Model Study to Investigate the Toxic Interaction between Spirotetramat and Myclobutanil on Pheasant Embryos in the Early Phase of Development

A spirotetramat és a miklobutanil közötti toxikus kölcsönhatás vizsgálata fácánembriókon, a fejlődés korai szakaszában

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Abstract: The toxic effects of the Movento insecticide (100 g/l (9.29 m/m%) spirotetramat) applied alone or in combination with Systhane 20 EW fungicide (200 g/l myclobutanil (19.4 m/m%) were studied on pheasant embryos, in the early phase of embryonic development. The test materials were injected in 0.1 ml volume into the eggs' air chamber on the first incubation day. Subsequently, on the third day of incubation, permanent preparations were made using the embryos to study the early developmental stage. Embryos fixed on microscope slides and stained with osmium tetroxide solution were studied by light microscopy. The embryonic mortality and the occurrence of developmental anomalies were analysed statistically by the Fisher test. Based on the experiment's results, the embryonic mortality of pheasant embryos increased in every treated groups (individual or combined administration of the chemicals) compared to the control. The differences in the individually treated groups could not be statistically proven. The simultaneous application of Movento and Systhane 20 EW significantly increased the mortality of pheasant embryos compared to the control. Both test substances were embryotoxic in pheasants, and an additive toxic interaction was revealed between Movento and Systhane 20 EW.

Keywords: *spirotetramat; myclobutanil; interaction, embryonic mortality, pheasant embryo*

Összefoglalás: Munkánk során a Movento inszekticid (100 g/l (9,29 m/m%) spirotetramat) és a Systhane 20 EW gombaölő szer (200 g/l miklobutanil (19,4 m/m%) toxikus hatásait vizsgáltuk, a növényvédő szereket önmagukban és kombinációban alkalmazva fácánembriókon, az embrionális fejlődés korai szakaszában. A vizsgálati anyagokat 0,1 ml térfogatban a tojások légkamrájába fecskendeztük az inkubáció első napján. Ezt követően az inkubáció harmadik napján az embriókból tartós preparátumokat készítettünk a korai fejlődési szakasz tanulmányozására. A tárgylemezeken rögzített és ozmium-tetroxid oldattal megfestett embriókat fénymikroszkóp alatt vizsgáltuk. Az embriómortalitás és a fejlődési rendellenességek statisztikailag Fisher-féle egzakt teszttel elemeztük. A kísérlet eredményei alapján a fácánembriók embriómortalitása minden kezelt csoportban (az egyedileg és az

anyagok kombinációjával kezelve) nőtt a kontrollhoz képest, a különbségek az egyedileg kezelt csoportokban statisztikailag nem igazolhatók. A Movento és Systhane 20 EW egyidejű alkalmazása szignifikánsan növelte a fácánembriók mortalitását a kontrollhoz képest. Mindkét vizsgálati anyag embriótoxikus volt fácánban, közöttük additív toxikus kölcsönhatás igazolódott.

***Kulcsszavak**: spirotetramat; miklobutanil; interakció, embriómortalitás; fácánembrió*

1. Introduction

The increase in the Earth's population places an ever-greater task on agriculture, as the food needs of the rapidly increasing population must be produced in an ever-smaller agricultural area, and the highest possible amount of crops in proper quality must be harvested from the available areas.

Integrated plant protection, which includes mechanical and chemical agrotechnical solutions, dramatically contributes to production safety. The use of pesticides is essential in the control of harmful organisms present today, and for the time being, no alternative in the future could replace chemical control to a greater extent than at present.

The solution for harmonising plant protection product use and the interests of environmental protection and safe food production is the reasonable, specific use of pesticides with the lowest environmental impact.

Pesticides and various chemical substances released into the environment pose a danger to plants, animals, and humans through the food chain. Sprayed agents can have a negative effect not only on adult wild birds, causing direct (immediate death) or indirect (reproductive biology) issues, but also on the embryo already developing in the egg (Nagy, 1984; Faragó, 1997; Szabó et al., 2003). Cunningham and Woodworth-Saigo (1995) investigated the problem of the decrease in shell thickness of bird eggs, which they attributed to calcium metabolism disturbances caused by chemicals. Wild bird species are exposed to the effects of agricultural chemicals, especially during reproduction and rearing. Since their reproduction period coincides with the time of pesticide spraying, extra attention should be paid to the ecotoxicological examination of the harmful effects of pesticides on living organisms. The possibility of direct exposure to wild bird eggs is increased by, among others, the non-prescribed application of the preparations, the inappropriate spraying technique, and the dangers arising from drifting (Keserű et al., 2004; Várnagy et al., 2003; Szabó et al., 2020).

