. An injection window was prepared by drilling a hole in the pointed end and injection of 100 µl of PM or normal saline was performed by 100 µl micropipette in to the air sac. The lethal concentration for 50% killing (LD_{50}) values was computed based on probit analysis, (Finney, 1964) and was found to be 40 ppm. The doses $1/10^{th}$ LD_{50} & $1/5^{th}$ LD_{50} of PM (4 ppm and 8 ppm, respectively) were chosen to be the

tested doses. 30 fresh fertile eggs weighing 54±1 gm were separated in to 3 batches, each batch contain 10 eggs. One batch of embryos was injected with normal saline. The other batches of embryos injected with 4 or 8 ppm PM each alternative day, starting from the day 7 of incubation for 2 weeks. The injection hole was sealed with plastic tap after each injection. On day 21 after 12 hours of the last dose an amount of 200 µl blood was collected from the blood vessels surrounding the embryonic membranes and the heamolyzate was used for the assessment of the enzyme activity calorimetrically, *(*Sadasivam & Manickam 1996).

Statistical method:

Statistical analysis was performed with the aid of the Graph Pad software package. Values are presented as means ± standard error of means (SEM). Student "t" test used for the analyzing of the data.

RESULTS AND DISCUSSION

The systemic toxicity of OPIs reflects the symptoms related to cholinergic hyperstimulation consequent to the irreversible loss of AChE catalytic activity, which typically emerges when inhibition exceeds 70% (Clegg and van Gemert 1999). Result of this study indicate that repeated exposure of embryo to $1/10^{th}$ of the LD_{50} (4 ppm) of PM inhibited the activity of the embryo blood AChE by (40.6%), well below the 70% threshold for the emergence of any symptoms of irreversible recovery of the AChE activity (Clegg and van Gemert 1999). And $1/5^{th}$ of the LD_{50} (8 ppm) inhibited embryo blood AChE by 69%, which is about the threshold of irreversible recovery of the AChE activity, (Table 1). The results indicate that $1/10^{th}$ of the LD_{50} had only marginal effects of AChE inhibition, whereas $1/5^{th}$ of the LD_{50} of PM caused significant inhibition of AChE (69%) which could not be reversible and may associate with prolonged behavioral changes and memory impairment, (Hong Zhu *et al*. 2001).

Table 1: Effect of pesticides mixture (PM) on the Acetylechlinestrase activity in chick embryo (U/ L).

Dose (ppm)	**Control**	**Treated**	**P value**
4	561.61 ± 42.62	334.45 ± 1.8	< 0.05 *
8		173.112 ± 2.1	< 0.05 *

Values are expressed as mean ± SEM (n = 10), Student 't' test.
*p value is significantly different from control

The result of this study is in consistent with other studies; Miwa Misawa *et al*. 1981, reported that injection of diazinon on day 3 of incubation inhibited AChE of chick embryo at the age of day 6 and day 8. Slotkin *et al*. 2006b and Song *et al*. 1997, reported that treatment of neonatal rats with 1 mg/kg chloropyrifos produced 10–20% of AChE inhibition. Hughes *et al*. 1997, also reported the drastic inhibition of carp AChE activity after 5 hours exposure to 2 ppm methidathion which associated with muscular and neural disturbances. Amina *et al*., 2006 and Mohan Snigh *et al*. 2006, reported that dimethoate separately inhibited AChE activities in maternal and fetal brain. Considering all the functions of AChE, we must ask whether PM might harm developing embryos by hindering the architectural development of their nervous systems. This would be rather speculative, especially when it involved

