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Reproductive toxicological changes in avian embryos due to a pesticide and an environmental contaminant

JÓZSEF LEHEL^{1*} , GÉZA SZEMERÉDY², RITA SZABÓ²,
LÁSZLÓ MAJOR², ADRIENN GRÚZ² and PÉTER BUDAI^{2**}

¹ Department of Food Hygiene, University of Veterinary Medicine, István u. 2, H-1078, Budapest, Hungary

² Institute of Plant Protection, Hungarian University of Agriculture and Life Sciences, Georgikon Campus, Deák F. u. 16, H-8360, Keszthely, Hungary

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RESEARCH ARTICLE

ABSTRACT

Single and simultaneous toxic effects of glyphosate (Amega Up, 360 g L⁻¹, 4%) and copper sulphate (0.01%) were studied in avian embryos treated either with injection directly into the air chamber or by immersion application for 30 min on day 0 of incubation. Alterations of the chicken embryos were evaluated during necropsy performed on day 19 of incubation, together with mortality, body weight and the type of developmental abnormalities. Based on the results, the injection application appeared to be more toxic than the immersion method, as it induced increased mortality and reduced the average body weight, and resulted in a higher incidence of congenital anomalies. Supposedly, a toxicodynamic interaction occurs between copper sulphate and glyphosate, which may reduce the vitality of embryos and thus decrease the number of offspring in wild birds.

KEYWORDS

chicken embryo, immersion, injection, copper sulphate, teratogenicity, glyphosate

INTRODUCTION

Pesticides are used in agriculture mainly to control pests, fungi, and weeds. Their application in agriculture helps to increase the yield, improve the quality as well as extend the storage life of food crops. As plant protection products are biologically active, they have the potential to affect non-target organisms, particularly in terrestrial environments where exposure may be continuous. The excessive use of pesticides may lead to the destruction of biodiversity. Many birds, aquatic organisms and animals are under the threat of harmful pesticides for their survival (Mahmood et al., 2016).

Besides adult birds, embryos in the eggs are threatened, because these chemicals can penetrate the porous eggshell and increase the mortality or development anomalies of embryos as well (Kertész, 2001).

An ecosystem can be contaminated by pesticides sprayed alone or applied together with other xenobiotics (e. g. potentially hazardous elements). Different pesticides can be used together as a mixture at the same time, can be applied alone step by step, one after the other, or metals may be present naturally in high amounts in a given area. Glyphosate and glyphosate-based herbicides are among the most frequently applied pesticidal active substances/pesticide products, while copper with fungicidal action is sprayed extensively for different purposes. Thus, the chemical load can generally appear in a complex way, therefore the simultaneous toxic effect and interaction of chemical agents can be expected at the same time.

*Corresponding author. Tel.: +36-1-478-4155.

E-mail: lehel.jozsef@univet.hu

**Corresponding author. Tel.: +36-83-545-226.

E-mail: budai.peter@uni-mate.hu

Over the past 30 years, there has been a significant increase in the number of studies on the effects of pesticides including their accumulation and their impact on wildlife. In the 1960s and 1970s a dramatic decline occurred in wildlife populations due to the use of pesticides based on organochlorine compounds, which could be traced back to the death of birds of prey (Köhler and Triebbskorn, 2013). The components can modify each other's toxic effect. Therefore, the examination of the combination of potentially hazardous elements and other chemicals gained significant ground in both avian (Fejes et al., 2001; Kertész and Hlubik 2002) and mammalian (Institóris et al., 2001; Pecze et al., 2001) toxicological research studies. Furthermore, the interaction effects are studied not only in the field of ecotoxicology, but also in all other areas dealing with healthcare and chemical safety issues (Oskarsson, 1983; Danielsson et al., 1984; Speijers and Speijers, 2004).

Copper has been used in agriculture due to its fungicidal and bactericidal properties for over a century (La Torre et al., 2018). It is essential in trace amounts for the normal development of living organisms, as it is a cofactor in many enzymes responsible for important processes in cells. On the other hand, it can trigger the production of free radicals, which can damage proteins and DNA (Ogórek et al., 2017). Copper is strongly bioaccumulative. Biological activity is a major factor in determining the occurrence and distribution of copper in an ecosystem (Aaseth and Norseth, 1986; Hejnal et al., 2018). It can be found in agricultural soils, in varying degrees originating from agrarian sources, e.g. manure, sewage sludge, fertilisers, and pesticides (Mantovi, 2003). Copper is important in integrated pest management. In organic farming it is almost exclusively used to control many bacterial and fungal diseases. However, the long-term consequences of its accumulation in the soil should be considered (La Torre et al., 2018) since it can be transferred easily to animals and humans as well through the food chain. Quantities exceeding the essential amount can be toxic, but sensitivity to copper toxicosis is species dependent. Generally, poultry resist chronic toxicosis better than most mammals (NRC, 1977). The contamination of agricultural soil with copper is a concern for the state of our environment and food safety (Komárek et al., 2010).

