

## Evaluation of Ag, Cu, Fe and MnO<sub>2</sub> nanoparticle mixture effect on histomorphological state of internal organs and tissues in laying hens

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The article presents the results of an impact assessment of a mixture of nanoparticles (MNPs) of metals (Ag, Cu, Fe) and manganese oxide (MnO<sub>2</sub>) on the histomorphological state of internal organs and tissues of laying hens. The study of a subchronic effect of an experimental sample of MNPs on the organism of laying hens was conducted on a Hayrase Brown cross-breed (n=112), weighing 1.5-1.8 kg at the age of 365 days. Colloidal solutions of MNPs (group II – 0.3 mg/kg, group III – 4.0 mg/kg body weight) and a mixture of salts of a corresponding metals (group I – 0.3 mg/kg body weight) were daily administrated to experimental groups of poultry as feed compound additives during 30 days. Distilled water was added to the compound feed for a control group of hens. Euthanasia of chickens (n=7) was performed by using inhalation chloroform anesthesia on a 15<sup>th</sup> and 30<sup>th</sup> day after exposure as well as 7 and 15 days after a termination of the drug exposure. Internal organs were selected for histomorphological studies. It has been found that changes of the morphological structure of the liver and kidneys established in chickens under the influence of MNPs in a toxic dose and occurred on the 30<sup>th</sup> day after the start and on the 7<sup>th</sup> day after the termination of it's a exposure. There were a thickness of the walls and lesions of the epithelial cells of the bile duct, a desquamation of a part of epithelial cells, a presence of granular dystrophy of the epithelium of the urinary tubules with elements of necrosis and hemorrhages. This testifies to the presence of dystrophic changes in hepatocytes, bile ducts, bile passages and urinary tubules.

**Key words:** nanoparticles, dose, laying hens, internal organs, histological examination.

### Introduction

A Copper, Iron and Manganese are vital trace microelements according to the classification of V.I. Georgievsky (1979). Nowadays these microelements are essential components of compound feed. They are mainly used in the form of sulfates or oxides and less used in the form of metal chlorides and carbonates that based on calculations by metal at the range from 1.2-2.0 mg/kg for Cu and to 8.0-12.0 mg/kg for Mn. It is generally possible to use Iron in concentration up to 60.0 mg/kg of body weight (Kim et al., 2016; Olukosi et al., 2019). Along with this, organic forms of metals known as chelates (complexonates) of trace elements are increasingly being used as substitutes for salts. They are produced by combining metal cations (microelements) with organic acid molecules (chellants) in such a way that stable compounds (chelates) are formed (from the Greek «chele» - claw), since they have higher bioavailability (Swiątkiewicz et al., 2014; Carvalho et al., 2015). However, there is another poorly studied form of microelements called nanoparticles (NPs) of metals and metal-containing compounds (mainly oxides) (Hassan et al., 2020). Advances in nanobiotechnology have had a significant impact on animal husbandry and veterinary medicine, where nanomaterials are widely used (Hill & Li, 2017; Bai et al., 2018). At present, nanomaterials in general and nanoparticles of essential metals with their oxides in particular are promising components of feed additives and premixes for animals and poultry (Saleh & El-Magd, 2018; Ognik et al., 2019). One of the main advantages of metal nanoparticles is their low toxicity compared to the relevant salts and their higher bioavailability relative to organic forms according to the opinion of some scientists (Sengupta et al., 2014; Ng et al., 2015).

Contamination of feed by pathogenic microflora with the subsequent formation of mycotoxins is an urgent problem in poultry farming today (Greco et al., 2014; Kehinde et al., 2014). It can be solved by addition of nanoparticles in feed, namely silver and copper nanoparticles have a pronounced fungicidal and fungistatic effect (Roman'ko et al., 2016).

However, there are sufficient data indicating a toxic effect of metal nanoparticles on the living organism. This effect is manifested depending on the route of nanoparticles' entry by the following factors: hepato- and pulmonary toxicity with signs of inflammatory and cytological (necrotic) reactions, hematopoietic disorder, a carcinogenic effect, ability to generate active forms of Oxygen and metabolic disorders (Oberdorster et al., 2005; Lynch et al., 2007).

There are studies concerning the influence of metal nanoparticles on the histomorphological structure of internal organs of laboratory animals among the data on the toxicological assessment and application of nanoparticles in medical practice (Thakur et al., 2014; Yang et al., 2017). Silver is one of the most studied nanomaterials in this regard because silver nanoparticles exhibit antimicrobial and antifungal properties. It is used in surgery, particularly in the treatment of wounds of various etiologies. Although, there is experimental evidence that silver ions extracted from surgical therapeutic means can enter the bloodstream, accumulate in the organs and cause toxic effects, especially on the liver and kidneys. However, oral or transdermal administration of Ag nanoparticles (Ag NPs) at doses of 2000 mg/kg of body weight did not lead to significant clinical signs, mortality, acute irritation or pathological reactions of the mucous membrane of the eyes and skin in rats, guinea pigs and rabbits. Therefore, the authors have conducted the following study to identify ultra-structural changes in organs. The two-fold intra-abdominal administration of Ag NPs (25.1-58.3 nm) at a dose of 2.000 mg/kg of body weight resulted in swelling of the renal tubule epithelium with cytoplasmic vacuolization, thickening of the basement membrane and destruction of some mitochondrial cristae. The narrowing of sinusoids with endothelial hypertrophy, swelling of hepatocytes with hypertrophied nuclei, the accumulation of fat globules in the nucleoplasm and cytoplasm were recorded in the liver (Sarhan & Hussein, 2014). Copper nanoparticles (Cu NPs) synthesized and widely used as catalysts, heat carriers in machine tools, semiconductors and antibacterial preparations. Therefore, there are risks associated with the release of developed nanoparticles into environment, and various health consequences of their undesirable effects on human health have increased. This led to additional studies of the toxicity of mentioned nanoparticles, namely the influence on a histomorphological state of a liver. Steatosis was observed around the central veins of liver tissues when using a Cu NPs at a dose of 341 mg/kg of body weight in mice. In addition, pathology studies have shown that the kidneys and spleen along with liver are the target organs of toxic effects of Cu NPs (Chen et al., 2006). Another study examined the effects of intraperitoneal injection of Cu NPs at different doses (10, 100 and 300 mg/kg b. w.) on the liver and the effectiveness of liver enzymes in rats. A vascularization of the central veins and vessels of the portal triad and a disappearance of hexagonal structure of hepatocytes in all experimental groups were established (Doudi & Setorki, 2014).

