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MODEL STUDY TO INVESTIGATE THE TOXIC INTERACTION BETWEEN GLYPHOSATE AND COPPER SULPHATE ON CHICKEN EMBRYOS

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ABSTRACT

The toxic effects of the Taifun Forte herbicide (360 g/l glyphosate isopropylamine salt) applied alone or in combination with copper sulphate were studied on chicken embryos in the early phase of embryonic development. The test materials were injected in 0.1 ml volume into the air chamber of eggs on the first day of incubation. Subsequently, on the third day of incubation permanent preparations were made from the embryo in order to study the early developmental stage. Embryos fixed on slides and stained with osmium tetroxide solution were studied under light microscope. The embryonic mortality and the developmental anomalies was analysed statistically by Fisher test. According to the result of the statistical evaluation, the embryonic mortality was not influenced by the single treatment of copper sulphate. However, Taifun Forte and its combination with heavy metal significantly increased the early embryonic mortality. Developmental abnormalities were sporadically observed due to the single administration of copper sulphate. The incidence of it was increased due to the treatment with herbicide alone and in combination with copper sulphate. Based on the results, additive toxic interaction may occur between the copper sulphate and glyphosate that can highly reduce the viability of the embryos or can lead to extinction of wild birds in serious cases.

Keywords: *glyphosate, copper sulphate, interaction, embryonic mortality, chicken embryo.*

INTRODUCTION

The chemical plant protection process is one of the most important polluting activities in the agricultural production. Sprayed pesticides and other xenobiotics, e.g. heavy metals, due to the agricultural activities during the plant protecting processes, can contaminate the ecosystem of a given habitat simultaneously. Therefore, the chemical load can occur as a complex problem, so the combined toxic effect, i.e. toxic interaction of at least two substances can be expected and the components can modify the effect of each other.

For several years, our research team has been conducting animal experiments aimed at determining the embryotoxic and teratogenic effects of pesticides and heavy metals by the use of avian embryos (Budai *et al.*, 2001). In the framework of these studies, the embryotoxic effects of the materials tested were monitored primarily in the late phase of embryonic development. Recently, a new processing technique has been introduced, which makes it possible to evaluate also the early phase of embryonic development. By the use of staining with 0.1% osmium tetroxide it is now possible to make permanent preparations of the embryo in the early phase development (between days 1 and 4) to determine any changes in embryo morphology and viability by light microscopy (Várnagy, 2005).

The objective of this study was to determine the individual and combined embryotoxic effects of heavy metal (copper) modelling the heavy metal load of the environment and an optionally selected pesticide widely applied in the practice (Taifun Forte). As the ecotoxicological test methods used in the practice are mainly limited to study the toxic effect of compounds used alone, data on interactions between pesticides can be regarded as gap-filling information, especially in relation to the avian organism (Thompson, 1996). Furthermore, the interaction effects are examined not only in the field of ecotoxicology, but also in all other areas that deal with health care and chemical safety issues (Oskarsson, 1983; Danielsson *et al.*, 1984; Speijers and Speijers, 2004).

MATERIALS AND METHODS

For modelling the environmental copper load, 0.01% copper sulphate solution (Reanal-Ker Ltd., Hungary) was used in individual and combined treatment. At present, copper is used primarily for wire manufacturing, as a chemical catalyst and for the production of alloys. It is applied as a nutrient in plant cultivation and as a bactericidal, fungicidal and algicidal agent in chemical plant protection. In veterinary medicine copper is used as a feed additive, growth promoter and disease-preventing substance (Adriano, 1986).

The herbicide Taifun Forte (360 g/l glyphosate [isopropylamine salt], Adama Hungary Ltd., Budapest, Hungary) was used in individual and combined treatment in typical field application rate (2.5%). It is a phosphorous-containing pesticide containing 360 g/l glyphosate isopropylamine salt as active ingredient and assigned to marketing category III. It is used widely on arable land as well as in horticulture and viticulture, for perennial and seedlings of single and dicotyledonous plants, for drying and total weed control. The product is not toxic to bees and moderately toxic to fish (NFCSO, 2012).

The study was conducted on purebred fertile Farm hen's eggs derived from the stock farm of Goldavis Ltd. (Sármellék, Hungary). The eggs were incubated in a Ragus type hatcher (Vienna, Austria). During the incubation the appropriate temperature (37–38°C), air humidity (65–75%) and the daily rotation of eggs were provided (Bogenfürst, 2004). The treatment of eggs (n=10/group) was performed on the day of initiation of hatching. In case of individual treatment, solution and/or emulsion made from test chemicals in 0.1–0.1 ml end volume were used while 0.2

ml of the chemical agents were injected into the air chambers of eggs in case of combined application (Clegg, 1964; Várnagy *et al.*, 1996; Kertész, 2001; Palkovics, 2003). For the preparation of solution and/or emulsion as well as in the control treatment, distilled water was used. The incubation was started immediately after the treatments.

In order to study the early phase of development, permanent preparations were made from 10 embryos per group on day 3 of incubation. Above the air chamber the calcic eggshell and the shell membrane were removed, then the germinal disk was cut around and stained with 0.1% osmium tetroxide solution. The stained germinal disk was placed into avian physiological saline solution (0.75 w/v%) with 38°C temperature and it was floated on a slide and fixed with DPX histological adhesive. Finally the slide was covered with coverslip. The permanent preparations were then examined by light microscopy (Sinkovitsné and Benkő, 1993; Kertész, 2001). In case of the biometric processing of the embryonic mortality and developmental anomalies, exact test according to Fisher was used.

