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Combinational Toxic Effect of Insecticide and Fungicide on Hematological Indicators in Mice

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Abstract

Exposure to two or more compounds in a living system may result in unexpected outcomes. The present study was planned to investigate the effect of two pesticides, Imidacloprid (IMI) and Carbendazim (CBZ), when exposed alone and in combination, on hematological indicators. All mice were equally divided into six different groups consisting of normal control, IMI-L & IMI-H (45.5 & 90.0 mg/kg feed, respectively), CBZ-L & CBZ-H (1.4 and 2.8 g/kg feed, respectively) alone, and in combination (IMI-L+CBZ-L). Blood was collected, and hematology was performed upon completion of the 28-day feeding period. Results revealed that exposure to IMI and CBZ did not show apparent signs and symptoms of toxicity except for the IMI high dose alone. The net average feed and water consumption per mouse were comparatively less in the IMI-H and CBZ-H groups compared to the control group. A dose-dependent significant change ($p<0.05$) in TLC, TEC, PCV, and Hb values was noticed in treatment groups. Non-significant changes ($p>0.05$) in the percent lymphocyte, eosinophil, and monocyte were noticed between all the treatment groups. No significant change ($p>0.05$) in the values of Hb, PCV, MCV, and MCH of the IMI-L+CBZ+L treated group was noticed when compared with the respective IM-L and CBZ-L groups. A non-significant change ($p>0.05$) in studied parameters was noticed when compared between IMI-L+CBZ-L treated groups with corresponding CBZ-L and IMI-L alone treatment groups. Based on the interactive index, the study concluded that a combination of IMI and CBZ exposure via the dietary medium has no definitive additive or synergistic toxic effect on hematological indicators.

Keywords: Carbendazim, Hematology, Imidacloprid, Interactive Index

1. Introduction

A pesticide is a chemical agent intended to prevent, kill, repel, or reduce pests such as insects, bacteria, fungi, nematodes, plants, rodents, and more[1]. In the 1950s, the use of pesticides in agriculture was deemed advantageous, with little concern for the potential risks these chemicals posed to the environment and human health[2]. However, in recent decades, the demand for and utilization of synthetic pesticides has escalated due to intensified agricultural practices aimed at meeting market demands for human and animal consumption in India. Total pesticide consumption was highest in

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Maharashtra, followed by Uttar Pradesh, Punjab, and Haryana. Moreover, per-hectare pesticide consumption was found to be highest in Punjab (0.74 kg), followed by Haryana (0.62 kg) and Maharashtra (0.57 kg) during the year 2016-17. Insecticides, fungicides, and herbicides are extensively utilized in India, with insecticides comprising the largest share. India currently holds the title of the largest pesticide producer in Asia and ranks twelfth globally for the suspension, manufacture, and application of these chemicals[3]. Moreover, in the fiscal year 2018-19, pesticide production in India reached 217 thousand metric tons[4,5].

Carbendazim (CBZ; methyl-2-benzimidazole carbamate) is a systemic fungicide belonging to the benzimidazole class of pesticides[6]. It is one of the most commonly used fungicides in the agricultural sector, with its main metabolite being benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate). CBZ is utilized in horticulture, agriculture, and as a fruit preservative to safeguard crops against decay caused by various fungal infestations[7]. CBZ has been associated with deleterious effects on testicular function, sexual maturity, fetal development, and hormonal regulation by directly affecting the hypothalamic, pituitary, thyroid, and other endocrine glands[6,8,9]. Its presence induces oxidative stress and upregulates gene expression associated with apoptosis pathways in various organ systems[10,11].

Neonicotinoids represent the most important and fastest-growing class of synthetic neuroactive insecticides, widely employed for controlling sucking insects on plants and animals. Members of this pesticide class include imidacloprid (the principal compound), acetamiprid, thiacloprid, nitenpyram, thiamethoxam, and others[12]. Imidacloprid (IMI), the pioneering member of the neonicotinoids group, was invented in 1985 by Dr. Shinzo Kagabu[13]. Since then, its usage has surged, and it is now among the most extensively utilized insecticides worldwide, registered for use on hundreds of crops in over 100 countries[14]. Now, countless uses of IMI responsible for inducing oxidative stress and lipid peroxidation in mammals[11,15,16]. IMI exposure is also linked to organ damage in the heart, liver, kidneys, testes, and other organs. IMI is responsible for teratogenicity, mutagenicity, neurotoxicity, reproductive issues, and immunotoxic effects in various animal species[17,18].

