Dose-Dependent Embryotoxicity and Teratogenicity Study of Prochloraz on Chicken Embryos

A prokloráz hatóanyagú Faxer fungicid dózisfüggő embriótoxicitási és teratogenitási vizsgálata házityúk-embriókon

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Abstract: The aim of the present study was to determine the dose-dependent embryotoxic and possible teratogenic effects of Faxer fungicide (450 g/l prochloraz) on the development of chicken embryos. The pesticide was applied at a practical spray concentration (3.33 µl/ml), and at twofold (6.66 µl/ml) and fivefold (16.65 µl/ml) doses. Emulsions of the test material were injected in 0.1 ml volume into the air chamber of the chicken eggs before starting the incubation. The chicken embryos were examined on day 19 by the followings: rate of embryo mortality, body weight, prevalence and type of developmental anomalies by macroscopic examination. The body weight of the live embryos was evaluated statistically by one-way ANOVA, the embryo mortality and the developmental anomalies were analysed by Fisher's exact test. Based on the results, we found that the embryotoxic effect increased with higher concentrations. In the fivefold dose (16.65 µl/ml), the average body weight of the chicken embryos was significantly lower ($p<0.05$) than that of the control group. The highest concentration of prochloraz-containing fungicide significantly increased ($p<0.001$) the mortality of chicken embryos compared to the control. Treatment induced growth retardation and embryonic minor structural anomalies were sporadic. No teratogenic effect was confirmed even in the fivefold dose.

Keywords*: prochloraz; chicken embryo; dose-dependent embryotoxicity; teratogenicity; injection*

Összefoglalás: Vizsgálatunkban a Faxer (450 g/l prokloráz) gombaölő permetezőszer házityúk-embrió tesztszervezetre gyakorolt embriókárosító hatását tanulmányoztuk. A növényvédő szer engedélyokiratában rögzített legmagasabb koncentráció (3,33 µl/ml) mellett, a túldozírozás által kiváltott méreghatás megítélése céljából a gyakorlati permetlé-töménység kétszeres (6,66 µl/ml) és ötszörös (16,65 µl/ml) dózisa is beállításra került. A keltetés megkezdése előtt a fungicid három koncentrációjából készült emulziókat 0,1 ml végtérfogatban a tyúktojások légkamrájába injektáltuk. Az inkubáció 19. napján a tojások feltárását követően rögzítettük az embriómortalitás alakulását, valamint a fejlődési rendellenességek előfordulási gyakoriságát és típusát, illetve lemértük és feljegyeztük a testtömegüket. Az embriómortalitási adatok és a morfológiai elváltozások biometriai értékelését Fisher-féle egzakt teszttel, míg a testtömeg

adatok statisztikai vizsgálatát egytényezős varianciaanalízissel hajtottuk végre. A prokloráz hatóanyagú gombaölő szer gyakorlati permetlé-töménységben (3,33 µl/ml), továbbá annak kétszeres dózisában (6,66 µl/ml) fokozta ugyan az embrióelhalást és a malformációk előfordulási gyakoriságát, valamint csökkentek az élő házityúk-embriók testtömegei a kontroll csoporthoz képest, de statisztikailag igazolható eltérés a vizsgált paraméterekben nem volt megállapítható. Az eredmények alapján feltételezhető a dózisfüggő embriótoxikus hatás, mivel az ötszörös dózisban (16,65 µl/ml) a fungicid szignifikáns mértékű embriómortalitás-emelkedést ($p<0,001$) és embrionális testtömegcsökkenést ($p<0,05$) indukált a kontrollhoz viszonyítva. Teratogén hatás nem volt igazolható, mert fejlődési rendellenesség - a legmagasabb dózis esetében is - csak sporadikusan jelentkezett, végtagdeformitás, nyitott mellkas és hasüreg formájában.

***Kulcsszavak**: prokloráz; házityúk-embrió; dózisfüggő méreghatás; teratogenitás; injektálás*

1. Introduction

Prochloraz is an imidazole fungicide widely used worldwide in agriculture and horticulture against phytopathogenic microorganisms. Like triazoles, imidazoles are used in veterinary and human medicine to treat fungal infections. Inhibits the enzyme lanosterol 14α-demethylase (CYP51A1), which is involved in the biosynthesis of ergosterol. The end result is damage to the fungal cell membrane and destruction of the fungal cells (Haselman et al., 2018; Heise et al., 2018).