RESULTS AND DISCUSSION

Embryonic mortality

On day 3 after treatment, only a single dead embryo (10.0%) was found in the control group (Table 1).

As a result of treatment with copper sulphate, the rate of embryonic mortality was 10.0%. The difference was not significant (Table 1).

The single administration of Taifun Forte herbicide increased the embryonic mortality up to 60.0%. This change was significant ($p<0.05$) as compared to the control group (Table 1).

The combined administration of herbicide and copper sulphate resulted in an embryonic mortality rate of 80.0%. According to the statistical evaluation, the change was statistically significant as compared to both the control group ($p<0.01$) and the group treated with copper sulphate alone ($p<0.01$) (Table 1).

Table 1. Embryonic mortality from teratogenicity test of Taifun Forte and copper sulphate in chicken embryos after single and combined administration

Treatment	Death No / No fertile eggs	Rate of embryonic mortality (%)
Control	1/10	10.0
Copper sulphate	1/10	10.0
Taifun Forte	6/10 [a1]	60.0
Taifun Forte + Copper sulphate	8/10 [a2, b]	80.0

[a]Significant difference as compared to the control group ([a1]$p<0.05$; [a2]$p<0.01$)

[b]Significant difference as compared to the group treated with copper sulphate alone ($p<0.01$)

Developmental anomalies

During the light-microscopic evaluation of permanent preparations stained with osmium tetroxide, no embryos showing developmental anomalies were found in the control group (Table 2 and 3).

One of the embryos treated with copper sulphate showed developmental anomaly (11.1%). This rate was not significantly different from that found in the control group (Table 2). The developmental anomaly consisted of retarded development of the embryo and its vascular system (Table 3).

Two embryos (50.0%) showed abnormal development as a result of the treatment with Taifun Forte herbicide alone. This change was not significant as compared to the control group (Table 2). The developmental anomaly consisted of retarded development of the embryo and its vascular system (Table 3).

Due to the combined treatment, the rate of developmental anomalies was increased to 50.0%. The change was not significant as compared to both the control group and the groups treated with either Taifun Forte or copper sulphate alone (Table 2). The type of developmental anomaly was retarded development of the embryo and its vascular system (Table 3).

Table 2. Developmental anomalies from teratogenicity test of Taifun Forte and copper sulphate in chicken embryos after single and combined administration

Treatment	**No of embryos showing developmental anomalies / No of live embryos**	**Rate of developmental anomalies (%)**
Control	0/9	0.0
Copper sulphate	1/9	11.1
Taifun Forte	2/4	50.0
Taifun Forte + Copper sulphate	1/2	50.0

Table 3. Types of developmental anomalies diagnosed in the teratogenicity test of Taifun Forte and copper sulphate in chicken embryos after single and combined administration

Treatment	**Types of developmental anomalies (incidences of developmental anomalies)**
Control	No anomaly
Copper sulphate	Poorly developed vasculature (1) Poorly developed body (1)
Taifun Forte	Poorly developed vasculature (2) Poorly developed body (2)
Taifun Forte + Copper sulphate	Poorly developed vasculature (1) Poorly developed body (1)

The results of study on the toxic effects of copper sulphate and glyphosate containing herbicide used alone or in combination in the early phase of embryonic development allow us to draw the following conclusions.

It can be established that the embryonic mortality found in the group treated with copper sulphate alone was not significantly different from that seen in the control group.

The embryonic mortality was higher in the group treated with Taifun Forte herbicide, than in the control or group treated with copper sulphate alone.

At the same time, it can be stated that the combined treatment with copper sulphate and the herbicide clearly resulted in enhanced embryo toxicity, since the rate of embryonic mortality found in the combination treatment group was significantly higher than that obtained in the control group or in the group treated with copper sulphate alone.

This is in harmony with the results of previous studies in which treatments were performed at different times of the incubation period and the eggs were opened and the results evaluated on day 19 of the incubation period. It was concluded that the combined treatment resulted in increased embryotoxic effect in comparison with the individual embryo damaging effect of the used components (Budai *et al.*, 2002).

The intravenous injection of copper salts into pregnant hamsters on day 8 of gestation caused an increase in embryonic resorptions as well as the appearance of developmental malformations in surviving offspring (Ferm and Hanlon, 1974).

Glyphosate containing RoundUp herbicide was examined by other researchers in Wistar rats. Rat dams were treated orally with 500, 750 and 1000 mg/kg glyphosate via drinking water. Results showed a 50% mortality rate for dams treated with 1000 mg/kg glyphosate. Skeletal alterations were observed in all treated groups. Based on the data the authors concluded that the glyphosate containing RoundUp is toxic to rat dams and induces developmental retardation of the fetal skeleton (Dallegrave *et al.*, 2003).

In view of the increased sensitivity of wild fowl species, the study reported in this paper should be extended to seed-eating birds (pheasants, Japanese quail) and waterfowl (mallards). We also recommend that the interaction studies should be complemented with hatchability studies and investigations performed at the postembryonic stage of development, so that the harmful effects of the chemicals under study can be explored more precisely.

CONCLUSIONS

Based on the results of our avian teratological study performed by applying of Taifun Forte (in typical field rate) and copper sulphate (inducing relatively low environmental copper load which could be less embryo toxic in itself) individually and simultaneously, it was established that joint effects of both chemical agents additively increased the embryonic mortality under the circumstances used in our experiments. According to the published literature, the join toxic effect of many pesticide combinations is at least additive. In some cases, pesticide mixtures, if

they particularly contain insecticide component, have been shown to be synergistic, with reported increase in toxicity up to 100-fold. However, these effects are species, time and dose dependent and are therefore difficult to predict routinely (Thompson, 1996).

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