Glyphosate came on the market in the 1970s, to become one of the most often used herbicidal active ingredients in the world at present. It is a broad-spectrum herbicide, used in weed control in agriculture, and vegetation control in non-agricultural areas (Solomon et al., 2007.) Thus, based on its widespread use, glyphosate may have negative effects on animal and human health due to its possible residues in feeds and foods (Cox, 1995; Krüger et al., 2014; Blaylock, 2015; Faria, 2015). It disrupts the Shikimate pathway of plants and some microorganisms by inhibiting the enzyme EPSP (5-enolpyruvylshikimate-3-phosphate) synthase (Lipok et al., 2010). This process reduces the biosynthesis of aromatic amino acids (tyrosine, tryptophan, phenylalanine) in target organisms, resulting in their death (Nielsen et al., 2018). This pathway is not found in animal cells and, thus, glyphosate is considered

relatively non-toxic to animals and is also environmentally safe (Giesy et al., 2000; Duke et al., 2012; Helander et al., 2012; Annett et al., 2014).

A teratological test performed on avian embryos is quick and accurate and allows investigation of the chemical impact on fetal development. Further advantages of this method include its low cost, its sensitivity to various agents, as well as its high degree of similarity to the morphological development of mammals (Korhonen et al., 1982). The major disadvantage of this type of study is the lack of maternal-fetal relationship and the high sensitivity of the embryo (Wilson, 1978).

Different application methods are recommended to investigate the toxic effects of chemical agents on the egg and the avian embryo (Hoffman and Gay, 1981). The most frequently used administration is the injection of the test substance into different parts of the egg, e. g. into the air chamber. Its advantages are that physical injuries of the embryo can be avoided during the application, the exact dose can be applied and thus the teratogenic alterations caused by the given concentration can be precisely calculated, and the results of the study can be properly evaluated (Meinzel, 1977; Várnagy et al., 1982). However, the injection method does not reflect field exposure, and the residues of the chemicals cannot appear at the same stage of embryonic development as after immersion treatment (Varga et al., 2002). However, in the case of the immersion method the amount of chemicals entering the egg cannot be exactly calculated because their penetration can be influenced by the permeability of the eggshell that is characteristic to a given species, as well as by different environmental factors (Tyler, 1955; Tullett and Deeming, 1982).

Teratological tests carried out on avian embryos provide useful data for environmental protection and for the protection of wild birds and their progeny, and facilitate the development of environmental-friendly chemical plant protection techniques (Várnagy et al., 1996).

The current study was designed to examine the individual and combined embryotoxic effects of potentially hazardous elements such as heavy metal (copper) modelling the heavy metal load of the environment and a glyphosate-based herbicide formulation (Amega Up, glyphosate isopropylamine salt) widely applied in the practice, on the development of chicken embryos.

MATERIALS AND METHODS

Test system

Three hundred and twenty, mixed-use Farm hen eggs (Goldavis Ltd., Hungary) with good fertility were used in the experiment (Table 1). They were randomised into eight 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.

Table 1. Experimental design

Group	No. of eggs	Treatment	
		Copper sulphate	Glyphosate (Amega Up)
Injection method			
I (control)	40	–	–
II	40	0.01%	–
III	40	–	4%
IV	40	0.01%	4%
Immersion method			
V (control)	40	–	–
VI	40	0.01%	–
VII	40	–	4%
VIII	40	0.01%	4%

Test substances

During the injection and immersion treatment, copper sulphate (Reanal-Ker Ltd., Budapest) was applied in a concentration of 0.01% using the results of a previous study (Fejes, 2005). The glyphosate-containing herbicide (Amega Up, glyphosate isopropylamine salt, 360 g L⁻¹, Nufarm Hungary Ltd.) was administered as a 4% emulsion corresponding to that used in plant protection practice. The test substances were used by individual and simultaneous application in these concentrations.

Avian physiological saline solution (0.75% sodium chloride) was used to dilute the test substances and for the treatment of the control eggs. The experimental design is presented in Table 1.

