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STUDY OF THE INDIVIDUAL AND COMBINED TOXIC EFFECTS OF HEAVY METALS (COPPER, CADMIUM) AND HERBICIDES (DUAL GOLD 960 EC, STOMP 330 EC) POLLUTING THE ENVIRONMENT IN AVIAN EMBRYOS

THESES OF DOCTORAL (PhD) DISSERTATION

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CONTENTS

1. SCIENTIFIC ANTECEDENTS

One of the basics of the existence and development of humankind is the agricultural activity, which creates the energies of human life through food production. At the beginning low yields characterized agriculture due to the low level of soil utilization and the use of primitive tools. In the period of feudalism the situation did not change much, the slow development of sciences and the small-scale industrialization did not help the development of agriculture. However at the beginning of capitalism conditions and possibilities changed completely. The spectacular development of biology, the appearance of industry and its extensive need for raw materials contributed to the development of agriculture enormously. The appearance of plant species with higher yields, pesticides and herbicides, and automated machines resulted in deep and fundamental changes in production. Yields and production safety grew significantly due to the appearance of new tools and technologies (NYÍRI, 1993).

When pesticides appear and were used extensively, the long wished dream of humankind came true, the prevention of pests, which often caused social tension and severe starvation. The vital condition of protection with chemicals and the production of pesticides were to get more and detailed information about the life of pests, pathogens and weeds. According to this we can speak about purposeful and aimed protection only in the second half of the 19th century.

Nowadays, besides food production, agriculture must pay close attention to the economical use of non-recyclable raw materials and the decrease or complete elimination of the damage to the environment, which affects wildlife.

The traditional mass production with its huge need of energy and chemicals is not suitable to solve these multiple tasks at the same time. This kind of production spoils the environment continuously by polluting the water and the soil and by endangering wildlife, and the consumption of the polluted products largely contributes to the health impairment of the population.

In agricultural production chemical plant protection pollutes the environment most. Although the licensing process of pesticides has changed a lot in the past few decades, huge amounts of chemicals, the biological activity of which is not completely known, escape to the environment. It is thought provoking that while over 6 million synthetic

materials have been created according to the surveys, the IARC, International Agency of Research on Cancer, has been able to examine only 900 materials thoroughly and issued certificates of them (ÁNGYÁN, 2003). During the examinations we must pay attention to the extent of accumulation and decomposition of pesticides in the soil, the spread of water pollution and the influence of each new compound on the food chain.

The land used for agricultural production is the food source, shelter and breeding place of our wild birds. However the risk is high because the pesticides out there are dangerous for them. The pesticides sprayed during the plant protection activities might have an influence not only on mature birds but also on the embryos in the eggs. In the environment which is contaminated by pesticides, these materials change the chemical environment of the plants and animals, populations and communities and make poisoning possible. The harmful effects of pesticides can be seen in the acute damage, or destruction of living creatures at a lower or higher level. The destruction of the mature animals can cause the death of the offspring who remained without food and care, even if they are not poisoned. Those who survived the acute poisoning but their resistance decreased can become the victims of different environmental pollutions (VÁRNAGY and BUDAI, 1995).

The rapid increase of environmental pollution can endanger the whole society, especially the compounds which can be traced in the soil-plant-animal-human food chain. Materials as hazardous factors which can cause harm to people by consuming food are partly natural by origin; partly they get into food in the course of production (GYALMOS and MOLNÁR, 1999).

During the chemical plant protection work, there is a great chance that the person who carries out spraying or the whole ecosystem of a habitat gets polluted with pesticides or xenobiotics with the combination of heavy metals among others, which can be found in big quantities in cultivated agricultural areas and can present harm themselves, they can even cause the deformation of the offspring to be born as a consequence (DARVAS, 2003). Unfortunately, the general toxicological an eco-toxicological consequences are not explored due to the high number of combinations, despite the fact that the study of especially the combination of heavy metals with other compounds has had an increasing importance recently in toxicological research in the field of experiments with birds (FEJES et al., 2001; KERTÉSZ, 2001), mammals (INSTITÓRIS et al., 2001; PAPP et al., 2001).

As a result of the demographic explosion the demand for food is bigger and bigger and this demand must be satisfied. As the chance to increase the cultivated areas is less and less

what is more we must face the decrease of agricultural land, we cannot give up the use of agricultural chemicals.

At the toxicological examination of the different xenobiotics and similarly at the eco-toxicological tests of the pesticides the chemical agents are applied one by one but we cannot ignore the fact that the chemical load generally appears in a complex way, therefore we can expect the simultaneous, complex toxic effect and interaction of the chemicals present and as a consequence the components can modify each other's toxic effect. The interest of the researchers turned to the effects of interaction gradually, not only in the field of ecotoxicology but also in any other fields that deal with the question of health care and chemical safety (OSKARSSON, 1983; DANIELSSON et al., 1984; SPEIJERS and SPEIJERS, 2004; YOUN-JOO et al., 2004).