Iron nanoparticles in the form of oxide are used as contrasting substances in magnetic resonance imaging. They are also used for stem cell marking and tracing. Due to their physiochemical properties, they can be used *in vivo* as drug carriers in the treatment of cancer cells. Their influence on the histomorphological structure of animal organs is contradictory, which led to relevant studies. Thus, it has been found a light portal inflammation of hepatocytes, centrilobular hyperemia, intravascular and portal aggregation of erythrocytes and centrilobular necrosis under intraperitoneal administration of Fe<sub>2</sub>O<sub>4</sub>Zn nanoparticle solution (<100 nm) for 7 days at doses of 100 and 200 mg/kg of body weight of liver tissue in rats. These phenomena were more pronounced when Fe<sub>2</sub>O<sub>4</sub>Zn was administered at a dose of 200 mg/kg body weight (Saadi & Hooshmandi, 2016).

Histological examination of the liver hepatocyte damage, inflammation, infiltration, accumulation of mononuclear cells and a liver beams degeneration after 14 days of an iron oxide nanoparticles intratracheally injection during 14 days at a dose of 40 mg/kg of body weight in rats (Vasili et al., 2016).

Manganese dioxide-based nanoparticles (MnO<sub>2</sub>) are a promising nanotherapeutic carrier for use in magnetic resonance tomography and in facilitating of tumor hypoxia. However, the complexity and potential toxicity limited clinical application of these NPs. The research results showed that administration of MnO<sub>2</sub> NPs (30-60 nm) for 15 days caused oxidative stress and reduced catecholamines in the hippocampal tissue, at the same time causing a dose-dependent increase in the number of apoptotic and necrotic cells in rats (Sadeghi et al., 2018; Fang et al., 2019).

However, the study of the impact of nanoparticles as promising ingredients of feed additives on the organism of productive animals and poultry is limited to the determination of individual clinical and biochemical blood parameters.

A mixture of nanoparticles has been developed with our previous research, which consisted of *in vitro* studies. In this case the genotoxic, mutagenic, and general toxic properties of Ag NPs, Cu NPs, Fe NPs, MnO<sub>2</sub> NPs, Zn NPs, Co NPs and cobalt hexacyanoferrate (C<sub>6</sub>CoFeN<sub>6</sub>) NPs were separately studied. At the same time Ag NPs, Cu NPs, Fe NPs and MnO<sub>2</sub> NPs did not exhibit the above-mentioned negative effects on eukaryotic cell models, CHO-K1 and *Allium cepa* test cells in contrast to Zn NPs, Co NPs and C<sub>6</sub>CoFeN<sub>6</sub> NPs. The range of harmless concentrations of these nanoparticles was 0.22-1061.0 µg/ml (Roman'ko, 2017). We did not investigate the separate effect of Ag NPs, Cu NPs, Fe NPs, and MnO<sub>2</sub> NPs on the organism of laboratory animals grounded on the above established facts, but we worked with the composite mixture (Ag, Cu, Fe, and MnO<sub>2</sub>) (MNPs) by choosing the concentration of each metal from a safe range according to 100 µg/ml. The range of metals' MNPs doses of 0.3; 1.0; 2.0 and 4.0 mg/kg b. w. was tested in a series of acute tests on rats. It was found that MNPs had a positive effect on the body at a dose of 0.3 mg/kg (biotic dose). A dose of 1.0-4.0 mg/kg had a negative effect, which is almost the same in the entire range (Orobchenko et al., 2013). The main objective of our work was to develop a prototype of a biologically active supplement for poultry. Therefore, it was necessary to test the biota dose in comparison with metal salts (therapeutic efficacy) in a chronic experiment on rats, and to show a toxic effect (a dose 4.0 mg/kg b. w. of MNPs was selected). As a result, we confirmed the possibility of using a KCHMe at a dose of 0.3 mg/kg for poultry (Kutsan et al., 2014).

In addition, the mechanism of the toxic effect of a composite mixture of metal nanoparticles (Ag, Cu, Fe, MnO<sub>2</sub>) at a dose of 4.0 mg/kg of body weight on organism of laying hens was established in our previous studies (Orobchenko et al., 2014). It was

characterized by hepato- and nephrotoxicity, changes in the proteinogram, expenditure of energy and antioxidant resources with the partial formation of oxidative stress and the development of inflammatory processes with clinical signs of immunosuppression ( $p \leq 0.05$ ), which resulted in conduction of this experiment.

The aim of our study was to assess the effect of a mixture of metal nanoparticles (Ag, Cu, Fe and  $MnO_2$ ) on the histomorphological state of the internal organs and tissues of laying hens.