2. Materials and Methods

Our experiment was carried out in 2021. The 0.75% water suspension of Movento (Bayer Hungária Ltd., Hungary) insecticide with 100 g/l (9.29 m/m%) spirotetramat active ingredient and the 0.225% fungicide Systhane 20 EW (Dow AgroSciences Hungary Ltd., Hungary) with 200 g/l myclobutanil (19.4 m/m%) active ingredient of oil emulsion was used, which corresponded to practical spray concentration. The embryotoxic effect of their single and combined administration was investigated in the early embryonic stages of the pheasant as a test organism.

Forty fertile pheasant eggs derived from the main colony of the Hubertus Hunting Association (Abádszalók, Hungary) were used in the experiment. The eggs were incubated after transportation and rested for 24 hours. The eggs spent 23–24 days within a Ragus-type incubator (Vienna, Austria). During the incubation, the appropriate temperature (37.5–37.8°C), air humidity (48–65%) and the daily rotation of eggs were provided (Bogenfürst, 2004).

The treatment of eggs (n=10/group) was performed on the first day of incubation. In the individual treatments, suspensions and emulsions made from test chemicals in 0.1–0.1 ml end volume were used, while in the case of combined treatments, 0.2 ml of the test materials were injected into the air chambers of eggs (Clegg, 1964; Lutz, 1974).

A hole was punched in the calcic eggshell, and the shell membrane above the air space, and then the proper quantity of the suspensions and emulsion of the test materials was injected into the air chamber (Clegg, 1964; Várnagy et al., 1996). After the injection, the hole was sealed with paraffin, and the eggs were returned to the incubator. In the control group, avian physiological saline solution (0.75 w/v%) was injected into the air space of the eggs in the manner described above. The incubation was started immediately after the treatments.

To study the early development phase, permanent preparations were made from 10 embryos per group on the third day of incubation. Above the air chamber, the calcic eggshell and the shell membrane were removed. The germinal disk was cut around and, with a filter paper placed on it, was put into avian physiological saline (0.75 w/v %) at 38ºC temperature. After blotting up the saline solution, the embryo placed on a slide was stained with 0.1% osmium tetroxide solution and fixated, then mounted with DPX histological adhesive and covered with a coverslip. The permanent preparations were examined by light microscopy (Sinkovitsné and Benkő, 1993).

The number of embryonic deaths and developmental abnormalities of embryos were recorded.

In the case of the biometric processing of embryonic mortality and malformations, an exact test, according to Fisher, was used.

3. Results

Three days after treatments, no dead embryo was found in the control group.

As a result of the treatment with Movento, the rate of embryonic mortality was 10.0%, but the difference was insignificant compared to the control group.

The single administration of Systhane 20 EW increased the embryonic mortality up to 30.0%. This change was also not significant as compared to the control group.

The combined administration of the insecticide and the fungicide resulted in a 40.0% embryonic mortality. According to the statistical evaluation, the change was statistically significant as compared to the control group ($p<0.05$) (Table 1).

Table 1. Embryonic mortality from teratogenicity test of Movento insecticide and Systhane 20 EW fungicide in pheasant embryos after single and combined administration

Treatment	Death No. / No. of fertile eggs	Rate of embryonic mortality (%)
Control	0/10	0.00
Movento	1/10	10.00
Systhane 20 EW	3/10	30.00
Movento + Systhane 20 EW	4/10[a]	40.00

[a]*Significant difference as compared to the control group ([a]$p<0.05$)*

During the light-microscopic evaluation of permanent preparations, only a single developmental anomaly (10.0%) was found in the control group (Table 2-3).

Two embryos treated with Movento insecticide showed developmental anomaly (22.22%). This rate was not significantly different from the control group's (Table 2). The developmental

anomaly was diagnosed as a retarded development of the embryo and its vascular system (Table 3).