2. RESEARCH OBJECTIVES

The aim of my examinations was:

- To reveal the individual and possible common embryo damaging influence of heavy metals (copper, cadmium), which are regarded as especially dangerous for living organisms, and can be found in the environment in larger quantities and
- That of the two widely used herbicide (pendimethalin, S-metilachlor).
- I examined the toxic influence of the materials in the experiment in the early and late embryonic development,
- The first step of which was to estimate the individual embryo damaging influence of heavy metals and pesticides in the examination system, I applied.

These series of examinations are suitable to study how the toxic effect of the examined pesticides is present in the developing bird embryos that were chosen as test organisms, besides the environmental heavy metal load. As the eco-toxicological examination methods, which are used in practice, are mainly restricted to the examination of individual toxic effects, the data in connection with the interactive effects of pesticides can be regarded as supplementary.

3. MATERIALS AND METHODS

3. 1. Test materials

3. 1. 1. Herbicides

- Stomp 330 EC (33% pendimethalin)
- Dual Gold 960 EC (960g/l S-metolachlor)

3. 1. 2. Heavy metals

- Copper sulphate
- Cadmium sulphate

3. 2. Concentrations applied

In the case of heavy metals the concentration was 0.01%, based on the results of a prior examination (FEJES, 2005).

On the basis of the available data of the literature, the herbicides were injected into the eggs in concentrations corresponding to those of the spray solutions used in the practice of chemical plant protection.

3. 3. Experimental animals

I made my experiments on the eggs of the hen (*Gallus gallus f. domestica).* At the beginning of my experiments I applied Ross 308 fertile eggs that I acquired from the incubation plant of Mezőtek Plc. in Zalaapáti. The Ross 308 is a white-feathered meat hybrid, which is the most popular species of the international market. Later because of

reasons beyond me I did not have the opportunity to acquire this type of egg continuously and safely therefore I started to use Shaver Rusticbro eggs, which were bought in the incubation plant of Goldavis Ltd. in Sármellék. The Shaver Rusticbro meat hybrid has similar good qualities and fertility features to the Ross 308 species. During the series of experiments I used 540 hen eggs altogether.

3. 4. Incubation

Incubation of the eggs was started after a 24-hour resting period following transportation in a Ragus type table incubator. The required temperature (37-38°C), relative humidity (65-70%) and daily rotation of the eggs were ensured during incubation in order to avoid the adhesion of embryos (BOGENFÜRST, 2004).

3. 5. Time and method of treatments

In my experiments I chose the starting day of the incubation for the day of the treatments. I started to model what effect can be induced if the chemical load is applied at the beginning of the development of the hen embryo. Before starting the treatments, I randomly divided the eggs into groups, making efforts to form groups that are as homologous as possible with respect to egg size and weight. In addition, all eggs were marked with black-lead pencil. On day 0, 60 eggs per group.

Injection was selected as the method of treatment, as this method allowed me to introduce the components to be tested in accurately measured doses into the air space of the eggs in a manner that ruled out any mechanical injuries (CLEGG, 1964; LUTZ, 1974; MEINIEL, 1977; VÁRNAGY et al., 1982). Before injection, a hole was drilled in the calcic eggshell above the air space, and then the test materials were introduced with an Ovijector automatic injector.

During the individual treatments the chemicals were introduced separately in a final volume of 0.1 ml each, while for the study of combined toxicity 0.1 ml of each component of a combination (i.e. a total of 0.2 ml solution or emulsion) was injected into the air space of the eggs. For the preparation of solutions and emulsions, distilled water was used in all

cases, and the eggs belonging to the control group were treated with distilled water of 0.1 ml final volume. At the end of the treatment procedure the holes were closed with paraffin and the eggs were placed into the incubator.

3. 6. Processing

During my studies the hen's eggs were opened and processed at two different times. In the case of eggs treated on the day of starting the incubation, permanent preparations were made from 10 embryos per group by staining with 0.1% osmium tetroxide on days 2 and 3 of incubation in order to study the early stage of development (SINKOVITSNÉ and BENKŐ, 1993; KERTÉSZ, 2001). The other eggs treated during the experiment were opened two days before hatching, on day 19 of incubation, when the embryos were examined according to the following criteria:

- The embryos were weighed and the number and type of developmental anomalies, if any, as well as the times and number of embryonic deaths were recorded.

- For histological processing, samples were taken from the liver and the long muscle of the neck (*m. longissimus colli*). The organs were fixed in 4% formaldehyde, embedded in paraffin, stained with haematoxylin and eosin, and evaluated by light microscopy (KRUTSAY, 1980; VETÉSI, 2002).

