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EFFECT OF COMBINED EXPOSURE OF DICHLORVOS AND MONOCROTOPHOS ON NEUROTRANSMITTERS AND ACETYLCHOLINESTERASE IN RAT

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ABSTRACT: Organophosphate pesticides are associated with neurotoxicity. Organophosphates are cholinesterase-inhibiting chemicals used predominately as pesticides. These have played a very important role in sustainable production of food, animal feed and as protection against disease vectors. They are also used as chemical warfare agents (nerve agents). The effects of dichlorvos and monocrotophos on brain neurotransmitters are well established. The combined effect of organophosphates (dichlorvos and monocrotophos) was supposed to have synergistic or additive effect on brain neurotransmitters. Combined exposure of dichlorvos and monocrotophos has significant inhibition in acetylcholine esterase activity during both studies of 15 days and 30 days. Levels of nor-epinephrine, dopamine and serotonin (5-hydroxytryptamine) increased significantly in dichlorvos and monocrotophos exposed animals. However, the increase was more pronounced in monocrotophos alone exposed animals. Thus, contrary to hypothesis, the combined exposure to these toxicants did not produce more pronounced toxicity as compared to their individual exposure except in case of nor-epinephrine.

INTRODUCTION: Easy availability and use of many organophosphates pesticides has been very common in the insecticide markets in the developing countries and it has resulted in an increase in the prevalence of mixed toxicity. Pesticides have great impact on all fields of economics, environment, and public health. Use of pesticides is helpful in enhanced productivity and availability of food to meet demands. They also enhance the storage life by controlling vector borne diseases and thus play a crucial role in today's world [1].

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Pesticides can be classified on the basis of chemical structure and modes of action. Organophosphate pesticides (OPs) are one of the most widely used classes of pesticides. OPs such as dichlorvos (DDVP) and monocrotophos (MCP) contain phosphorus and derivatives of phosphoric acids [2]. They inhibit the enzymatic activity of acetylcholine esterase (AChE), the key enzyme that hydrolyzes the neurotransmitter acetylcholine an enzyme responsible for the termination of action of acetyl choline [3]. Inhibition of AChE enzyme results in the accumulation of acetylcholine that causes over stimulation of cholinergic receptors, which in turn stimulates neurological activity in the organism [4].

Organophosphates are the most frequently used pesticides detected in the house dust and indoor air of homes [5, 6]. Widespread use of these has resulted in exposure to these pesticides to large sectors of population, which include agriculture workers and

their families, besides the common population who may be exposed through home application of pesticides or *via* residues on food [7, 8]. The use of many or one after the other may provide rapid action and more residual effect than any of them if applied singly in sequence. Multiple exposures of pesticides may affect the efficacy, toxicity profile of pesticides and effects thus produced may be additive or synergistic or far dangerous in comparison which occurs on single exposure to any pesticide. Occurrence of prolonged multiple exposures to chemicals in society are common. Still only, few studies have been carried out to find the effect of interaction between toxic substances like pesticides that damage the nervous system [9].

According to draft efficacy guidelines 607, use of pesticides should be the minimum and the dose used should be appropriate. Therefore this type study should be included as part of efficacy evaluation of multiple exposure [10]. Damage occurring by pesticides involves many mechanisms [11]. Generation of free radicals is one of the most important mechanisms behind toxicity of organophosphates [12, 13]. There is production of metabolites which cause oxidative damage by carrying out oxidation of fatty acid of membrane lipids and inactivation of protein and enzymes [14]. Organophosphates are detoxified by the action of carboxylesterase [15]. Carboxylesterases (β-esterase or non-specific esterases) are group of hydrolytic enzymes important in the metabolism and subsequent detoxification of many xenobiotic and endogenous compounds, including Ops [16, 17]. They can hydrolyze ester-containing compounds to the corresponding alcohol and acid [18]. Due to increased affinity with OPs, carboxylesterases protect the organism against OP toxicity [19]. The effect produced by a single pesticide can be predicted fairly well based on our understanding of the mechanism of toxic action but effect of multiple exposure of pesticides are more difficult to predict and understand.