## Materials and methods

The studies were performed in the Laboratory of Toxicological Monitoring of the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" (NSC "IEVCM"). Experimental studies were carried out on poultry with the respect to a basic principles of bioethics: keeping and carrying out of chickens and their feeding was conducted in accordance with the norms and diets recommended for this poultry cross. Euthanasia was performed by inhalation of chloroform anesthesia (Strasbourg, 1986). Planning and conducting experiments were carried out in accordance with the guidelines "Preclinical study of veterinary medicines" (Kotsiumbas et al., 2006).

Experimental samples of the following metal nanoparticles were used:

- Argentum (initial concentration –  $86.4 \mu\text{g}/\text{cm}^3$  for metal, average size  $31.5 \pm 0.9 \text{ nm}$ );
- Iron (initial concentration –  $3174.0 \mu\text{g}/\text{cm}^3$ , average size  $100.0 \pm 10.0 \text{ nm}$ );
- Manganese dioxide (initial concentration –  $2785.0 \mu\text{g}/\text{cm}^3$ , average size  $50.0 \pm 3.0 \text{ nm}$ );
- Copper (initial concentration –  $2678.0 \mu\text{g}/\text{cm}^3$ , average size  $70.0 \pm 4.0 \text{ nm}$ ).

Prototypes of metal nanoparticles were synthesized by the chemical condensation method through the reducing a corresponding metal salts in an aqueous medium. Above mentioned nanoparticles were standardized by stability and size criteria at the Institute of Biocolloid Chemistry named after F.D. Ovcharenko of the National Academy of Sciences of Ukraine. Argentum nanoparticles were synthesized by the reduction of nitrate ( $\text{AgNO}_3$ ) in reaction with a 1.0% solution of Tannin and a 0.03 N potassium carbonate solution ( $\text{K}_2\text{CO}_3$ ). Iron nanoparticles were obtained by reducing iron chloride (III) ( $\text{FeCl}_3$ ) in reaction with sodium borohydride. Manganese dioxide nanoparticles were synthesized by reduction of potassium permanganate ( $\text{KMnO}_4$ ) in reaction with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Copper nanoparticles were synthesized by reduction of copper sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) in reaction with sodium borohydride. The synthesis was carried out in the alkaline environment. A dextrin solution (30.000-40.000 Da) was used as a stabilizer in 0.9% sodium chloride solution. The average size of the test samples of metal and metal-containing nanoparticles was calculated using the method of laser correlation spectrometry (LCS). Measurements were conducted on laser-correlation spectrometer Zetasizer-3 ("Malvern Instruments Ltd", United Kingdom). The experimental samples of nanoparticles were visualized by transmission electron microscopy using a JEOL JEM-1230 electron microscope (Tokyo Boeki Ltd, Japan).

A test sample of MNPs was prepared based on the above mentioned nanoparticles. This mixture was used as a prototype of a nano nutraceutic for use in poultry production, containing colloidal dispersions of Ag NPs, Cu NPs, Fe NPs and  $MnO_2$  NPs at a concentration of  $100 \mu\text{g}/\text{cm}^3$  for each metal, respectively.

This formulation was selected based on the results of previous *in vitro* studies of nanoparticles, namely experimental samples did not have genotoxic, mutative and membrane-toxic effects (Roman'ko, 2017).

As a comparison, we used a solution of a mixture of salts of the corresponding metals:  $\text{AgNO}_3$ , ( $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ ), ( $\text{MnSO}_4 \times 5\text{H}_2\text{O}$ ) and ( $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ ) at a concentration of  $100 \mu\text{g}/\text{cm}^3$  for each metal in the experiments.

The experiment was carried out to study the sub-chronic effect of the test sample of the MNPs on the organism of the laying hens of the cross Hisex Brown (egg direction of productivity) in the conditions of vivarium NSC "IEVCM". Four groups of laying hens (one control group and three experimental groups) were formed for this purpose. Weight of tested hens was 1.5-1.8 kg, age 365 days, 28 individuals in each group (Table 1).

**Table 1.** Experimental scheme for studying the effect of a composite mixture of metal nanoparticles on the organism of laying hens of the cross Hisex Brown ( $n=112$ ) in the conditions of a subchronic toxicological experiment

Groups	Dose per kg body weight, mg/kg	Terms of study, days			
		15	30	After cessation of administration	
				7	15
		The number of animals for study			
Control group	Distilled water	7	7	7	7
First experimental group	Mixture of metal salts, 0,3 $\mu\text{g}/\text{kg}$ body weight	7	7	7	7
Second experimental group	MNPs, 0,3 $\mu\text{g}/\text{kg}$ body weight	7	7	7	7
Third experimental group	MNPs, 4,0 $\mu\text{g}/\text{kg}$ body weight	7	7	7	7

Hens of the experimental groups (I, II, III groups) was daily exposed for an experimental sample of MNPs and a mixture of salts of the corresponding metals (I group) for 30 days. Nanoparticles were used as feed additives after 15 days of exposure of experimental chickens of all groups on a standard diet (equalizing period).

Solutions of mixtures of metals in nano- and macrodispersed forms were added to compound feeds immediately before feeding and by subsequent thorough mixing. A solution of a mixture of salts of the corresponding metals was administered at a dose of 0.3 mg/kg of body weight to the hens of the first experimental group, MNPs was administered at a dose of 0.3 mg/kg

of body weight (b. w.) (biotic dose) to the second experimental group, and MNPs was administered at a dose of 4.0 mg/kg of body weight (toxic dose) of the third experimental group.

Distilled water was administered to the control group of hens in addition to the combined feed under similar regulations.

The MNPs prepared *ex tempore* every day before being added into the feed.

Solutions of nanoparticles were stored in dark glass in the refrigerator (at 4°C).