- Skeletal preparations were made by the use of alizarin red staining to detect any possible developmental abnormalities occurring in the skeletal system of the embryos (DAWSON, 1926). Skeletal preparations were then examined by stereoscopic microscope.

- I took liver, brain, beak, claw and feather samples in order to process them with the methods of FTIR and FT-Raman spectroscopic. With the applied methods we could follow how much the experimental materials built in the organism of developing hen embryo. I examined the beak, claw, and feather samples of the chicken without any preparation applying a diamond cell and a FTIR microscope. I examined the

liver and brain samples of the chicken after liofilization with FTIR Drift technology and FT-Raman method.

3. 7. Statistical analysis

The body weight data were evaluated by analysis of variance (ANOVA). For the statistical evaluation of embryonic mortality and developmental anomalies the RXC Chi^2 test was used (BARÁTH et al., 1996).

4. RESULTS OF THE EXPERIMENT AND THEIR EVALUATION

4. 1. Study of early embryonic development

During the examination of the early embryonic development I concluded that heavy metals intensified the frequency of the occurrence of mortification but neither the use of copper nor cadmium sulphate resulted in the significant increase of the parameters, which was justified by the results of other authors (FEJES, 2005). Of the pesticides and heavy metals, which got into the eggs individually, the Stomp 330 EC proved to be the most toxic for the development of hen embryos, which was used in the degree of the concentration of the spray. None of the individual treatments resulted in significant deviation comparing them to the control group.

Of the groups which were treated collectively, the ratio of the mortified embryos increased significantly in both the control group and in the group which was treated by Dual Gold 960 EC individually, when they were treated with the combination of copper sulphate and Dual Gold 960 EC.

Summarizing the developmental disorders we can conclude that there were embryos with developmental disorders in each group, but I did not perceive significant deviation during the statistical processing. Developmental disorders could be observed in development of the blood vessels, the somites the brain bladder and the eye bladder.

4. 2. Results of gross pathological processing

During the processing on the 19^{th} day heavy metals increased the rate of mortifications significantly, moreover copper sulphate decreased the body mass of the animals significantly, which is proved by the results of FEJES (2005), who also made experiments with hen embryos.

In comparison with the values of the control group, the rate of mortified embryos also increased significantly in the groups which were treated by herbicides and heavy metals individually. When the herbicides (Dual Gold 960 EC, Stomp 330 EC) were used individually, one quarter of the treated animals died in comparison with the values measured in the control group. Similarly to the results of the examination of the early stage of development, the embryo toxic effect of the products could be felt in the case of both pesticides right after the period of incubation.

In the groups which were treated individually, the frequency of development orders remained at a relatively low level (2.33-8.57%) during the whole period. On the basis of the values of the body mass I concluded that when herbicides were used individually, the Stomp 330 EC decreased the body mass of the embryos significantly.

Of the jointly treated groups, we can conclude that in the case of the combinations which contained copper sulphate as a heavy metal component, the significant increase of the mortified embryos could be observed only in comparison with the control group. In the groups which were treated with cadmium sulphate and Dual Gold 960 EC or cadmium sulphate and Stomp 330 EC embryo mortification increased significantly in comparison with both the control and individually treated groups, therefore we can conclude that the embryo toxic effect increased undoubtedly in comparison with the individual embryo toxic effect of the used components. In the case of development disorders I experienced a significant deviation only in one case when the copper sulphate and the Stomp 330 EC were applied together, where one fifth of the embryos showed some development disorders. In the other groups, which were treated together, the occurrence of development disorders remained as low level as in the case individual treatments. Looking at the types of development disorders, the most frequent problems were the oedema, the shortening of the beak mandible, and the incorrect posture of the feet and the neck. As the collective result of the treatment with copper sulphate and Stomp 330 EC or cadmium sulphate and Dual Gold 960 EC, the body mass of the treated embryos decreased significantly in comparison with the control and individually treated groups, therefore we can conclude that the combined treatment resulted in increased embryo toxic effect in comparison with the individual embryo damaging effect of the used components.

4. 3. Results of processing by skeletal staining

The highest rate of development disorders were caused by the use of copper sulphate and Stomp 330 EC of all the applied chemical materials, which were mainly resulted in the incorrect posture of the feet and the neck and the lag of growth, proved by the pathological processing.

In the case of the groups which got a combined treatment, the number of the development disorders, which were shown by the Dawson painting technology, increased when copper sulphate and Stomp 330 EC or cadmium sulphate and Dual Gold 960 EC were used, in comparison with the individually treated groups. The types of disorders were the incorrect posture of the feet and the neck and the lag of growth.

4. 4. Results of histological processing

Histopathological examination did not reveal any hepatotoxic or myopathic effect of the chemical agents applied in any of the groups tested.

