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TERATOGENICITY TESTING OF CHLORPYRIFOS AND TEBUCONAZOLE IN CHICKEN EMBRYOS AFTER SIMULTANEOUS ADMINISTRATION

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ABSTRACT

The objective of this study was to determine the single and simultaneous toxic effects of chlorpyrifos containing insecticide formulation (Pyrinex 48 EC) and tebuconazole containing fungicide formulation (Mystic 250 EW) on the development of chicken embryos. Amount of 0.1 ml of 1% Pyrinex 48 EC and of 0.4% Mystic 250 EW was alone and concomitantly injected into the air chamber of eggs on the first day prior to incubation. The chicken embryos were examined on day 19 of incubation for the followings: number of embryonic deaths, body, liver and heart weight of the embryos, and type of developmental anomalies. The body, liver and heart weight data were evaluated statistically by One-Way ANOVA, Tukey and Dunnett tests, the embryonic mortality and the developmental abnormalities were analysed by Fisher's exact test. The combined administration of Pyrinex 48 EC and Mystic 250 EW pesticides on the chick embryo had shown to be embryotoxic to the chick. The rate of mortality and the incidence of developmental anomalies were increased due to the simultaneous application of them. The body and liver weight were significantly reduced. Our teratogenicity study revealed that the combined administration of both chlorpyrifos (Pyrinex 48 EC) and tebuconazole (Mystic 250 EW) pesticides on the chick embryo had shown to be embryotoxic to the chick. They had a slight addition effect on the rate of embryonic mortalities, however, the toxic interaction of both pesticides on developmental anomalies, liver and body weight was not proven.

Keywords: *chlorpyrifos, tebuconazole, teratogenicity, chicken embryo, developmental anomalies.*

INTRODUCTION

Pesticide has received great popularity in agricultural sector in enhancing the production by its purpose of eliminating and preventing pest infestation that may

cause great losses to the yield. Principally, pesticides are allowed to be registered if after demonstration, it will not remain or persist in the environment exceeding their period of its intended use. Generally, pesticides residue can be found abundantly in the natural environment. The degradation of these pesticides through microbial transformation such as oxidation and hydrolysis had caused contamination mainly in the water source and reservoir (Fenner *et al*., 2013).

Majority of pesticide show high degree of toxicity due to their key function to eliminate certain organism consequently creating some risk of harm. Humans may accidentally expose to pesticides by consuming contaminated foods and drinks or substantial exposure around the housholden vironment. Pesticides caused adverse effects to the environment (water, soil, and air) from leaching, runoff and spray drift while also caused detrimental effects on wildlife, fish, plants, and other non-target organisms. Thus, the main concern is not only on human health, but also negative impacts on wildlife such as avian species as one of the non-target organisms encompassing the sensitive ecosystems (Damalas and Eleftherohorinos, 2011). Organophosphate (OP) and carbamate (CB) are anti-cholinesterase (anti-ChE) insecticides that is generally more toxic to avian than to mammals. Narrowing down to animal or wildlife exposure, many research has shown that bird as one of the bio-monitoring organism for chemical toxicity in the environment, has been detected dangerous levels of heavy metals, pesticides and chemicals sourcing from water bodies and food sources for both humans and animals (Lightfoot and Yeager, 2008). There are vast records of accidental poisoning and mortality of non-target avian species caused by OP (Bartkowiak and Wilson, 1995). Many of the frequently used azole fungicides might cause endocrine disruption *in vivo*, although the profile of action *in vivo* varies (Taxvig *et al*., 2007). The residue of tebuconazole along with other plant protection products are also detected in grey partridge eggs, a gamebird in French cereal ecosystem (Bro *et al*., 2016). Eggs exposed to pesticide in laboratory setting is determined as a relevant exposure scenario in risk assessment procedure because of the potential to affect the reproductive success of wild bird in natural ecosystems (Ortiz-Santaliestra *et al*., 2020).

The aim of this study was to reveal the individual and possible common embryotoxic effects of organophosphate insecticide (Pyrinex 48 EC) and tebuconazole fungicide (Mystic 250 EW) evidently found in the environment which may jeopardize the ecosystems and living organisms.

MATERIALS AND METHODS

Test Materials

- Control: Distilled water
- 1% emulsion of Pyrinex 48 EC in distilled water, based on plant protection practice in the field. This pesticide is an organophosphate insecticide containing 44.4 ± 2m/m% chlorpyrifos as an active ingredient and assigned to marketing category I. It is used for the control of a wide range of insect pests on arable land as well as in orchards.

- 0.4% emulsion of Mystic 250 EW in distilled water, based on plant protection practice in the field. This pesticide is a triazole fungicide containing 26.0 ± 1.6 m/m% tebuconazole as an active ingredient and assigned to marketing category I. It is used to combat fungal diseases on grapes, cherries, almonds, cereals, and rapeseed or canola.

Experimental animals

The experiment was conducted on the eggs of a mixed-use hen breed called Farm (Gallus gallus f. domesticus) obtained from incubation plant of a local poultry farm, Goldavis Ltd. (Sármellék, Hungary). A total of 160 fertile hen eggs were used in the experiment.