Several toxicological studies have shown that prochloraz is an endocrine disruptor and can damage the reproductive system. Prochloraz has multiple mechanisms of action *in vitro*, including inhibition of the activity of other cytochrome P450 (CYPs) enzymes that catalyse crucial biochemical processes in higher organisms. It may affect aromatase (CYP19A1), a key enzyme in steroidogenesis, and thus interfere with the biosynthesis of estrogens. In addition, as an agonist of the aryl hydrocarbon receptor (AhR), it may also affect the expression of genes encoding cytochrome P450 enzymes, immunity regulation and cell differentiation. Screening tests have shown that prochloraz antagonises the androgen and estrogen receptors, and *in vivo* has been found to be antiandrogen in the Hershberger bioassay (in rats), as evidenced by a reduction in reproductive organs weight, an effect on androgen-regulated gene expression in the prostate and an increase in luteinizing hormone (LH) levels (Vinggaard et al., 2002; Stein et al., 2014).

In developmental biology and reproductive toxicity studies, prochloraz was teratogenic to zebrafish embryos (*Danio rerio*) as well as caused morphological and functional damage to the reproductive organs of adult fish. Furthermore, prochloraz may negatively affect the reproductive capacity of western honey bees (*Apis mellifera*) and thus the probability of population survival (Baumann et al., 2015; Glavinic et al., 2019).

The different agricultural areas offer sources of food, shelter and breeding places to wild birds (common pheasant, for example), therefore the sprayed pesticides can contaminate not only the adults, but the embryos developing in egg, as well. Their toxic effects can result in embryonic lethality and developmental abnormalities (Fejes et al., 2002; Várnagy et al., 2003; Szabó et al., 2020; Juhász et al., 2005). The aim of our study was to examine the dose-dependent embryotoxic and possible teratogenic effects of prochloraz-containing fungicide (Faxer) on the development of chicken embryos (*Gallus gallus domesticus*) after administration by injection technique. The relationship between dose and toxicity of pesticides and other xenobiotics is poorly understood in avian embryo (Szabó et al., 2003; Keserű et al., 2004).

2. Materials and Methods

One hundred and sixty, mixed-use Farm color hen eggs (Goldavis Ltd., Hungary) with good fertility were used in the experiment. They were randomised into four groups (40 eggs/group) based on their size and weight. The eggs were incubated in a Ragus type table incubator (Vienna, Austria), applying adequate temperature (37–38°C), relative humidity (65–70%) and daily rotation of the eggs during the incubation (Szabó et al., 2022). The prochloraz-containing fungicide (Faxer, Belchim Crop Protection Ltd., Hungary) was applied at a practical spray concentration (3.33 µl/ml), and at twofold (6.66 µl/ml) and fivefold (16.65 µl/ml) doses.

The experimental protocol of the study was approved by the local Committee of Animal Welfare at Hungarian University of Agriculture and Life Sciences, Georgikon Campus.

Emulsions of the test material were injected in a volume of 0.1 ml directly into the air chamber of the eggs with a micropipette on day 0 of incubation, and the hole was closed with paraffin after the treatment. Control eggs were treated with avian physiological saline solution (0.75% sodium chloride).

The eggs and the embryos were processed by necropsy on day 19 of incubation, and the following parameters were monitored for evaluation: mortality, body weight, and developmental abnormalities of the embryo (Juhász et al., 2006; Lehel et al., 2021).

Statistical analysis of the body weight of live embryos was performed by one-way analysis of variance (ANOVA). The mortalities and the developmental anomalies of embryos were analysed statistically using Fisher's exact test. Statistical analysis of the data was performed using R statistical software.

3. Results

The mortality and the developmental abnormalities are summarised in Table 1, and the body weight of the live embryos in the groups are shown in Figure 1. The embryonic mortality rate was 2.50%, and no embryos showing developmental anomalies were found in the control group (0.00%). The average body weight of the embryos was 24.30±1.44 g in the control group. Treatment with Faxer fungicide at practical spray concentrations resulted in an increase in embryo mortality rate to 12.50%. Developmental abnormalities were detected in two living embryos (5.71%). The differences were not significant. The treatment did not cause statistically significant change in the body weight (23.93±1.38 g). When prochloraz fungicide was applied at a dose of 6.66 µl/ml, the rate of embryonic mortality was increased to 15.00%, and two embryos shows teratogenic malformation (5.88%). The average body weight of the embryo was 23.86±0.85 g in this group. In the fivefold dose, the average body weight of the chicken embryos was significantly lower (23.51±1.24 g, $p<0.05$) than that of the control group. The highest concentration of prochloraz-containing fungicide significantly increased (30.00%, $p<0.001$) the mortality of the chicken embryos compared to the control, but the incidence of developmental anomalies was sporadic (10.71%). The types of the developmental anomalies were open abdomen and thoracic cavity and leg deformities in all cases.