The dopaminergic neuronal cell population has been hypothesized to be vulnerable to oxidative stress because of the auto-oxidation of dopamine (DA) itself *i.e.,* DA is metabolized to 3,4-dihydroxyphenyl acetic acid *via* monoamine oxidase [20, 21]. Mesencephalic GABAergic neurons are not at risk of such intrinsic oxidative stress, but are equally as vulnerable as DA. Intracellular oxidases, including xanthine oxidase, monoamine oxidase, and cyclooxygenase-2, are available to transfer electrons to exogenous ligands resulting in reactive oxygen species (ROS) generation. In addition, the oxidation of dopamine produces dopamine quinones, reactive species that can also cause damage to lipids, proteins, and DNA [21]. Manganese ethylene-bis-dithiocarbamate (Mn-EBDC), like mancozeb (MZ) and maneb (MB), can catalyze the oxidation of catechols [22]. If DA becomes available to MZ or MB in the cytosol or extracellularly, the EBDC-catalyzed oxidation of catecholamines denotes another potential source of highly reactive free radicals and ROS [23]. These studies provide strong evidence that administration of OP induces rapid and pronounced AChE activity in the striatum, hippocampus and brain stem [23, 24]. A general hypothesis for mechanism of neuro-chemical changes is the blockade of AChE leading to an early increase of ACh concentration which initiates seizures. These seizures lead to secondary changes in release and turnover of monoamines, in particular dopamine. This causes changes in levels of excitatory amino acid which leads to neuropathology and lethality and in inhibitory neurotransmitters which may reflect compensatory mechanisms [24].

The present study was aimed to evaluate the combined toxic effects of a commercial preparation of a pesticide Nuvan, which contains 72% dichlorvos (DDVP) and Kadett containing 36% monocrotophos (MCP). A comparison was done between the effects produced individually and in combination of DDVP and MCP. Cholinesterase (ChE) activity, monoamine oxidase (MAO), nor-epinephrine (NE), DA in male Albino rats was determined after 15 and 30 days of treatment. The study pointed out to the importance of performing biochemical tests on the combination of insecticides.

MATERIALS AND METHODS:

Chemicals and Reagents: The two organo-phosphorous compounds, dichlorvos (Nuvan) (76%) and monocrotophos (Kadett-36) were obtained from Syngenta Chemicals and P.I. Industries Limited respectively. All other chemicals and reagent used were of analytical grade. Ultra pure water prepared using a Millipore apparatus

(Millipore Company, New Delhi, India) was used throughout the experiment to avoid any contamination and for the preparation of reagents/ buffers used for various biochemical assays in our study.

Experimental Protocol:

Animals and Treatment: All experiments were performed on adult male albino rats weighing 190 ± 10 g. Animals were obtained from animal house facility of Defence Research and Development Establishment (DRDE), Gwalior. All animals received humane care in compliance with the guidelines of the "Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)". Animal ethical committee of DRDE, Gwalior, India also approved the protocols for the experiments. The Institutional Animal Ethical Committee (IAEC) registration number was 37/GO/Rbi/S/99-CPCSEA. Prior to dosing, they were acclimatized for 7 days to light from 600 to 1800 h alternating with 12 h darkness. The animals were housed in stainless steel cages in an air-conditioned room with temperature maintained at 25 ± 2 °C. Rats were allowed standard rat chow diet throughout the experiment. Twenty four animals were divided into four groups and were housed three per cage and treated for one month **Table 1**.

TABLE 1: DOSING OF THE GROUPS OF ANIMALS

Group	Number of animals	Drug, dose and route
Group 1 (control)	Six	Drinking water (Blank)
Group 2	Six	Dichlorvos (DDVP); 2.0 mg/kg, orally
Group 3	Six	Monocrotophos (MCP); 2.0 mg/kg, orally
Group 4	Six	DDVP; 1 mg/kg, orally + MCP; 1 mg/kg, orally

Animals in group I treated as control with distilled water. The animals were dosed once daily through the oral (MCP) and oral (DDVP) route. Dosing was carried out at the same time between 1100 h to 1200 h. The two doses were selected based on earlier published studies [25, 26]. Both the selected doses were $1/10^{th}$ of their reported LD_{50}. After four weeks, blood was collected in heparinized tubes and for serum in non-heparinized tubes. Animals were then euthanized by decapitation. Brain was removed, rinsed in cold saline, blotted, weighed, and used for various biochemical variables.