Treatments

Prior to the treatments, the eggs were randomly divided into four different groups which were made as homologous as possible with respect to egg size and weight. Forty eggs were used in each group for each treatment. In the individual treatments, emulsions made from test chemicals in 0.1–0.1 ml end-volume were used while in the joint treatments, 0.2 ml of the chemical agents were injected into the air chambers of eggs in each combination.

Methods

On the first day prior to incubation, the blunt end of the eggs was disinfected, a hole was drilled in the calcic eggshell to inject test material into the air chamber using micro-pipette (Fejes et al., 2002; Budai et al., 2003; Szabó et al., 2019). After injection, the hole was sealed with paraffin. The same manner was applied for control group but the test material was replaced with distilled water (Várnagy et al., 2003).

All eggs were placed into the incubator (Ragus type table incubator Vienna, Austria). They were incubated for 19 days with the incubation temperature of 37-38°C, relative humidity 65-75% and the eggs were turned three times a day until the day of final processing.

Processing

The assessment was made on day 19th of incubation (two days before hatching). The eggs were open and examined based on the following criteria (parameters):

- the body, liver, and heart weight of the embryo
- number and type of development anomalies, if any, and
- number and day of embryonic death

The identification or estimation day of embryonic death were determine based on detailed description on chick embryo development stages by Hamburger and Hamilton (Darnell and Schoenwolf, 2000).

Statistical evaluation

The body, liver, and heart weight data were evaluated statistically by the One-Way ANOVA after controlling of their distribution using Comparison-Quantile Plot. Comparative evaluation of the results of the different groups was carried out by Tukey and Dunnett tests. The statistical analysis of the results of embryo-mortality and development abnormalities were performed by Fisher's exact test.

RESULTS AND DISCUSSION

Embryonic mortalities

Lowest number of embryonic mortality (4) was observed in the control group after 19 days of incubation (Table 1). Significant number of embryonic death was recorded for embryos with single and combined administration of Pyrinex 48 EC and Mystic 250 EW compared to the control group ($p<0.001$). Embryo treated with 0.4% of tebuconazole fungicide (Mystic 250 EW) recorded the lowest number of dead embryos (21) among the groups of embryo treated with the pesticides (Table 1).

Embryos in the group with single administration of chlorpyrifos insecticide (Pyrinex 48 EC) at concentration of 1% shown second highest rate of embryonic death (25) (Table 1). Combined administration of 1% chlorpyrifos containing insecticide (Pyrinex 48 EC) and 0.4% tebuconazole pesticide (Mystic 250 EW) gave the highest rate of embryonic mortalities (31). The increase of embryonic mortality rate after combined administration was also significant ($p<0.05$) compared to the embryo treated with Mystic 250 EW (Table 1).

Developmental anomalies

There was no malformation in the control group (Table 1). Out of 15 alive embryos from the group treated with 1% chlorpyrifos (Pyrinex 48 EC), three embryos (20%) were observed with developmental anomalies. This rate was significant ($p<0.05$) compared to control group (Table 1). The embryos were shown with malformations on the legs and beak from the evaluation.

Leg deformation was observed on embryo (5.26%) in the group with single treatment of 0.4% tebuconazole (Mystic 250 EW). The rate was, however, not significant ($p<0.05$) compared to control group (Table 1).

The group with combined administration of 1% chlorpyrifos (Pyrinex 48 EC) and 0.4% tebuconazole (Mystic 250 EW) were observed to have developmental anomalies (open abdomen and beak malformation) on two embryos (22.22%), which was also the highest number of embryos to be malformed per number of alive embryo and had significant rate of developmental anomalies ($p<0.05$) compared to normal embryo in control group (Table 1).

Pathological processing

Body Weight

All groups with pesticides treatments gave significant reduction in body weight compared to control group. The body weight of embryo from single administration of 1% chlorpyrifos (Pyrinex 45 EC) was significantly different ($p<0.01$) from the control (Table 2). Embryo treated with tebuconazole fungicide (Mystic 250 EW) at concentration of 0.4% weighed significantly less compared to embryo in control group ($p<0.05$) (Table 2).

Combined administration of 1% chlorpyrifos (Pyrinex 45 EC) and 0.4% tebuconazole fungicide (Mystic 250 EW) resulted with the lowest body weight and was significantly differ ($p<0.01$) compared to embryo in the control group (Table 2). The mean weight was also lowest compared to embryo with single administration of chlorpyrifos or tebuconazole.

Liver Weight

Embryos from the control group had the highest liver weight (Table 2). Embryos that were administered with 1% chlorpyrifos (Pyrinex 45 EC), 0.4% tebuconazole (Mystic 250 EW) and the combination of both on the first day prior to incubation had caused significant reduced in liver weight.

Single administration of chlorpyrifos containing insecticide (Pyrinex 45 EC) at concentration of 1% resulted in significant reduced of mean liver weight compared to the control ($p<0.01$) (Table 2).

Embryos in the group that had been administered with tebuconazole fungicide (Mystic 250 EW) at concentration of 0.4% shown the lowest liver weight among all groups with significant decrease compared to control group ($p<0.01$) (Table 2).