The application of multiple pesticides during single crop cultivation is a common practice aimed at protecting crops from pest infestation. In reality, humans or animals are often exposed simultaneously or sequentially to numerous chemicals through various exposure routes. Many of these chemicals exist as mixtures, and their toxicity is primarily attributed to interactions among them, leading to unpredictable toxic effects[19]. However, assessing the potential health hazards of chemical mixtures is a very difficult and challenging task, and it remains a significant concern for both the scientific and regulatory communities[20]. Numerous researchers have emphasized the importance of studying interactions of chemical mixtures, focusing on oxidative stress, enzymatic effects, antioxidant status, gene expression, inflammatory markers, and neurobehavioral changes[6,16,21,22]. Mansour and Refaie introduced the concept that the joint action of chemical mixtures could be qualitatively and quantitatively estimated based on experimental data obtained from animal exposure to pesticide mixture and/or individual components[23].

Considering the reality of exposure to multiple chemicals, the present study focused on evaluating the toxic effects of CBZ and IMI, both alone and in combination, on simple hematological parameters. The selection of two different categories of chemicals aimed to assess potential drug interactions, with the expectation that they may influence each other's effects within the body.

2. Materials and Method

2.1 Pesticides and Chemicals

Technical grade carbendazim (97%) was procured from Sigma Aldrich, USA. Imidacloprid (94 %) technical grade was a kind of gift received from M/s Gharda Chemicals, Pvt. Ltd. Mumbai. All the chemicals of analytical grade used in the present study were obtained from established firms.

2.2 Feed formulation and Feeding

Selected dosages of test compounds (mg/kg body weight) were converted into dose required through feed (mg/kg feed) by standard formula[24]. Standard animal feed was ground to a fine powder. The technical grade test compounds were dissolved in the required quantity of acetone with different concentrations (Table 1) and sprinkled on powder form of feed. Finally, pellets were prepared, dried, and stored in air tied container until used. The control group feed was also formulated similar

fashion, only the vehicle was added. Finally, prepared pelleted feed was fed to the animals from different groups for a period of 28 days.

2.3 Animals

A total of 65 male Swiss Albino mice weighing about 25-30 gm were procured from the National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab. Mice were housed in the Small Laboratory Experimental House of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, Ludhiana. Mice were maintained in polyacrylic cages throughout the experiment. During the experiment animals were maintained under standard managemental practices (Pelleted feed; Ashirwad Pvt Ltd, Mohali Punjab and water *ad-libitum* with 12h light and 12h dark cycle and room temperature 22±3°C).

2.4 Experimental Design

The study was performed with partial help of sub-acute oral toxicity study guideline (OECD-TG 424). Mice were assigned to the different test groups based on a random sort program so that the mean body weight of each group would be comparable. Sixty male mice were randomly divided into six groups of 10 animals each. Group I served as control. Group II and, III were treated with low and high dose of Imidacloprid (IMI), respectively. Group IV and V was treated with high and low dose of Carbendazim (CBZ), respectively. Group VI was treated with a combination of low-dose IMI and low-dose CBZ. All animals received normal and test compounds for the period of 28 days through feed (Table 1).

2.5 Sign and Symptoms of Toxicity

All animals were observed two times in a day throughout the experiment to see any signs and symptoms of toxicity, if any, such as a change in gross observed behavior, motor activity, hyperactivity, tremor, convulsions, diarrhea, dyspnoea and death.

2.6 Effect on Haematological Parameters

Blood samples were collected at the end of day 28 in tubes containing heparin from all the mice, directly from the heart. Hematological parameters were performed manually following standard laboratory protocols[25,26], which include Hemoglobin (Hb), Packed Cell Volume (PCV), Total Erythrocyte Counting (TEC), Total Leucocyte Counting (TEC) and differential leucocyte count.

2.7 Calculation of Interactive Index

The Mansour's formula[23] was used to identify the type of interaction between pair of toxicants in terms of Interaction Index (I.I.):

I.I.= M+C/A1+A2

Whereas: M, C, A1 & A2 represent the mean values obtained from the biochemical estimation of a studied parameter.

M is the value of the mixture or combination-treated group; A_1 & A_2 for the values of the individual or alone compounds treated group; and C for the value of the control group.