All groups of hens were observed for 45 days (main period). The euthanasia of poultry was performed by total exsanguination under conditions of chloroform anesthesia inhalation. It was performed in two time intervals: on the 15<sup>th</sup> and 30<sup>th</sup> day after administration of a solution of experimental sample of MNPs and a mixture of salts of the corresponding metals and after 7 and 15 days of termination of the MNPs and metal salts.

Thereafter the pathological dissection of hens was carried out according to standard techniques (Bello et al, 2012). Pathological changes were studied and recorded during a post-mortem examination. These changes occurred after the exposure to the samples of metal mixtures in nano- and macrodispersed forms on the animal body. Samples of organs and tissues were taken during a postmortem autopsy to calculate weight coefficients and histomorphological studies.

A total number of 784 samples (lungs, heart, kidneys, spleen, liver, trachea and colon) were selected for hisomorphic examinations during the autopsy. Organ samples were fixed in a 10.0% solution of neutral formalin. The material was washed with running tap water to remove the fixing substance. Dehydration and condensation were carried out in alcohols according to a concentration gradient of 70.0, 80.0, 90.0, 96.0 and 100.0% ethanol, alcohol-chloroform and chloroform in order to conduct paraffin filling. Subsequently, histological slices were made to a thickness of 5-7 µm on a rotational microtome MPS-2 according to standard methods adopted in histological studies with further hematoxylin-eosin staining. All histological techniques were performed in accordance to the formulations set out in the guidelines for pathological and microscopic techniques. Histological specimens were investigated using the Axioskop 40/40FL (Carl Zeiss, Germany) followed by microscopic video photography. A quantitative morphometric evaluation was performed with VideoTest-Morphology 5.1 software.

Statistical processing of the results of studies carried out using Statistica 6.0 software (StatSoft Inc., USA). The accuracy of the results was assessed by Tukey test with Bonferroni adjustment.

## Results

There was no change in the general condition of the experimental laying hens during the chronic intake with feed (30 days) of the MNPs sample compared to the solution of the mixture of salts of the corresponding metals and after the termination of its administration (15 days). Chickens were active, well-received feed and water, had a characteristic cross appearance. The comb and earrings were bright, red in color, and the beak was yellowish. The plumage was brown in color, smooth, bright and dense, which fit well to the surface of the body. There were no detected significant changes within the anatomopathological study of hens with respect to the control during the entire period of examination. The third test group of poultry was an exception (MNPs, 4.0 mg/kg b. w.), where a slight swelling in the small and large intestines was found during the administration of the MNPs.

The results of the determination of the internal organ mass coefficients are given in Table 2.

Thus, no significant changes in the mass coefficients were observed in the brain, heart, spleen, and lungs in birds of all experimental groups on the 15<sup>th</sup> day of the experiment with respect to the control group. However, the excess of this indicator in the liver of hens of the third experimental group was 26.5% ( $P \leq 0.05$ ), and a tendency to increase the liver mass coefficients was observed in the first and second experimental groups. The muscle stomach mass coefficient increased in the first experimental group by 43.4% ( $P \leq 0.05$ ) at this time. This coefficient did not differ from the control in the second and third groups. A 17.7% increase of the liver weight ratio ( $P \leq 0.05$ ) was recorded in the first experimental group of poultry on the 30<sup>th</sup> day of microelements administration. Likely changes were not identified in all experimental groups in other organs at this term of the study.

There were no possible changes of mass ratios in the heart, spleen and lungs of hens of the experimental groups 7 days after the cessation of drug administration. However, the brain mass coefficient of hens in the first experimental group exceeded the control by 20.0% ( $P \leq 0.05$ ). The liver and muscle stomach mass ratios were reduced respectively by 18.7% ( $P \leq 0.05$ ) and 41.0% ( $P \leq 0.001$ ) in the second experimental group, whose chickens received MNPs at a biotic dose (0.3 mg/kg b. w.). It was only found a change of the mass coefficient of muscular stomach by 40,4% ( $P \leq 0,01$ ) in the third experimental group (MNPs 4.0 mg/kg b. w.).

It was found that the muscle stomach mass coefficient was reduced by 18.5% ( $P \leq 0.05$ ) and 27.2% ( $P \leq 0.05$ ) at the last stage of the study (15 days after termination of drug administration) in hens of the first and second experimental groups respectively, as well as a decrease of heart coefficient by 14.8% ( $P \leq 0.05$ ) in the third experimental group, respectively. The pathological changes have not been established during histological study of the liver in the control group of hens. The organ is covered by an external capsule of dense connective tissue, which deeply penetrates and divides it into lobules. The hepatic lobule consists of hepatic beams (plates) and sinusoidal capillaries, which radially converge to the central vein. Hepatic beams are built from hepatic parenchymal hepatocyte cells with rounded nuclei. The contacting surfaces of an opposite hepatocytes form the walls of the bile capillary. The layers of hepatocytes are surrounded by sinusoidal capillaries, the wall of which is made of endothelium. A Kupffer cells are located on the inner surface of the capillary especially in the areas of their branching. These cells are connected by their processes to the endothelium. Hepatocytes do not come into direct contact with the blood stream. As a result of the liver histological examination, changes in the morphological structure of the organ have been found in the third group of hens on the 30<sup>th</sup> day after the beginning of the MNPs administration and on the 7<sup>th</sup> day after termination of the administration. An increase in the number of neutrophilic leukocytes in the lumen of blood vessels and in connective tissue