Biochemical Assays: White blood cells (WBC), red blood cells (RBC), haematocrit (HCT), haemoglobin (Hb), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and platelets were measured on Sysmex Hematology Analyzer (model K 4500). Blood glucose, urea, creatinine and albumin were performed using Merck kits. Total tissue protein was measured by the method of Lowry *et al*., [27] Acetylcholinesterase activity was determined in serum and brain by the method of Ellman *et al*., [28] Dopamine (DA), norepinephrine (NE) and 5-hydroxytryptamine (5-HT) were estimated according to the procedure of Jacobwitz and Richardson [29]. Monoamine oxidase activity was studied in brain mitochondrial fraction following the method of Wurtman and Axelrod [30]. The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was assayed according to the method of Reitman and Frankel [31].

Statistical Analysis: The results are expressed as the mean ± SEM of number of observations. Comparisons of means were carried out using one way Anova followed by student 't' test to compare means between the different treatment groups. Differences were considered significant at $P<0.05$ unless otherwise stated in the text.

RESULTS:

AChE Levels in Serum and Brain after 15 Days: Effects of individual and combined exposure of DDVP and MCP on serum and brain AChE activity in rats is shown in **Fig. 1**. A significant inhibition in AChE activity was recorded in DDVP and MCP exposed group. Interestingly, MCP alone was found to have more pronounced inhibition as compared to DDVP which was almost similar to DDVP+MCP combination group.

Neurotransmitters Levels in Serum and Brain after 15 Days: Effects of dichlorvos and monocrotophos on whole brain biogenic amines like NE, 5-HT, DA either individually or in combination is shown in **Fig. 2**. Levels of NE, DA and 5-HT increased significantly in DDVP and MCP exposed animals however, the increase was more pronounced in MCP alone exposed animals. Combined exposure to these toxicants did not produce more pronounced toxicity as compared to their individual exposure except in case of nor-epinephrine.

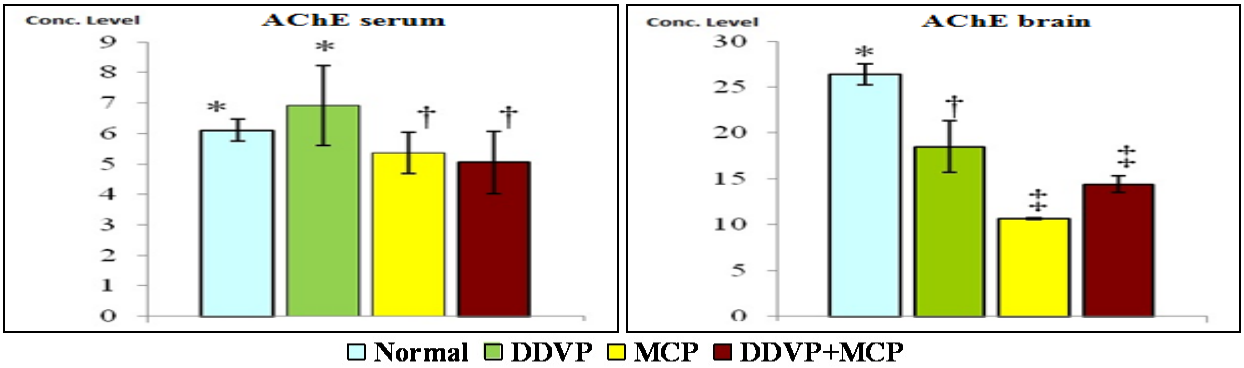

FIG. 1: EFFECT OF INDIVIDUAL AND COMBINED EXPOSURE TO DDVP AND MCP ON ACHE ACTIVITY IN RAT SERUM AND BRAIN AFTER 15 DAYS EXPOSURE. (AChE, acetyl cholinesterase as nmol/min/mg protein. values are mean ± SE; n = 5. *, †, ‡. Differences between values with matching symbol notations within each column are not statistically significant at 5% level of probability).