Two embryos (28.57%) showed abnormal development as a result of the treatment with Systhane 20 EW fungicide alone. This change was insignificant compared to the control group (Table 2). The developmental anomaly was identified as a retarded development of the embryo and its vascular system (Table 3).

Due to the combined treatment, the rate of developmental anomalies was 16.67%. The change was insignificant compared to the control group and the groups treated with either insecticide or fungicide alone (Table 2). The type of developmental anomaly was also a retarded development of the embryo and its vascular system (Table 3).

Table 2. Developmental anomalies from teratogenicity test of Movento insecticide and Systhane 20 EW fungicide in pheasant embryos after single and combined administration

Treatment	No. of embryos showing developmental anomalies / No. of alive embryos	Rate of developmental anomalies (%)
Control	1/10	10.00
Movento	2/9	22.22
Systhane 20 EW	2/7	28.57
Movento + Systhane 20 EW	1/6	16.67

Table 3. Types of developmental anomalies diagnosed in the teratogenicity test of Movento insecticide and Systhane 20 EW fungicide in pheasant embryos after single and combined administration

Treatment	Types of developmental anomalies (incidences of developmental anomalies)
Control	Less developed body (1)
Movento	Poorly developed vasculature and body (2)
Systhane 20 EW	Poorly developed vasculature and body (2)
Movento + Systhane 20 EW	Poorly developed vasculature and body, undeveloped embryo (1)

4. Discussion

Based on the experiment's results, it can be established that the embryonic mortality found in the groups treated with the insecticide or the fungicide alone was not significantly different from that seen in the control group.

At the same time, it can be stated that combined treatment with the pesticides enhanced the embryotoxicity since the rate of embryonic mortality found in the group receiving the combined treatment was significantly higher than that obtained in the control group.

As a result of the treatments, developmental delay appeared in the form of the retarded development of the vascular system and the body. Movento and Systhane 20 EW applied together proved to be embryotoxic for the pheasant embryo in the initial stage of its development. Based on the incidence of malformations, a teratogenic effect cannot be confirmed because the detected developmental disorders can later be compensated (Juhász et al., 2005).

These results are in harmony with the results of previous studies in which eggs were treated with various pesticides at different periods of incubation, and signs of embryotoxicity were detected at necropsy, but teratogenicity was not proven (Budai et al., 2002; Varga et al., 1999).

The interaction avian teratology test results also confirmed the pheasant embryo's increased sensitivity to the toxic effects of pesticides applied together, which may exceed the consequences of individual exposure. Furthermore, in parallel with the opinion of other authors, it can be stated that the interaction avian teratology studies indicate with appropriate sensitivity the unique toxic effects that are modified as a result of the joint exposure of different xenobiotics (Varga et al., 1999; Juhász et al., 2006).

There are differences in sensitivity between different species of wild birds to various chemicals (Kertész, 2001). The larger pore volume and specific surface area of some bird eggs can increase the exposure. Compared to the species of the *Phasianidae* family, including the pheasant, the species belonging to the *Anatidae* family (e.g. mallard duck) are more sensitive to spirotetramat (Maus, 2008).

The scientific and literary sources are convincing that the bird embryo can be used well and efficiently in first-line embryotoxicity, as it reacts with great sensitivity to the damaging effects of various physical and chemical agents affecting the body. The morphological and functional changes in the embryogenesis of birds show similarities with the embryonic development of mammals from many points of view, providing an opportunity for extrapolations (Pan and Fouts, 1978; Hill and Hoffman, 1984; Major et al., 2022).

5. Conclusions

Based on our experimental data, an additive effect was observed at the embryonic mortality due to the simultaneous injection treatment of Movento and Systhane 20 EW.

Besides the injection treatment method, it would be advisable to perform complete examinations with immersing treatments to model expositional circumstances during the plant protection practice.

In addition, the experiment could be supplemented with pathological processing, during which it is also possible to evaluate and examine histological samples from the blood and different organs (e.g., the liver). Moreover, the examination could also be supplemented with skeletal staining, revealing possible developmental abnormalities in the skeletal system. Also, using groups with a more significant number of elements would be recommended during the test replication.

The experiments that model the practical use more efficiently can provide a more accurate picture of the effects of chemical stresses on the environment. All information obtained can help to protect nature and wildlife in the future.

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