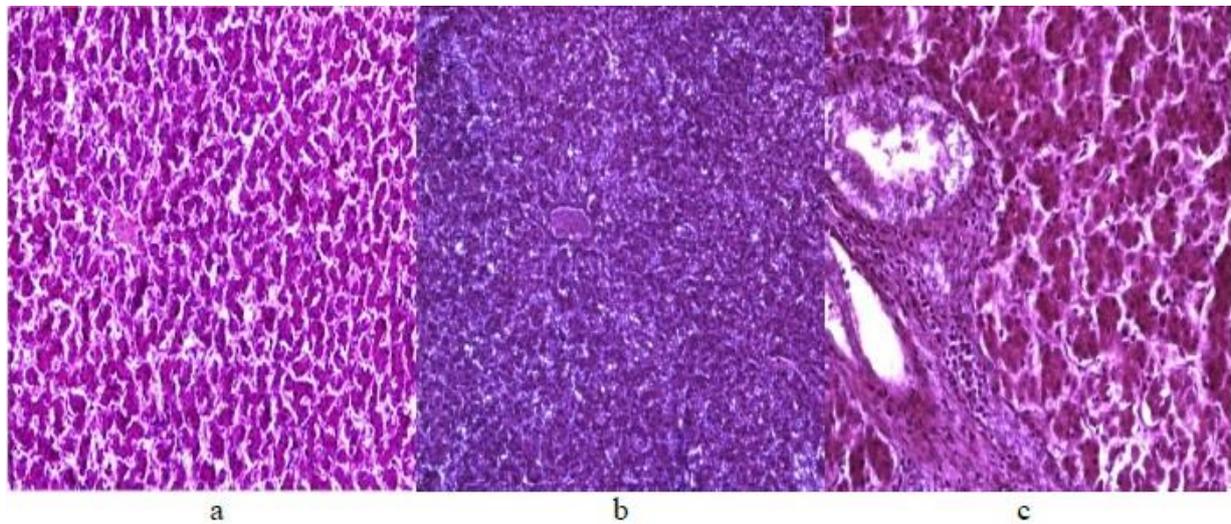
was established during histomorphological examination of the liver of chickens. Epithelial cells of the bile duct were also affected.

According to the results of the histomorphological structure, the liver of hens of the first and second experimental groups was the same and corresponded to the anatomical norm at all stages of the study. More intense changes were observed in the liver of a third experimental group of hens on the 7<sup>th</sup> day after the termination of the MNPs. Dystrophic changes of hepatocytes (Figure 1 a), bile ducts and biliary passage (Figure 1 b) was also noted in addition to those mentioned above. An increase in the number of macrophages was also observed. The walls of the bile ducts are thickened, infiltrated by mononuclear cells, some of the epithelial cells are desquamated, while the intensity of the changes was less than 15 days after the cessation of the administration of MNPs.

**Table 2.** The mass coefficients values of internal organs of laying hens during an alimentary administration of solutions of a mixture of metal salts and MNPs in the dynamics of a subchronic toxicological experiment ( $M \pm m$ ,  $n=7$ )

Term of study	Organ / Groups	Brain	Heart	Spleen	Liver	Lungs	Muscular stomach	
After the start of administration	Control group	0.28±0.02	0.52±0.02	0.15±0.01	2.11±0.22	0.55±0.06	2.95±0.28	
	15 days	First experimental group (salts of metals 0.3 mg/kg body weight)	0.25±0.01	0.48±0.02	0.13±0.01	2.44±0.10	0.43±0.01	4.23±0.37*
	Second experimental group (MNPs, 0.3 mg/kg body weight)	0.24±0.00	0.51±0.03	0.13±0.01	2.48±0.09	0.43±0.01	3.02±0.05	
	Third experimental group (MNPs, 4.0 mg/kg body weight)	0.24±0.01	0.52±0.01	0.15±0.02	2.67±0.02*	0.45±0.04	2.82±0.03	
	30 days	Control group	0.26±0.01	0.47±0.03	0.21±0.02	2.37±0.08	0.54±0.05	3.07±0.17
	First experimental group (salts of metals 0.3 mg/kg body weight)	0.27±0.01	0.45±0.03	0.16±0.02	2.79±0.12*	0.60±0.10	3.22±0.19	
	Second experimental group (MNPs, 0.3 mg/kg body weight)	0.25±0.00	0.49±0.02	0.14±0.03	2.35±0.11	0.49±0.05	2.90±0.37	
	Third experimental group (MNPs, 4.0 mg/kg body weight)	0.26±0.01	0.46±0.02	0.16±0.02	2.31±0.18	0.52±0.01	3.21±0.19	
	7 days after	Control group	0.25±0.01	0.49±0.05	0.10±0.01	3.10±0.23	0.48±0.03	4.26±0.27
	First experimental group (salts of metals 0.3 mg/kg body weight)	0.30±0.02*	0.52±0.03	0.12±0.01	2.89±0.19	0.55±0.06	3.63±0.30	
	Second experimental group (MNPs, 0.3 mg/kg body weight)	0.25±0.01	0.43±0.02	0.11±0.01	2.52±0.04*	0.58±0.06	2.51±0.05***	
	15 days after	Third experimental group (MNPs, 4.0 mg/kg body weight)	0.24±0.00	0.48±0.02	0.12±0.01	2.72±0.19	0.49±0.04	2.54±0.28**
Control group	0.27±0.01	0.54±0.02	0.12±0.03	2.74±0.06	0.43±0.02	3.68±0.16		
First experimental group (salts of metals 0.3 mg/kg body weight)	0.24±0.01	0.50±0.02	0.17±0.03	2.59±0.14	0.45±0.04	3.00±0.23*		
Second experimental group (MNPs, 0.3 mg/kg body weight)	0.25±0.02	0.50±0.03	0.12±0.01	2.79±0.25	0.44±0.01	2.68±0.07*		
Third experimental group (MNPs, 4.0 mg/kg body weight)	0.26±0.02	0.46±0.02*	0.16±0.03	2.57±0.09	0.47±0.02	3.10±0.37		

Notes: \* -  $P \leq 0.05$ ; \*\* -  $P < 0.01$ ; \*\*\* -  $P < 0.001$  - compared with the control group.