Combined administration of both pesticides Pyrinex 45 EC and Mystic 250 EW at concentration of 1% chlorpyrifos and 0.4% tebuconazole respectively on the first day prior to incubation resulted in significant decrease of liver weight compared to control group ($p<0.01$) (Table 2).

Heart Weight

The heart weight was rather sporadic among all groups. Lowest mean heart weight was recorded for the embryos from the group that had been administered with tebuconazole fungicide (Mystic 250 EW) at a concentration of 0.4% but, it was not significant compared to control group (Table 2). Combined administration of chlorpyrifos containing insecticide (Pyrinex 40 EC) at concentration of 1% and tebuconazole fungicide (Mystic 250 EW) at concentration of 0.4% resulted in insignificant higher heart weight compared to control group (Table 2).

Results obtained from this teratogenicity study shown that single administration of chlorpyrifos containing insecticide (Pyrinex 48 EC) at concentration of 1% had induced the toxicity to the embryo resulting in developmental deformities and increase the mortality. It also caused significant reduction in body and liver weight compared to the embryo in control group.

Findings from this study on the toxic effect of Pyrinex 48 EC can further support the findings from the experiment conducted by using the same chlorpyrifos insecticide on chick embryo which had caused significant reduction in body weight and increase rate of embryonic mortality (Budai *et al*., 2015; Lehel *et al*., 2014; Szabó *et al*., 2016).

In another experiment, the combination of insecticide (chlorpyrifos and cypermethrin) induced explicit alterations in the embryonic growth and development and resulted in malformations particularly to the axial and appendicular skeletal structures of chick embryos after it was administered as a single dose (0.005, 0.001, 0.01, 0.05, 0.1, or 0.5 µg) on day '0' of incubation (Uggini *et al*., 2012). The metabolites of chlorpyrifos is proved to be embryotoxic after it was evaluated using the chick embryo and were administered to 3-day embryos by the air cell method (Muscarella *et al*., 1984).

Fungicide (Mystic 250 EW) containing active ingredient tebuconazole was applied at 0.4% concentration as single administration on the chick embryo was proved to be embryotoxic. It increased the rate of mortality compared to normal embryo and

had cause embryo with developmental anomalies. The fungicide also caused significant reduction of body weight and liver weight of the embryo.

Similar experiment by using immersion method of the eggs treated with 0.1% tebuconazole pesticide (Mystic 250 EW) together with single and combined administration of 0.01% cadmium sulphate and 0.01% lead acetate shown significant reduction in body weight. Embryonic mortalities were higher in all treated groups except for the group treated with Mystic 250 EW with sporadic rate of developmental anomalies (Szemerédy *et al*., 2018).

An experiment was also conducted to observe the effect of tebuconazole on birds. Captive partridges were fed with 0%, 20% or 100% of tebuconazole application rate during 25 days in late winter. Birds fed with high dose of tebuconazole has reduction of hatching rate by 23% and the brood size is 1.5 times smaller compared with controls (Lopez-Antia *et al*., 2021).

Table 1. Mortality and developmental anomalies of embryos on day 19 of incubation

Treatment	No of embryos showing abnormality/No of live embryos	Death No/Total eggs
Control	0/36	4/40
Pyrinex 48 EC	3/15[c]	25/40[a]
Mystic 250 EW	1/19	21/40[a]
Pyrinex 48 EC + Mystic 250 EW	2/9[c]	31/40[a; b]

a=significant decrease compared to the control group ($p<0.001$)

b=significant decrease compared to the group treated with Mystic 250 EW alone ($p<0.05$)

c=significant decrease compared to the control group ($p<0.05$)

Table 2. Embryonic body, liver and heart weights (g) on day 19 of incubation

	Control	Pyrinex 48 EC	Mystic 250 EW	Pyrinex 48 EC + Mystic 250 EW
Number of embryos (n)	36	15	19	9
Average body weight (g)	25.42	22.24[b]	24.54[a]	20.97[b]
Average liver weight (g)	0.451	0.390[b]	0.373[b]	0.400[a]
Average heart weight (g)	0.141	0.144	0.126	0.157

a=significant decrease compared to the control group ($p<0.05$)

b=significant decrease compared to the control group ($p<0.01$)

CONCLUSION

Our teratogenicity study revealed that the combined administration of both chlorpyrifos (Pyrinex 48 EC) and tebuconazole (Mystic 250 EW) pesticides on the chick embryo had shown to be embryotoxic to the chick. It increased the rate of

embryonic mortality and the incidence of embryonic developmental anomalies. The body and liver weight were also significantly reduced. Pyrinex 48 EC and Mystic 250 EW had a slight addition effect on the rate of embryonic mortalities, however, the toxic interaction of both pesticides on developmental anomalies, liver and body weight was unjustified. Further investigation is recommended to study the toxic effects on the developing embryo and the hatching rate. The chick may allow to mature in order to evaluate its functional normality after the treatments during the embryo stage. Beside the injection treatment method applied during the studies it would be advisable to perform complete examinations with immersion treatment that can represent better the exposure realized in the environment and compare the results achieved from different treatment methods.

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