Treatments

The study and the treatments were performed in accordance with the methods described by Várnagy et al. (1981). All applicable international, national and/or institutional guidelines for the care and use of animals were followed. The experimental protocol of the study was approved by the local Committee of Animal Welfare at Pannon University, Georgikon Faculty (permission No.: MÁB-10/2019).

Injection method. The individually and/or simultaneously applied test substances and the saline solution were administered in a volume of 0.1 ml directly into the air chamber of the eggs with a micropipette on day 0 of incubation, depending on the treatment groups. Initially, the blunt ends of the eggs were disinfected with 70% ethanol, then the calciferous eggshell was drilled through (Forte 300 dental drill, Netdent Trade Ltd., Hungary) before the injection, and the hole was closed with paraffin after the treatment (Clegg, 1964).

Immersion method. For the immersion method, chicken eggs were dipped into the solution or emulsion of the test substances and/or saline solution for 30 min on day 0 of incubation at room temperature (Várnagy et al., 2000). The applied concentration of the test substances was the same as for the injection method.

Investigated parameters

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. Skeletal preparations of the embryos were made using alizarin red dye to stain the bones and cartilages and to detect any possible developmental changes in the skeletal system, followed by stereomicroscopic evaluation (Dawson, 1926).

Statistical analysis

Statistical analysis of the body weight of live embryos was performed by one-way analysis of variance. The homogeneity of variances was examined by the Shapiro–Wilk test. Comparative evaluation of the results of different groups was carried out by Tukey's test. The mortalities and the developmental abnormalities of embryos were analysed statistically using Fisher's exact test. Statistical analysis of the data was performed using R statistical software.

RESULTS

Injection method

Embryonic mortality. The results of embryonic mortality are presented in Table 2. The mortality rate was 7.5% in the control group. The single administration of copper sulphate increased the mortality up to 20% in Group II. The changes were not significantly different as compared to the control. Glyphosate caused an increase in mortality (35%) in Group III, which was statistically significant as compared to the control ($P < 0.05$). Due to the simultaneous administration (Group IV), a significant increase in embryonic mortality (up to 40%) was induced as compared to the control group ($P < 0.001$).

Developmental abnormalities. The rate of developmental abnormalities was 2.7% in Group I (Table 2). The single injection of copper sulphate induced leg deformation and open abdomen in a rate of 3.1% (Group II), which was not significantly different from the control group. Similarly, a

Table 2. Mortality and developmental anomalies of embryos due to single and combined treatment by injection method

Group	Treatment	No. of embryos	
		showing abnormality/No. of live embryos	No. of deaths/Total eggs
I	Control	1/37	3/40
II	Copper sulphate	1/32	8/40
III	Glyphosate	1/26	14/40 ^a
IV	Copper sulphate + Glyphosate	3/24	16/40 ^b

^a significant decrease compared to the control data ($P < 0.05$);

^b significant decrease compared to the control data ($P < 0.001$).

Fig. 1. Abnormalities: open abdomen (A) and leg deformation (B) caused by the simultaneous administration of copper sulphate and glyphosate

low malformation rate was observed in Group III. However, the simultaneous administration of copper sulphate and glyphosate (Group IV) caused an increase in the rate of developmental anomalies up to 12.5%, including open abdomen and leg deformation (Fig. 1).

Body weight. The average body weight of the embryos was 20.87 ± 1.61 g in Group II, which was significantly lower than in the control group (21.96 ± 1.16 g, $P < 0.05$). Glyphosate treatment (Group III) did not cause a statistically significant change in the body weight (21.04 ± 1.92 g). The simultaneous administration of copper sulphate and glyphosate resulted in a significant reduction ($P < 0.001$) of the average body weight (Group IV: 20.16 ± 1.96 g) as compared to the control (Fig. 2A).

Skeletal staining preparation. The highest rate of developmental disorders of the skeletal system (leg deformation, growth retardation; Fig. 3) were caused by the simultaneous use of copper sulphate and glyphosate; however, the anomalies were sporadic if the test substances were applied individually (Table 2).

Immersion method

Embryonic mortality. The results of embryonic mortality are presented in Table 3. The percentage of dead embryos was 5% in the control group. The single administration of copper sulphate increased the mortality up to 7.5% in Group VI. The changes were not significantly different from those in the control group (Group V). Application of glyphosate caused 10% mortality of the treated embryos in Group VII, which was also not significantly different from that found in the control. The simultaneous administration of copper sulphate and glyphosate (Group VIII) induced a statistically not significant increase in embryonic mortality (12.5%).