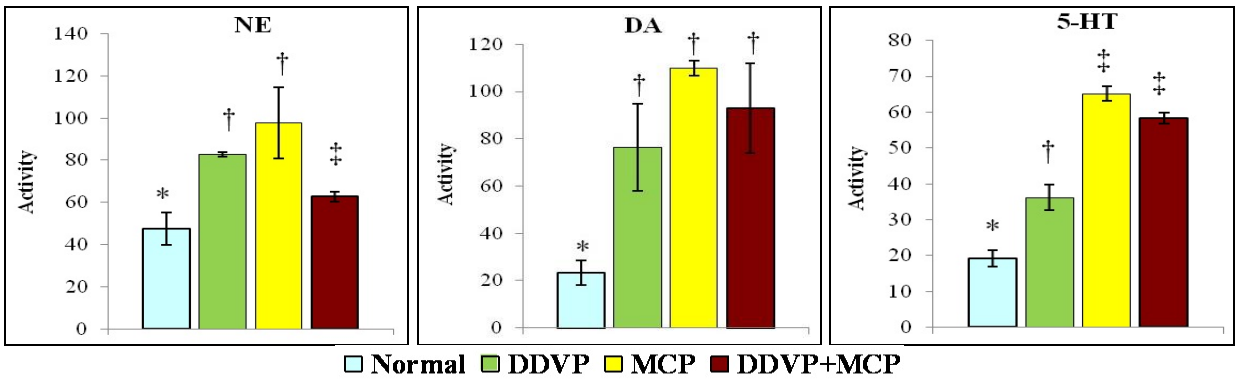

FIG. 2: EFFECT OF DDVP AND MCP EITHER ALONE OR IN COMBINATION ON ALTERED NEUROTRANSMITTERS LEVELS IN RAT BRAIN AFTER 15 DAYS EXPOSURE (NE, Norepinephrine as µg/g tissue; 5-HT, 5-hydroxytryptamine as µg/g tissue and DA, Dopamine as µg/g tissue. SE; n=6 values are mean ± *. †. ‡. Differences between values with matching symbol notations within each column are not statistically significant at 5% level of probability).

AChE Levels in Serum and Brain after 30 days: Effects of individual and combined exposure of DDVP and MCP on serum and brain AChE activity in rats is shown in **Fig. 3**. A significant inhibition in AChE activity in brain was recorded in DDVP and MCP exposed group. The serum AChE activity was however unaltered on individual exposure to DDVP. As observed in 15 days, MCP alone was found to have more pronounced inhibition as compared to DDVP alone. We also observed a marked difference in AChE serum activity on exposure to DDVP+MCP combination group as compared to the individual groups.