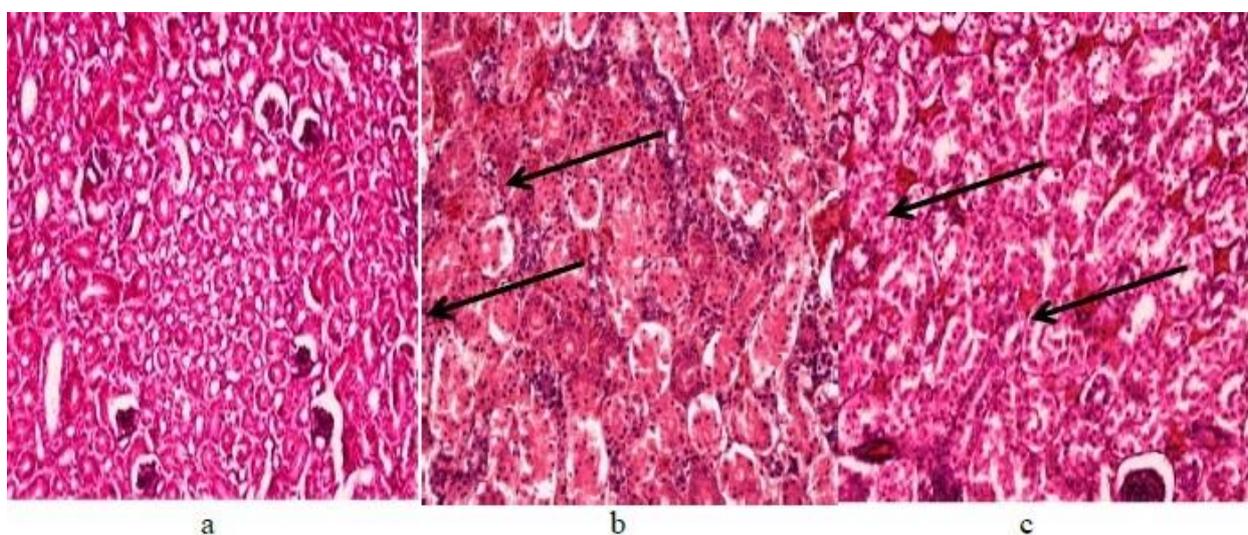
**Fig. 1.** Histomorphological changes in the liver of experimental laying hens when exposed to a toxic dose of MNPs, 4.0 mg/kg body weight (third experimental group). **a.** Control, **b.** Granular dystrophy of hepatocytes, **c.** Lesions of epithelial cells of the bile duct G + E,  $\times 100$ .

The organ was surrounded by a thin connective tissue capsule in a histological study of the kidneys of hens in the control group. Nephron is a structural and functional unit of the parenchyma, which consists of the renal corpuscle, urinary and collecting tubules. The renal body (malpighian body) is the initial section of the nephron and consists of the Bowman's capsule and the renal glomerulus. The renal corpuscle has a vascular pole, the area of which includes the afferent arterioles and efferent arterioles and the urinary pole exit, from beginning of the tubule. Glomerulus is a complex of arterio-arterial anastomoses. The urinary tubules are covered with high prismatic epithelium. The granularity that is contained in the protoplasm of cells closer to its apical part gives the protoplasm a cloudy appearance. Small collecting ducts covered with a low prismatic light epithelium. A granularity is absent in their protoplasm, and nuclei have a circular shape. Epithelial cells become higher and prismatic as the diameter of the collecting tubule increases.

As a result of a histomorphological study of kidneys of chickens of the first experimental group, it was found that their structure in all terms of study did not deviate from the anatomical norm.

It was found granular dystrophy of the epithelium of the urinary tubules in the kidneys of a second experimental group of hens 30 days after a start of the experiment which received MNPs in a biotic dose. The cytoplasm of epithelial cells was cloudy, the boundaries of cells were indistinguishable, the nuclei were differed in size, some of them showed signs of pycnosis. Signs of inflammation of the urinary tubules were observed. The wall is fluffed and infiltrated with mononuclear cells.

By this time, alterative changes were also observed (Figure 2 a) which had a more pronounced character of manifestation in the kidneys of the third experimental group of hens exposed to MNPs at a toxic dose. Thus, granular dystrophy of the epithelium of the urinary tubules and necrosis were recorded during the study. The lumens of individual tubules were filled with desquamated epithelial cells. A noted presence of hyperemia and hemorrhages indicates a hemodynamic disorder (Figure 2 b).



**Fig. 2.** Histomorphological changes in the kidneys of the studied laying hens under the influence of a toxic dose of MNPs, 4.0 mg/kg of body weight (third study group). **a.** Control; **b.** Alterative changes in the epithelium of the urinary tubules; **c.** Hyperemia of the microvasculature of the kidneys G + E,  $\times 100$ .

Hyperemia of the microvasculature and dystrophic changes were expressed in epithelial cells in the kidneys of hens of the third experimental group on the 7<sup>th</sup> day after the termination of the MNPs administration.

Dystrophic changes of the epithelial cells of the urinary tubules were established during the study of the kidneys of hens of this experimental group on the 15<sup>th</sup> day after a termination of the MNPs administration. There was an intensive infiltration with lymphoid cells, macrophages, granulocytes of connective tissue around the vessels.

It should be noted that histological changes were not observed in the kidneys of the first and second experimental groups of hens on the 7<sup>th</sup> and 15<sup>th</sup> days after the termination of trace element mixtures administration.