Based on the I.I. formula, the ratings of the interaction indices are interpreted as below:

i. In case of positive effect (i.e. increase of the concerned biochemical parameters above the control values due to effect of the individual compounds);

Where:

I.I.>1 means potentiation; I.I.=1 means additive; I.I.<1 means antagonism

Table 1. Experimental designs consist of grouping and dose conversion

Groups	Treatments	Standard Dose (mg/kg b.wt., PO)	Dose conversion for feed (ppm; mg/kg feed)
I	Control	–	Normal feed
II	IMI-L	6.5 mg/kg	45.5 mg/kg feed
III	IMI-H	13 mg/kg	91.0 mg/kg feed
IV	CBZ-L	200 mg/kg	1.4g/kg feed
V	CBZ-H	400 mg/kg	2.8 g/kg feed
VI	CBZ-L+IMI-L	6.5mg/kg+200mg/kg	45mg/kg+1.4g/kg

ii. In case of negative effect (i.e., decrease of the concerned parameters below the control values due to the effect of the individual compounds);

Where:

I.I.>1 means antagonism; I.I.=1 means additive; I.I.<1 mean potentiation

Finally, I.I. of all parameters were summarized in tabulated form and used to draw the conclusion of the type of interaction between compounds when they are present together in the body of living beings.

2.8 Statistical Analysis

Finally, all data was compiled and comparisons among different groups were done using statistical design. Data was analysed using IBM SPSS Statistics, Version 2.0 statistical software and Microsoft Excel. All the data were expressed as Mean ± SEM. All the data were compared by employing one-way Analysis of Variance (ANOVA) with Tukey's multiple comparisons as post hoc test. A p-value of <0.05 was considered as statistically significant.

3. Results

3.1 Effect on In-Life Parameters

Administration of CBZ, IMI and their combinations during the study period one mortality in IMI-H treated group was noticed and otherwise, no any-other signs of toxicity in mice from other groups were noticed. A rough body coat in CBZ-treated mice, and slight hair falls on the snout in the high-dose IMI-treated group was observed. These changes were quite prominent from mid to end of the experiment. Control, low-dose CBZ, IMI and their combination groups did not exhibit any signs of toxicity and all animals appeared normal during the study period.

3.2 Effect on Hematology

Effect on hematological parameters was evaluated at the end of the last dose 28th day of post-dietary exposure to CBZ and IMI alone and in combination. IMI-L+CBZ-L treatment shows a significant decrease ($p \leq 0.05$) in the level TLC as compared to the control group but significantly higher ($p \leq 0.05$) than the IMI-L and CBZ-L alone treated groups. A dose-dependent significant change ($p \leq 0.05$) in TLC was noticed in both the alone-treated groups (Table 2 and 3).

A significantly lower ($p \leq 0.05$) value of TEC was noticed in the IMI-H-treated group as compared to all other groups. The combination group did not show a significant ($p > 0.05$) change in TEC value. A percent neutrophil count was significantly ($p \leq 0.05$) higher noticed in IMI-L+CBZ-L treated group as compared to the CBZ-L treated group. Non-significant changes ($p > 0.05$) in percent lymphocyte, eosinophil and monocyte were noticed between all the treatment groups (Table 2 and 3).

A significant ($p \leq 0.05$) change in PCV, HB values was noticed with exposure to CBZ and IMI exposure as compared to the control group. Significantly ($p \leq 0.05$) less HB and PCV value was noticed in IMI-H treated group as compared to all other groups (Table 2 and 3).

A dose-dependent significant increase ($p \leq 0.05$) in the level of MCH value was calculated in CBZ and IMI

Table 2. Effect on haematological parameters in mice exposed to carbendazim, imidacloprid alone and their combination for 28 days.

Group	Treatment	TLC*	TEC*	Differential leukocyte count (%)			
				Lymphocyte	Neuttrophil	Eosinophil	Monocyte
I	Control	6.71±0.06^{d}	26.53±0.93^{d}	73.16±0.87^{a}	19.00±0.81^{ab}	3.00±0.58^{a}	4.50±0.42^{a}
II	IMI-L	2.41±0.04^{b}	22.70±0.98^{bc}	72.33±0.42^{a}	17.50±0.88^{a}	3.00±0.36^{a}	4.50±0.61^{a}
III	IMI-H	1.90±0.03^{a}	19.20±0.84^{a}	73.00±0.73^{a}	18.83±0.79^{ab}	2.50±0.22^{a}	4.66±0.66^{a}
IV	CBZ-L	2.53±0.09^{b}	25.65±0.48^{cd}	72.85±0.46^{a}	20.50±0.42^{ab}	3.00±0.31^{a}	3.66±0.49^{a}
V	CBZ-H	1.68±0.07^{a}	20.99±1.02^{ab}	71.66±0.42^{a}	20.14±0.79^{ab}	2.16±0.40^{a}	4.71±0.42^{a}
VI	IMI-L+CBZ-L	3.35±0.05^{c}	24.61±0.67^{cd}	70.80±0.37^{a}	21.20±0.73^{b}	2.60±0.24^{a}	4.00±0.31^{a}

Values (Mean ± SEM, n=10) bearing different superscripts in the same column differ significantly (p<0.05) in Tukey's multiple comparison post hoc test.