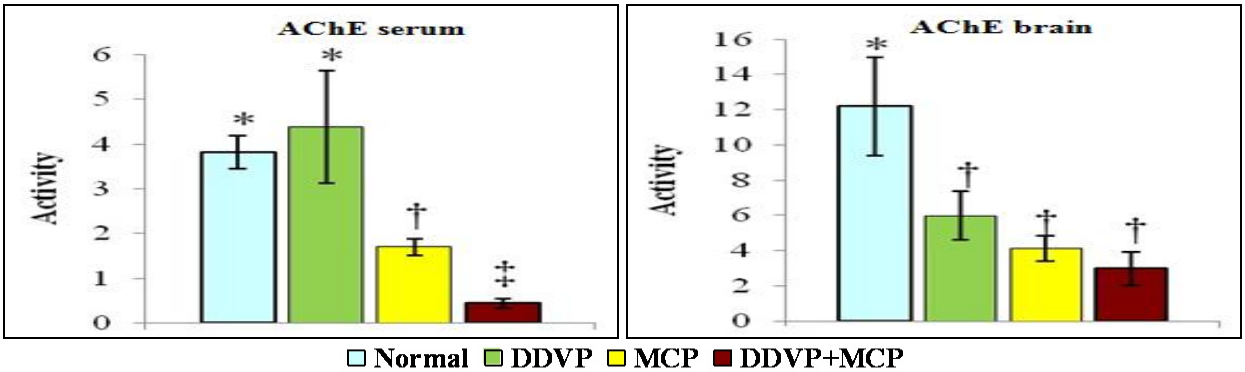

FIG. 3: EFFECT OF INDIVIDUAL AND COMBINED EXPOSURE TO DDVP AND MCP ON ACHE ACTIVITY IN RAT SERUM AND BRAIN AFTER 30 DAYS EXPOSURE. (AChE, acetyl cholinesterase as nmol/min/mg protein. values are mean ± SE; n = 5. *. †. ‡. Differences between values with matching symbol notations within each column are not statistically significant at 5% level of probability).

Neurotransmitters Levels in Brain after 30 Days: Effects of dichlorvos and monocrotophos on whole brain biogenic amines like NE, 5-HT, DA either individually or in combination is shown in **Fig. 4**. Levels of NE, and DA did not show any alteration on DDVP exposure, although a significant decrease in 5-HT levels were observed. MCP was found to have more pronounced effect than DDVP on brain neurotransmitters as observed by a decrease in NE and DA levels. The combination of DDVP and MCP was also found to alter the brain NE and DA levels which was almost similar to MCP alone. 5-HT levels showed no change in activity on co-exposure.

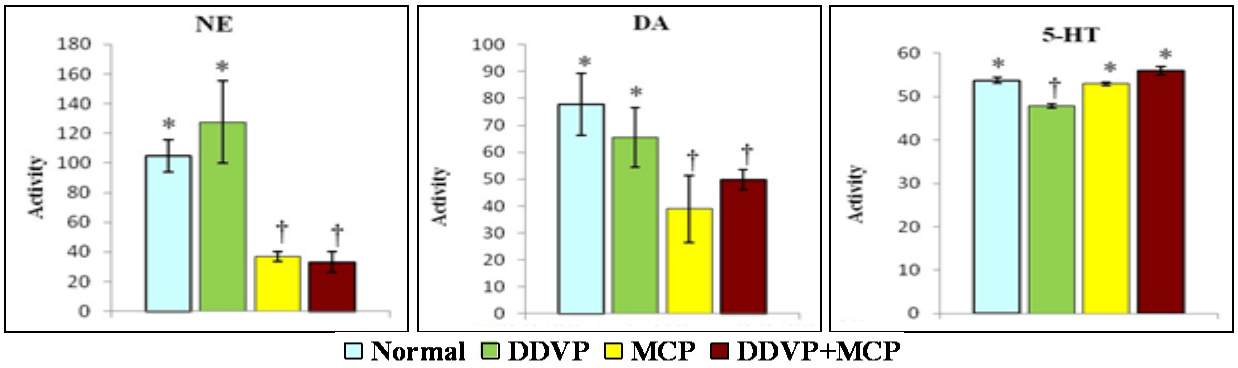

FIG. 4: EFFECT OF DDVP AND MCP EITHER ALONE OR IN COMBINATION ON ALTERED NEUROTRANSMITTERS LEVELS IN RAT BRAIN AFTER 30 DAYS EXPOSURE. (NE, Norepinephrine as µg/g tissue; 5-HT, 5-hydroxytryptamine as µg/g tissue and DA, Dopamine as µg/g tissue. SE; n=6; values are mean ± *. †. ‡. Differences between values with matching symbol notations within each column are not statistically significant at 5% level of probability).

DISCUSSION: Exposure to organophosphate pesticides has become one of the major causes of death due to poisoning [32]. DDVP and Mono-crotophos are two OPs insecticides that are increasingly being used in agriculture and easy availability as well access has lead to misuse in homicidal and suicidal poisoning cases. Both DDVP and MCP produce typical signs and symptoms of organophosphate poisoning soon after the exposure [33]. The present study provides data for changes in Acetyl cholinesterase (AChE) activity, NE, DA in male albino rats and the parameters were determined after 15 and 30 days of exposure to DDVP and MCP alone and in combination. Thus toxicity of these OPs was studied to assess their effects on brain neurotransmitters and to find out possible interaction between these two widely used organophosphates.