the risk of low level of AChE inhibition by PM at the dose of 4 ppm. As AChE has extra-synaptic, non-cholinergic role, morphogenic role, and has a cell adhesive function during neural development, (Layer 1995, Massoulie *et al*. 1991 and Robertson 1993). Therefore as consequences of the AChE inhibition by PM, it could interfere with the morphogenic function of AChE. This has effect on the neural development and axial skeletal defects in addition to behavioral changes such as embryonic convulsive and uncoordinated movements (Peddrick Weis and Judith S. Weis, 1975 & Meneely and Wyttenbach, 2005). David and Louis, 1984 reported the inhibition of brain AChE of chick embryo treated with the organophosphorus insecticide EPN associated with cervical and axial scoliosis as well as severe edema. Similar results were reported by (Siddiqui *et al*. 1991 & Zahran *et al*. 2005) on animals treated with OPIs. In vitro studies employing a variety of neuronal cell types have reported retardation of neurite outgrowth in response to AChE inhibition in chick embryo, (Layer *et al*. 1993). As the cholinergic system is involved in the growth of the axial length of the eye, (Young FA., 1965 and Stone RA *et al*., 1991), inhibition of AChE during embryo developmental process may result in ocular developmental defects. Previous work on other organophosphate insecticides such as chloropyrifos showed that dosing of chick embryo with chloropyrifos from day 2 to day 9 of incubation yielded an inhibition of 45% acetylcholinesterase in brain. This is resulted in a significant degree of myopia in form-deprived eyes resulting from significant lengthening of the vitrial chamber of the eye, (Andrew M. Geller *et al*. 1998). AChE also occurs in migrating neural crest cells (Miki *et al*., 1983) and inhibition of AChE by PM during early embryo development will definitely lead to a defect on the neural tube and on the CNS development. Moreover inhibition of AChE by PM may interfere with fetal developmental process that are largely dependent on the AChE ability to hydrolyze ACh (Soreq and Seidman 2001), such as neural outgrowth (Bigbee *et al*. 2000), synaptogenesis (Sternfeld *et al*. 1998), cell adhesion (Bigbee and Sharma 2004), and neuronal migration (Byers *et al*. 2005 & Dori *et al*. 2005). Indeed, the inhibition of fetal AChE may be deleterious to coordinate development of the CNS given the postulated novel role for the AChE in nervous system development (Lassiter *et al*., 1998). Acetylcholine is released in response to nerve stimulation and binds to post-synaptic acetylcholine receptors, resulting in muscle contraction or gland secretions. Its action is rapidly terminated by hydrolysis with AChE via the serine hydroxyl in the catalytic triad of AChE, (Koelle GB, 1946). So the indirect effect of PM exert in its ability to induce toxicity through the inhibition of AChE, leading to accumulation of acetylcholine and subsequent activation of cholinergic, muscarinic, and nicotinic receptors (Bagchi *et al*., 1995). Accumulation of acetylcholine due to inhibition of AChE by PM may induce bradycardia, which is a common effect of acetylcholinesterase inhibitors, (Lin *et al*., 2007). However, the neuro toxicity of dimethoate seperatly depends on mixed function oxidase (MFO) catalyzed activation to its corresponding oxygen analog, which is direct inhibitor of AChE (Maroni *et al*., 2000). Therefore, PM may exert its effects through electrophilic attack on the cellular constituents of the developing tissues (Samanta and Chainy, 1995) with simultaneous generation of reactive oxygen species (Lemaire *et al*., 1994; Sharma *et al*., 2005). On the other hand, inhibition of AChE by PM may affect the antioxidant enzyme system which accelerate the developmental toxicity of PM, (Olga L´opez *et al*.,

2007). Mohamed Abou-Donia 2003, reported extensive neuropathological alterations and neurological deficits of the CNS accompanied by sever inhibition of AChE due to exposure of human to sarine.

CONCLUSION

This study illustrates that chick embryo represents a promising model system for evaluation of developmental neurotoxicity of organophosphate insecticides. The chronic exposure of chick embryo to low doses of PM induced inhibition of AChE activity. Due to the nonenzymatic functions of AChE during critical embryo CNS development PM may disturb the neuro-development so memory impairment and behavioral changes may prolong throughout the life span of the organism.

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