A depletion of the lymphoid structures of the white pulp was established as a result of a histomorphological study of a hen's spleen in all experimental groups which was set at the end of the experiment (15 days after the termination of the administration of trace element preparations). At the same time, the periarterial lymphoid couplings were narrow and loosely filled with lymphoid cells. Lymph nodules were single in the field of view. The histomorphological structure of the spleen of hens of all groups was at the same morphofunctional level.

The histological structure of the lungs, trachea and large intestine of hens of the first-third research groups in morphological state approached the structure of the organs of hens of the control group at all periods of study.

Histomorphological studies revealed that the following structural changes were found in the liver of hens on the 30<sup>th</sup> day after a start and on the 7<sup>th</sup> day after a termination of administration the MNPs in a toxic dose: an increase in the number of neutrophilic leukocytes in the lumen of blood vessels and in connective tissue, thickening of the walls and lesions of epithelial cells of the bile duct, an increase in the number of macrophages, desquamation of some epithelial cells. The above mentioned facts indicate a presence of degenerative changes in hepatocytes, bile ducts and biliary passages.

Histomorphological changes were found in the kidneys of hens who received MNPs in a toxic dose up to the 7<sup>th</sup> day after the termination of an administration. They were expressed in the presence of granular dystrophy of the epithelium of the urinary tubules with elements of necrosis as well as signs of inflammation of the urinary tubules. It was accompanied by hyperemia and hemorrhages.

Isolated cases of granular dystrophy of the epithelium of the urinary tubules were observed in the kidneys of hens who received MNPs in a biotic dose 30 days after the beginning of the experiment in contrast to hens who received metal salts.

The histomorphological structure of lungs, tracheal wall and large intestine of hens of all experimental groups was at the same morphofunctional level with a control group.

## Discussion

The mucous membrane of the digestive tract is the first barrier of the poultry's body that comes into direct contact with nanoparticles given the oral route of MNPs administration. As it can be seen from the results of studies, the MNPs did not cause inflammatory reactions from the digestive tract, either during the thirty-day administration or after the termination of administration at doses of 0.3 and 4.0 mg/kg of body weight, which indicates its high bioavailability and biocompatibility. The obtained data are confirmed a fact, that there are no toxic effects from the digestive tract in rats, which were perorally administered by Argentum nanoparticles with polyvinylpyrrolidone coating at doses of 50, 100 and 200 mg/kg/day for 90 days (Garcia et al., 2016).

The presence of swelling in different parts of the intestines of chickens under the conditions of the MNPs administration in dose 4.0 mg/kg of body weight is possibly associated with a change in the microflora of the digestive tract, which is consistent with a literature data. Thus, mice were fed granulated feed with Ag NP in doses of 46, 460, and 4600 µg/kg of feed (11.4, 114 and 1140 µg/kg, respectively) for 28 days. There were no reported cases of toxicity. However, an increase in the ratio between Firmicutes and Bacteroidetes bacteria has been found, due to an increase in the number of Firmicutes and a decrease in Bacteroidetes (van den Brule et al., 2016). An increase in the number of Firmicutes-type bacteria can lead to excessive gas production due to the fermentation of the intestinal substrate (Rowland et al., 2018). Feeding rats with Cu nanoparticles (40-60 nm) at a dose of 6.5 mg/kg of feed during 5 weeks resulted in inhibition of the enzymatic activity of the intestinal microbiota (Cholewińska et al., 2018).

In addition, a decrease in the weight coefficients of the muscular stomach of experimental groups of hens which receiving MNPs after the cessation of administration was recorded. This can be explained by the prolonged effect of metal nanoparticles on the peristalsis of the digestive tract. As an example, the motor activity index increased and there was also a significant increase in colon contractions in rats who were intragastrically administered cerium nano-dioxide for 10 days at a dose of 3.0 mmol/rat/day (Yefimenko et al., 2015). However, nanoparticles can be able to penetrate the intestinal wall, entering the bloodstream and distributed to all organs and tissues (Fischer & Chan, 2007; Wang et al., 2015), causing various pathological changes in them.

The hepato- and nephrotoxic effects of various metal and metal-containing nanoparticles are shown in the following works. For example, the most typical target tissues for accumulation of Argentum nanoparticles upon repeated administrations were the kidneys and liver (Park et al., 2010), that is consistent with the results of our studies. Shamsutdinova and Derxo (2015) studied the effect of Ag NPs on the body of rats. Aqueous dispersion of Ag NPs in a daily dose of 4.25; 6.61 and 12.81 mg/kg of body weight were added to the drinking water of the animals of the experimental groups for 30 days, respectively. According to the research results, it was found that the oral intake of an aqueous dispersion of Ag NPs into the rat organism had a more significant effect on the level of AST activity in blood plasma, liver and kidney supernatant compared with ALT activity. This fact confirms their participation in mitochondrial functioning and energy exchange. Enzymes shifts are more pronounced in the liver supernatant than in the kidneys, which is associated with a greater participation of this organ in the elimination of metal. Changes in the activity of aminotransferases in the blood as well as in the liver and kidney supernatants are dose-dependent.

The intake of an aqueous dispersion of Ag nanoparticles at a daily dose of 12.81 mg/kg caused membranotoxic effect on kidney cells.

The nephro- and hepatotoxic effect of copper nanoparticles is also evidenced by other data. Thus, Cu NPs 25 nm in size at doses of 312, 625, 1250 and 2500 mg/kg of body weight were administered once perorally to rats of both sexes. Histopathological changes were found in the liver, kidneys and spleen of male rats receiving Cu NPs at a dose of 1250 mg/kg, as well as in female rats receiving nanoparticles at a dose of 2500 mg/kg of body weight. There were detected following changes: mild inflammation and cellular infiltration and enlargement of sinusoids in the liver, mild and moderate inflammation along with cellular infiltration, hyaline inclusions, cell debris in the tubules, tubular dilatation, and glomerular atrophy in the kidneys. The frequency and degree of manifestation of these changes in male rats were higher than in female rats (Lee et al., 2016).