The study pointed out to the importance of time dependent toxicity of these pesticides. Significant changes in brain neurotransmitter levels on exposure to MCP and the DDVP+MCP combination was seen. In particular, the changes were observed in brain dopamine levels which hint towards the involvement of dopaminergic neurons in pesticide induced neurotoxicity. Our present study underlines the importance of alterations in the dopamine system as a possible causative mechanism behind the behavioral and functional changes associated with delayed neurotoxicity. The effects of OPs on AChE results in the accumulation of acetylcholine and this ultimately produces cholinergic actions. Both brain and blood AChE activity have been used to monitor the effect of OPs. As in human beings, brain AChE activity cannot be measured, red blood cell AChE is used as a surrogate for brain AChE to assess human risk exposure to MCP and DDVP [34]. OPs block the hydrolysis of bound acetylcholine and results in accumulation of ACh in nerve synapses. This causes the post synaptic cell to remain excited which in turn induces excitability, tremors, paralysis and finally death [35].

Both DDVP and MCP are AChE inhibitors can cause convulsions, status epilepticus, convulsions, muscle fasciculations and injury to neurons and muscles. Excitotoxicity arises due to excessive activation of cholinergic and glutamatergic receptors and as per recent findings suggest oxidative stress is one of the possible cause of excitotoxic injury. Dopamine is a neuro-transmitter naturally produced in the body and plays major roles in regulation of movement, emotion, motivation, feeling of pleasure, stabilization of the brain activity, addiction, flow of information to other parts of the brain *etc*. [36, 37, 38]

Since dopamine is a chemical messenger, it activates the dopaminergic receptors. This occurs in the frontal lobes and disorders in this part of the brain can cause a decline in memory, problem solving and attention and downfall in neuro-cognitive functions. Dopamine is connected with the pleasure system of the brain which leads to feelings of enjoyment and reinforcement and motivates us to do certain activities.

Many evidences of parkinson`s disease can be seen in recent years in the rural areas where the pesticides are being used heavily and mitochondrial dysfunction, oxidative stress and proteasomal dysfunction are proposed as contributing factors for development of this disease.

In the present investigation we hypothesize the possible involvement of the dopaminergic neurotransmitter system in the development of OP induced delayed neurotoxicity in the rat. The altered changes in the neurotransmitters are consistent with the past reported literature in which there was possible involvement of dopaminergic neurotransmitter system in dichlorvos induced delayed neurotoxicity was seen [39].

In correlation with the results obtained in earlier studies, oxidative stress cannot be ruled out as a major factor underlying the toxicity of both the toxicants. Previous reports have illustrated that chronic dichlorvos exposure lead to nigrostriatal dopaminergic degeneration along with loss of 60-80% of the nigral dopamine neurons and reduction in striatal dopamine and tyrosine hydroxylase levels. Swollen, dystrophic neuritis, dystrophic neuritis and mitochondrial abnormalities were observed in dichlorvos exposed animals. The changes observed confirm the evidence of oxidative stress, including increased mitochondrial ROS levels and increased lipid peroxidation [40].

CONCLUSION: Concerns regarding the impact of human health on exposure to OPs either occupationally or environmentally have long been raised. These were prompted specially by the fact that OPs do indeed affect the peripheral and central nervous system because of inhibition of AChE. Therefore studies have been conducted to ascertain whether besides the acute cholinergic syndrome OPs might cause other neurological effects either as a consequence of such inhibition or as a consequence of other mechanisms of toxicity. The present study led us to conclude that additive or synergistic effects have to be considered while dealing with the toxicity of pesticides, especially when same or different classes of pesticides like an OP (organophosphate) and a CM (carbamate) are present. This may be of prime importance in infants and children where developmental stages of toxicity may influence behavioral aspect during further stages of life.

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CONFLICT OF INTEREST: There is no conflict of interest.

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