CBZ: carbendazim, IMI: imidacloprid

Total erythrocyte count (TEC) - *10^{6} cells/µL; Total leucocyte count (TLC) - *10^{3} cells/µL

Table 3. Effect on haematological parameters in mice exposed to CBZ, IMI alone and their combination for 28 days.

Group	Treatment	PCV	HB	MCHC	MCH	MCV
I	Control	56.67±0.159^{d}	18.08±0.248^{d}	31.91±0.493^{a}	6.86±0.29^{a}	21.50±0.79^{a}
II	IMI-L	51.60±0.373^{b}	17.28±0.119^{b}	33.49±0.361^{b}	9.08±0.36^{b}	27.12±1.17^{b}
III	IMI-H	41.11±0.264^{a}	13.75±0.091^{a}	33.46±0.042^{b}	6.19±0.25^{a}	18.26±0.72^{a}
IV	CBZ-L	53.00±0.223^{c}	17.62±0.110^{bc}	33.24±0.223^{b}	8.53±0.49^{b}	25.64±1.38^{b}
V	CBZ-H	52.49±0.315^{bc}	17.51±0.123^{b}	33.35±0.244^{b}	6.84±0.16^{a}	20.51±0.48^{a}
VI	IMI-L+CBZ-L	52.61±0.406^{bc}	17.17±0.080^{b}	32.64±0.314^{ab}	6.99±0.18^{a}	21.43±0.54^{a}

Values (Mean ± SEM, n=10) bearing different superscripts in the same column differ significantly ($p<0.05$) in Tukey's multiple comparison post hoc test.

CBZ: carbendazim, IMI: imidacloprid, Haemoglobin (Hb): g/dl; Packed cell volume (PCV): %; Mean Corpuscular Volume (MCV): fL; Mean Corpuscular Haemoglobin (MCH): pg; Mean Corpuscular Haemoglobin Concentration (MCHC): g/dl.

Table 4. Net combinational effect (Interactive Index) on haematological parameters in mice exposed to CBZ and IMI for 28 days.

L	N	E	M	TLC	TEC	PCV	HB	MCHC	MCH	MCV
(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(+ve)	(+ve)	(-ve)
0.99	**1.06**	**0.93**	**1.04**	**2.04**	**1.06**	**1.04**	**1.01**	**0.97**	**0.79**	**0.81**
PO	**AN**	**PO**	**AN**	**AN**	**AD**	**AD**	**AD**	**AN**	**AN**	**PO**

L: lymphocyte; N: neutrophil; E: Eosinophil; M: Monocyte. Hb: Haemoglobin; PCV: Packed cell volume; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin; MCHC: Mean Corpuscular Haemoglobin Concentration.

Numerical value indicate type of interaction and interactive index. AN: antagonism; AD: additive; PO: potentiation. Positive (+) and negative (-) signs are indicative of the effect drugs on particular parameters with respect to control.

alone treated animals as compared to control animals but non-significant as compared to IMI-L+CBZ-L treated animals (Table 2 and 3). Moreover, significantly higher ($p \leq 0.05$) values of MCV and MCH were noticed in CBZ-L and IMI-L treated groups as compared to all other groups. No significant ($p > 0.05$) change in the values of Hb, PCV, MCV and MCH of IMI-L+CBZ+L treated group was noticed when compared with respective IM-L and CBZ-L groups.

3.3 Effect on Interactive Index

An interactive index was calculated for the hematological parameters. The majority of the parameters showed the antagonistic effect (5 parameters) followed by potentiation (3 parameters) and additive (3 parameters). Overall interactive index indicates that interaction between the CBZ and IMI is antagonistic in nature (Table 4).

4. Discussion

Typically, hematological parameters are analyzed to assess the functional status and internal environment of animals[27,28]. In the present study, treatment with CBZ and IMI alone induced dose-dependent reductions in PCV, HB, TLC, and TEC, indicating that these chemicals are capable of inducing toxic effects. The decline in red blood cells suggests that CBZ disrupts erythropoiesis or enhances red blood cell death[29]. Similarly, a substantial decrease in the mean values of RBCs, Hb, PCV, MCHC, and elevated neutrophils in MCV, WBC level, lymphocytes, and eosinophils, responsible for macrocytic hypochromic anemia in animals exposed to CBZ and mancozeb alone or in combination reported[30].