Tang et al. (2019) established hepatotoxicity and a mechanism of action upon subchronic oral administration of equivalent doses of nano copper 50, 100, and 200 mg/kg/day to rats while the development of a feed additive from nano copper. The addition of nano copper (200 mg/kg) resulted in a significant increase in alanine and aspartic aminotransferases in the blood serum, which indicates the presence of oxidative stress and inflammation in the liver, as confirmed by the corresponding histopathological changes. Subchronic administration of the above dose also significantly disrupted the function and structure of the kidneys and led to an increase in oxidative stress and cytokines, which significantly reduced the level of mRNA, protein, and CYP450 s activity (Xu et al., 2018).

The scientists also used Fe nanoparticles with a size of  $70 \pm 10$  nm, 42 male white outbred mice and 18 male white outbred rats. The experiment was conducted in two stages. At the first stage, the animals were divided into 3 groups and received the drug intramuscularly: the first group – at a dose of 7.4 mg/kg, the second – 11.0 mg/kg, the third – 14.8 mg/kg. At the second stage, 2 groups of animals were formed, 6 male mice and 6 rats, who received a Fe nanoparticle solution one-time perorally at a concentration of 5.0 mg/kg. There was a significant increase in the size of the kidneys and the liver compared to the control in mice and rats after oral administration of nanoparticles. After intramuscular injection of Fe nanoparticles the changes in the internal organs (lungs, spleen) were dose-dependent and manifested by signs of circulatory disorders and cell dystrophy. The severity of established changes increased with an increase a dose from 7.4 mg/kg to 14.8 mg/kg. The changes were manifested in the form of impaired blood circulation, cell damage was practically not observed under conditions of oral administration. Morphological changes were not found in the brain matter and in the heart of animals in both stages of the experiment (Navolokin et al, 2011).

Negative effects have been established in a study with a single intragastric administration of an aqueous suspension of nanodispersed MnO with a particle size of 15-29 nm to nonlinear white mice. They are manifested in the form of enlargement and plethora of veins, pathological inclusions in erythrocytes and increased platelet aggregation, the severity and prevalence of which increases with increasing dose (Zaiceva et al., 2012). Histopathological analysis showed that single oral administration of nano MnO<sub>2</sub> (45 nm) to rats caused changes in the liver, spleen and brain at doses of 500 and 1000 mg/kg body weight (Singh et al., 2013).

The increase of the action effect of nanoparticles is evident and a dose-dependent, when we compare the norms of administration of microelements in the form of salts (for metal from 1.2-2.0 (for Cu), 8.0-12.0 (for Mn) and up to 60 (for Fe) mg/kg of body weight) with MNPs used by us (Ag, Cu, Fe, MnO<sub>2</sub>) (biotic dose 0.3 and toxic 4.0 mg/kg body weight for each metal). Less intensive changes in liver and kidney tissues were recorded 15 days after the termination of the administration of MNPs in a toxic dose. Also, the presence of pathological changes in organs hasn't been observed when the biotic dose was administered on the 7<sup>th</sup> day after the termination of the administration of MNPs. Thus, our data on the influence of a mixture of metal nanoparticles on the histomorphological state of the internal organs and tissues of laying hens are consistent with the data about dose-dependent toxicity described in scientific literature worldwide, which has a recoverable nature in poultry. The results of the experiment have made it possible to establish a safe period of a MNPs administration for laying hens, which is 30 days.

Thus, there is a risk for living systems, which can be manifested by the development of various pathological states. The reason for the risk is in the peculiarities of the action of nanoparticles, namely, the ability of easy penetration into all organs and tissues of the body and stimulation of metabolic processes in biotic doses. Therefore, there is a need for certification and approbation of nanometals on a living organism, especially on a productive animal organism at the present stage of nanotechnology development.

## Conclusions

Negative changes were observed in the kidneys only on the 30th day of administration when studying the effect of MNPs (Ag, Cu, Fe, MnO<sub>2</sub>) as a promising feed additive for poultry in a biotic dose (0.3 mg/kg of body weight for each metal). These changes were manifested in the form of granular dystrophy of the epithelium of urinary canals and signs of inflammation of the uriniferous tubules. A restorative character of observed changes has been established, pathological changes were not observed in organs on the 7<sup>th</sup> day after the termination of administration. The results of the experiment made it possible to establish the possibility of using MNPs in feeding. The safe period of administration for laying hens is 30 days.

The histomorphological state of internal organs and tissues was determined under the conditions of administration of MNPs (Ag, Cu, Fe, MnO<sub>2</sub>) to laying hens in a toxic dose (4.0 mg/kg of body weight for each metal). It was found that changes in the morphological structure of the liver and kidneys occurred on the 30<sup>th</sup> day after the beginning and on the 7<sup>th</sup> day after the termination of the administration. There were thickening of the walls and lesions of epithelial cells of the bile duct, desquamation of part of the epithelial cells, the presence of granular dystrophy of the epithelium of the urinary tubules with

elements of necrosis and hemorrhages, which persisted 15 days after cessation of administration with a lower intensity. This indicates dystrophic changes in hepatocytes, bile ducts and urinary tubules.

The obtained data shows the mechanism of the toxic action of MNPs and can serve as one of the diagnostic criteria in the case of poultry poisoning by nanoparticles. The histological structure of the lungs, trachea walls and colon of hens of all experimental groups had no difference from the control group, which indicates the selective effect of nanoparticles in the case of use the MNPs (Ag, Cu, Fe, MnO<sub>2</sub>).

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