Developmental abnormalities. Developmental abnormalities were not recorded in the control group (Table 3). Single treatment with copper sulphate induced a 2.7% rate of

Fig. 2. Body weight (average $\pm$ SD; g) of the embryos after single and simultaneous administration of glyphosate and copper sulphate by injection method (Fig. 2A) and by immersion method (Fig. 2B). ^asignificant decrease as compared to the control data ($P < 0.05$); ^b significant decrease as compared to the control data ($P < 0.001$)

Fig. 3. Skeletal staining preparation. Normal development of control embryo (A) and growth retardation of an embryo (B) caused by the simultaneous use of copper sulphate and glyphosate

anomalies including leg deformation and open abdomen (Group VI), which was not significantly different as compared to the control group. The percentage of teratogenic malformations was 2.8% in Group VII due to the application of glyphosate. A similar rate of developmental abnormalities was found in Group VIII (2.9%) due to the

Table 3. Mortality and developmental anomalies of embryos due to single and combined treatment by immersion method

Group	Treatment	No. of embryos showing abnormality/No. of live embryos	Number of deaths/Total eggs
V	Control	0/38	2/40
VI	Copper sulphate	1/37	3/40
VII	Glyphosate	1/36	4/40
VIII	Copper sulphate + Glyphosate	1/35	5/40

simultaneous administration of copper sulphate and glyphosate. Generally, leg deformations and growth retardation were induced by the glyphosate-containing herbicide applied alone or in combination (Group VIII). The rate of developmental anomalies was similar in all groups treated with the investigated chemicals.

Body weight. The average body weight of the embryos was 20.26 ± 1.83 g in Group VI. It was not significantly reduced compared to the control group (20.97 ± 1.29 g). Due to the glyphosate treatment (Group VII) the body weight was 19.89 ± 1.28 g, which was significantly lower than in the control group ($P < 0.05$). The simultaneous administration of copper sulphate (0.01%) and glyphosate (Group VIII) resulted in a significant decrease ($P < 0.001$) of the average body weight (19.61 ± 1.68 g) compared to the control group (Fig. 2B).

Skeletal staining preparation. The skeletal development disorders (incorrect posture of the feet) induced by the individual or simultaneous application of the test substances were sporadic (Table 3).

DISCUSSION

Agricultural areas provide feedstuffs, living and hatching territory for wild birds, and thus the study of the environmental effect of different chemical agents including pesticides is very important. Depending on the exposure level they can induce acute damage or even destruction of living organisms.

The results of individual teratogenicity studies on copper sulphate in chickens are in accordance with those of toxicity studies in other species. Depending on its dose, copper has embryotoxic potential and may cause developmental anomalies (Várnagy and Budai, 1995).

According to our findings during the evaluation on day 19, copper sulphate significantly increased the mortality rate and decreased the body mass of the embryos compared to the control group. These results are similar to those obtained in the experiment performed by Fejes (2005), where the eggs were treated by the injection of copper sulphate into the air chamber on days 1 and 12 of incubation.

In the injection groups, adverse effects were more pronounced and the rates of mortality and developmental anomalies were higher than in the immersion groups. This is in line with the findings of a study conducted on chicken embryos by Kertész (2001), indicating a slower onset of toxicity because the compounds must penetrate the eggshell and the shell membranes.

According to our findings, the single administration of the herbicide caused a significant increase in the rate of embryonic mortality and developmental abnormalities.

Some studies have labelled herbicide formulations containing glyphosate as teratogens (Dallegre et al., 2003), but it should not be overlooked that adverse effects may vary among different animal species (Tompa, 2005; Hued et al., 2012; Mottier et al., 2013), and are affected by the duration of exposure and the dose (Várnagy and Budai, 1995).

Roongruangchai et al. (2018) studied the effect of 0.1 ml volumes of 0.01%, 0.05%, 0.3% and 0.5% w/v glyphosate solution injected into the yolk sacs of eggs at 21 h of incubation and repeated with a volume of 0.05 ml on day 3 of incubation. They found that glyphosate was toxic to chick embryos. It caused mortality, growth retardation and malformations on day 3 and skeletal alterations on day 6, which also indicates its teratogenic effect.