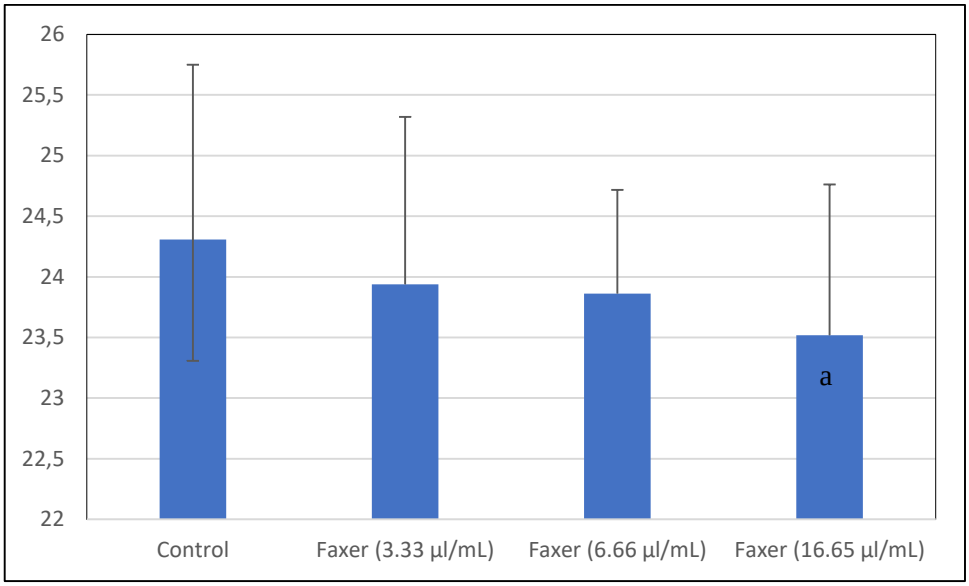

Figure 1. *Body weight of live embryos (average ± SD; g) with increasing concentrations*

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

Table 1. Mortality and developmental anomalies of embryos with increasing concentration.

Treatment	No. of embryos showing abnormality/No. of live embryos	No. of deaths/Total eggs	Rate of developmental anomalies (%)	Mortality (%)
Control	0/39	1/40	0.00	2.50
Faxer (3.33 µl/mL)	2/35	5/40	5.71	12.50
Faxer (6.66 µl/mL)	2/34	6/40	5.88	15.00
Faxer (16.65 µl/mL)	3/28	12/40[a]	10.71	30.00

[a] *Significant decrease as compared to the control data ($p<0.001$)*

4. Discussion and Conclusions

The results of our study showed that the embryotoxic effect of the fungicide Faxer, containing prochloraz as active substance applied with increased concentration, was manifested in significant decrease of embryonic body weight, and that of higher embryonic mortality compared to the control group. The embryotoxicity was dose-dependent. However, no teratogenicity was observed at the concentrations tested (3.33 µl/ml; 6.66 µl/ml; 16.65 µl/ml).

Haselman et al. (2018) investigated the effects of chronic prochloraz exposure on *Xenopus laevis* amphibians over several life stages. Treatments were initiated in embryo at concentrations of 0; 6.7; 20; 60 and 180 µg/l. In the pathogenetic study, the hepatotoxic and nephrotoxic lesions detected by the researchers were positively correlated with increasing dose. Furthermore, the study confirmed the antiandrogenic and endocrine disrupting effects of prochloraz with involvement of the hypothalamic-pituitary-gonadal (HPG), thyroid (HPT) and adrenocortical (HPA) axes.

Domingues et al. (2013) studied differences in sensitivity between zebrafish embryos (*Danio rerio*) and adult zebrafish to prochloraz concentrations between 4.6 and 8.5 mg/l after 96 hours of exposure. They conclude that the early life stage is more sensitive to sublethal dose than the adult stage. In their study, prochloraz was found to be teratogenic at medium concentrations. The sensitivity of zebrafish is therefore highly dependent on the developmental stage.

In our study on chicken embryos (*Gallus gallus domesticus*), the dose-dependent embryotoxicity of a prochloraz-containing fungicide induced fatal, significant embryo damage in the fivefold dose (16.65 µl/ml).

Our teratological research supports our thesis that the avian embryo is well suited for developmental biology studies because it is sensitive enough to indicate the damaging effects of different chemical agents (Várnagy et al., 1996; Varga et al., 1999; Budai et al., 2003).

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