The decrease in lymphocyte counts in CBZ, IMI, and IMI-L+CBZ-L treated groups indicates an immunosuppressive effect on the mammalian system and blood-forming organs. The WBC count is a reliable and well-standardized biomarker of systemic inflammation, as several species include the cells in controlling immunological activity[31]. Neutrophils are usually detected at the onset of illnesses or sensitivity to environmental toxins in the bloodstream[32]. The increased count of neutrophils in treated animal groups may be partially a compensatory reaction to the functional deficiency

caused by CBZ and IMI treatments at different doses. Further, oral administration of IMI caused a reduction in TEC, Hb, PCV, MCV, MCH, and MCHC values. The DLC revealed a significant increase in neutrophil count and a significant decrease in lymphocyte count in treated animals[33].

The results of the present research are consistent with previous reports, which have shown that CBZ treatment induces a substantial reduction in RBC levels, Hb, PCV, and TEC, and an increase in MCV, MCH, and platelet count in laboratory animals[9,34]. CBZ exposure in male rats resulted in reductions in RBC levels, Hb concentration, and hematocrit values[35]. Reductions in RBC levels or Hb in the blood can indicate impaired Hb biosynthesis or impaired erythrocyte development[36]. The decrease in RBC level, Hb concentration, and PCV probably indicates erythrocyte hemolysis and an increase in the pace at which Hb concentration may be reduced or a decrease in Hb synthesis[37]. The decrease in PCV is due to a decrease in RBC count induced by either degradation or a decrease in size. Anemia in such species can be attributed to enzyme inhibition by CBZ, thereby interfering with RBC precursors and hemoglobin biosynthesis, among other markers[38]. In the present case, a change in cell size could be attributed to the swelling of red blood cells, as indicated by the rise in Mean Corpuscular Volume (MCV) due to an increase in size[39]. While there are many causes associated with an increase in erythrocyte size (MCV), it is usually regarded as a reaction to stress[40]. Further, CBZ has been shown to have an impact on hematological parameters in the African giant rat, particularly by increasing packed cell volume, hemoglobin concentration, and the number of red blood cells[41].

Pesticide exposure, whether singular or multiple, is an ongoing process. When two or more pesticides from different groups, each with distinct mechanisms of action, coexist, they may produce unexpected outcomes. Direct prediction of their impact is challenging[42,43]. Interactions between these agents can occur during both the toxicokinetic and toxicodynamic phases within organisms, and they may manifest varying degrees of effect, ranging from antagonism to potentiation. The data from this study, encompassing generalized and hematological observations, indicate that a mixture consisting of CBZ and IMI influences activity. CBZ and IMI individually produced dose-dependent toxic effects on hematological parameters. However, when they coexist in the body, they did not reveal any significant effects when compared with their respective control groups. Combinations predominantly demonstrated antagonistic effects, followed by potentiation and additivity. Interestingly, the combined treatment of IMI and CBZ exhibited a less pronounced impact on the studied parameters compared to the sum of their individual effects, suggesting a prevalent antagonistic effect similar to prior pesticide studies[44,45].

Our earlier laboratory findings also clearly suggested that the co-existence of CBZ and IMI at high doses had a potentiating effect, while low-dose combinations of CBZ and IMI had antagonistic effects or weak interactions on bone marrow-derived mesenchymal stem cells[11,46]. Similarly, when mixture toxicity for ecotoxicological endpoints was evaluated, approximately 70% of the aquatic toxicity studies showed effects within 10-15 % of the results exhibiting more effect and 10-15 % showing less toxicity, with 5% of the studies showing effects larger than a factor of 2.5 and in 1% more than a factor of 5[47]. When combinations of pesticides were tested on duckweed, the combination of acifluorfen with mesotrione showed large antagonistic effects[48]. However, when herbicides and organophosphorous compounds were tested on duckweed and algae, mixtures of bentazone with either malathion, endosulfan, or chlorpyrifos were found to be non-toxic (antagonistic) when compared to concentration addition and independent action[49]. Predicting toxicity in mixtures is complex due to factors such as dose, dose ratio, common cellular targets, and metabolic pathways that influence interactions[50]. Interactions are more complex than simple similar actions and independent joint actions, which are usually expected[47].

5. Conclusion

Continuous exposure to different categories of pesticides in animals or humans may or may not produce effects equivalent to the sum of their individual effects. The resultant effect may even be less or more than the sum of their individual toxic effects. CBZ and IMI were administered via the natural route (fed through feed), instead of a fixed dose via oral gavage. The findings suggested that the co-existence of CBZ and IMI in the animal body does not lead to additivity or potentiation as expected, while individual drugs produced dose-dependent toxic effects on hematological parameters. The net effect was lesser than the sum of their individual

effect on most of the parameters, indicating antagonism between IMI and CBZ within the body of an animal.

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