Winnick (2013) investigated the toxic effect of glyphosate and Roundup (a glyphosate-containing product, glyphosate potassium salt, ether alkylamine ethoxylate detergent) on developing chicken embryos, when applied at a concentration of 19.8 and 9.9 mg of active ingredient glyphosate/kg egg mass in both preparations. Survivability was significantly lower in chickens treated with Roundup than in the case of glyphosate-treated embryos. The body weight of the embryos was significantly reduced due to treatment with 9.9 mg kg⁻¹ glyphosate in Roundup and 19.8 mg kg⁻¹ glyphosate. A significant reduction in tibiotarsus and beak length was observed in embryos treated with Roundup (9.9 mg kg⁻¹ glyphosate). Supposedly, the high interaction potential is due to the ethoxylated detergent component, and the combined use of glyphosate and the surfactant in the same pesticide product.

Ruuskanen et al. (2020) studied the effects of a glyphosate-based herbicide applied as contaminated food on feathering and body weight gain in Japanese quails (*Coturnix japonica*) in order to collect more data about the effects of glyphosate-containing herbicides in avian taxa, because it is a poorly understood area. The results showed that the consumption of the contaminated food caused delayed plumage development, and residues of the herbicide were present in the eggs, muscles, and liver.

The glyphosate in Roundup induced reproductive changes (reduced levels of testosterone and oestradiol, morphological alterations of the testis and the epididymal region) in male ducks (*Anas platyrhynchos*) (Oliveira et al., 2007).

Basically, a pesticide product including all components was used in our study. However, other components than the active substance of the product (e.g. solvent, detergent, surfactant etc.) may have their own effect (Székács, 2017).

Glyphosate and glyphosate-based herbicides induce a cytotoxic effect in testis and kidney cells (Vanlaeys et al., 2018; Tsatsakis et al., 2019), disturb the oestrogen pathway as endocrine disruptors (Mesnage et al., 2017), reduce testosterone production (Romano et al., 2012), and produce teratogenic malformations (Fagan et al., 2012). Furthermore, neurological alterations (e.g. anxiety, depression-like behaviours, decreased locomotor activity and learning abilities) (Ait Bali et al., 2017; Zheng et al., 2018; Ait Bali et al., 2019; Eriguchi et al., 2019), digestive problems (dysbiosis, liver fibrosis, fatty liver, altered metabolome) (Ait Bali et al., 2018; Mesnage et al., 2018; Pandey et al., 2019), cardiovascular problems (e.g. anaemia) (Wang et al., 2019), and carcinogenic effects (e.g. melanoma, leukaemia, myeloid lymphoma, myeloma (de Roos et al., 2005; Andreotti et al., 2018; Leon et al., 2019) may be produced. Glyphosate has immunotoxicological effects that affect the complement cascade, phagocytic function and the lymphocyte response and cause inflammation (Wang et al., 2019; Watanabe et al., 2019; Qiu et al., 2020).

Phenoxyethanol (POE) exerts toxic effects on human cells (hepatic, embryonic and placental cell lines), including negative effects on cellular respiration and membrane integrity, leading to necrosis and apoptosis (Mesnage et al., 2013).

Based on the scientific results, researchers stated that there is an antagonistic interaction between glyphosate and POE detergent. Although the surfactant is markedly more toxic than the glyphosate itself, their combined use can amplify or change their toxic effects (Mesnage et al., 2013, 2019; Peillex and Pelletier, 2020).

The toxic effect resulting from the interaction of a glyphosate-based herbicide and copper sulphate on chicken embryos is a quite rarely investigated topic. However, the results of combined administration in our experiment by either of the two methods showed that embryonic mortality was increased and the body weight was decreased significantly compared to the control groups.

Similarly, a glyphosate-based herbicide (Gliaalka Star, glyphosate potassium salt) and heavy metals (cadmium, copper) were applied alone and/or simultaneously to embryonated chicken eggs using different treatment methods to investigate their teratogenic effects. Their combined administration induced significant reduction in the body weight of the embryos and increased the rate of embryo mortality (Szemerédy et al., 2017; Szabó et al., 2020).

Due to individual and simultaneous application, developmental abnormalities (leg deformation, growth retardation, opened abdomen) were sporadically observed in our studies including both treatment methods. Our experience is in line with the scientific literature indicating that the incidence of gross developmental anomalies is sporadic and not characteristic, and abnormalities most frequently occur in the skeletal system. This is not surprising as the skeletal system has outstanding importance in experimental teratological studies (Tompá et al., 1971). However, in some cases developmental changes may also occur in the soft tissues.

Toxic interaction, at least additive action, may occur between two pesticides if they act together in the same organism at the same time. Especially if the combination contains an insecticidal component, this undesirable effect may be more severe and even synergistic by increasing toxicity up to 100-fold. However, these effects depend on the animal species, the exposure time and the dose, and thus their routine prediction is difficult (Thompson, 1996).

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