4. 5. The result of the FTIR and FT-Raman spectroscopic processing

In the course of the examination with the spectroscopic technique I studied the intensity of the stripes taken during the analysis of the colour pictures, especially the cholesterol and bilirubin stripes that appeared in the liver, which indicate the restructuring of the molecular structure influenced by a chemical. The shifts of the stripes are caused by the infiltration of the test material into the tissues. I could observe the change only in one group as the effect of Dual Gold 960 EC. The analysis of the liver indicated the increase of the level of bilirubin, and the decrease of the level of cholesterol. Analyzing the histological samples of the brain I could observe less intensive stripe intensity.

4. 6. Recommendations

In my opinion the examinations I carried out can be extended with the examinational methods that I want to introduce below and other processing methods. In this way we can get more information about the special features of the chemical agents in the experiment and their behaviour in the living organism.

The series of examinations can be completed by:

- The weighing of the removed organs (heart, liver) for histological processing and other organs.

- The pathological examinations of the long neck muscle, liver and other organs.

- The biochemical and disintegration dynamic examination of the applied chemical materials and their metabolites.

- The weighing of the dry and wet body mass and the detailed evaluation of the embryos on the basis of different development of the body and the organism in the middle period of the embryonic development when it is not possible to make a durable preparation with the above mentioned painting method due to the size of the embryos.

- The test of the blood samples, obtained by the help of a glass capillary from the umbilicalis of the artery, (VÁRNAGY, 1981; KERTÉSZ and HLUBIK, 2002) for certain blood plasma parameters (glucose, calcium, magnesium, inorganic phosphate, AST, ALT, ALP, LDH, pChE, total protein, albumin).

- As the evaluation with the spectroscopic analysis showed the growth of the concentration of bilirubin that appeared in connection with the toxic effect when Dual Gold 960 EC was applied, I think it is advisable to examine the concentration of gall paint in the plasma when the blood plasma parameters are tested.

- However, owing to the high sensitivity of wild fowl species, the same studies should be performed on waterfowl species (mallard) as well as on seed-eating birds (pheasant, Japanese quail).

- Besides the injecting treatment method that was applied during the experiments it would be advisable to complete the performed examination with a spraying, (bathing, immersing) treatment that can model the expositional circumstances during the plant protection practice better and with the comparison of the results that were achieved during the different treatment technologies.

- The experiments can be completed with incubation and post-embryonic examinations, which can give further information about the individual and collective behaviour of the used materials in biological systems.

On the basis of the results of the examinations of the widely used copper sulphate as pesticide and other herbicides in practical spray concentration in chemical plant protection, I suggest introducing the means of biological protection (FISCHL, 2000) and the technologies of integrated plant protection in plant protection practices on a large scale in order to protect wild life that surrounds people. On the basis of the research into agronomy and agro-technology for example on horticultural plantations, the use of chemical saving technology –spraying machines with plant sensors- can reduce the amount of sprayed pesticides by as much as 75% and as a result the load on the environment can be reduced significantly (DIMITRIEVITS, 2004). In order to protect the health of the person who sprays pesticides, it is worth examining the possibilities of the use of Raman spectroscopy I dealt with in my essay because it allows to detect the accumulation of the micro amounts, the agents and their metabolites which get into the human body, from biological samples, for example from the hair.

All in all, we can say if the new methods and procedures, which were advised to be applied during processing, are introduced and the examination of the environment damaging materials are extended, the human effect on the environment can be more recognizable and with the practical use of the acquired results there is a bigger chance to preserve our environment for ourselves and our grandchildren in its original condition.

5. NEW SCIENTIFIC RESULTS

1. Using FTIR and FT-Raman spectroscopic technique I provided new basic data concerning the accumulation of certain chemicals in embryonic stage, furthermore I was also focusing on lesions as a result of these agents.

2. Studying the toxic substances individually and jointly I concluded that in the groups treated with Stomp 330 EC, embryo-mortality, backwardness in development was much higher than in the control groups and in those treated with herbicides individually; consequently the agent „pendimethalin" proved to be highly toxic by itself and also when used in combinations with heavy metals.

3. Having completed the FTIR and FT-Raman spectroscopic analysis, it can be stated that – as a result of injection – treatment, only Dual Gold 960 EC herbicide was found to have caused lesions on molecular level in the liver and brain tissues of the embryos. The herbicide induced a decrease in cholesterol level, while an increase in bilirubin level in the samples.

4. I also concluded that toxicity might greatly increase when heavy metals and pesticides are used jointly as compared to the toxic-effect of the components used individually. Death-rate in early development stage reached 10-40% when heavy metals and pesticides were applied in combination, while during the 19^{th} day of evaluation it came near to 50%.

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