Toxic Effects of the Magnesium Nitrate on Liver of the Embryos from Species Gallus Domesticus

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Fertilized chicken eggs (from hybrid ROSS 308), procured from Agricola Bacau – Romania, have undergone incubation, after previously being mechanically cleaned, disinfected with UV rays, numbered, weighed and measured. 3 lots were made from these eggs of which one witness (LM) and two experimental (EG1 and EG2). All these groups were homogeneous in terms of weight, having 59.47 to 62.371 g.The eggs of the two experimental groups were injected 0.1 mL solution of magnesium nitrate. The concentration of the solution was 0.016% for the first experimental group and 0.61% for the second group.In both experimental groups, the injection of the solutions was made after 2, 4, 6 and 8 days after of the incubation process.At the end of the incubation period were studied both embryos and the chicken resulted. They were weighed, measured, evaluated in terms of necropsy, and some of them were killed and dissected to extract some of their body organs, such as heart, liver, stomach, intestinal mass.Histological samples were extracted from the liver and 20 trichromic stained blades (HEA) were prepared by the paraffin sectioning technique.These blades were studied in MO (Leica DM-750) and the following results were obtained: the weight of the chickens hatched from the eggs of LM group was 43.321 ± 1.305 g, representing 70.765± 1.39% of the weight of respective eggs.For EG1A and EG1B groups, the hatched chickens weighed 36.953 ± 3.481g and 36.233 ± 1.267g respectively, these values being reduced by 14.7% and 16.36% as compared to that of chickens from the LM group.For all other experimental groups, the embryos were stopped in different stages of growth HH, with low and very low weights (0.093, 0.0323 g, etc.). At the level of liver tissue were found severe dystrophic processes for the chickens and the embryos of the experimental groups (steatosis, hepatocytes with the destroyed membrane, pyknosis of the hepatocyte nuclei, intracytoplasmic protein coagulation, etc.)

Keywords: magnesium nitrate, embryos, liver tissue

Normally, nitrate and nitrite are found in soil and plants, they being a part of the nitrogen cycle in nature where otherwise a good balance is established. Anthropogenic activities, often unconscious and irresponsible, spoiled the natural balance resulting in massive and extremely dangerous pollution of the environment (water, air, soil, plants), with serious and long-term effects on health and biological integrity of living beings [1]. Thus, the pollution of soil, groundwater and surface waters, plant and air with nitrate and nitrite was produced using intensive, massive and uncontrolled synthetic fertilizers containing nitrogen as well as organic products through inadequate management of other synthetic (pesticides, etc.) or natural pollutants. From water and soil, nitrates accumulate in plants and later they can reach the human and animal body. After their ingestion, nitrates turn into nitrites, under the action of enzymes existing in gastric and intestinal juices, being absorbed into the blood and then exercising their toxic action on many tissues and organs (liver, heart, nervous, glandular) of the animal and human body. Thus, in humans, ingestion of water and foods that contain high amounts of nitrates can cause: hypertension, headache, hives, disorders of the blood circulatory system and thyroid gland, severe cyanosis and even malignancies [2, 3]. At humans, the most sensitive and vulnerable to nitrates and nitrites action are young children (infants), whose body is severely affected. As consequence, digestive disorders can appear (infectious diarrhea), intolerance to certain proteins, decreased ability of the immune defense system, but especially affecting the hemoglobin levels in the blood. Hemoglobin is altered by nitrite, which has as effect the increasing levels of methemoglobin in the blood (methemoglobinemia), resulting in the apparition of the newborn blue syndrome (BBS Blue Baby Syndrome). This

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disease manifests clinically as skin cyanosis (especially at the level of lips and eyelids) and mucous membranes but can eventually lead to infant death [4, 5]. In the animal organism, the toxic action of the nitrates is similar and quite well-known [5, 6].

This kind of studies has been made on many species of fish and mammals at various concentrations of sodium and potassium nitrates [7]. Although the effect of nitrites was well studied, their teratogenic effects are not demonstrated in all cases. As new results of scientific research on this topic are published, the European legislation regarding permissible levels of nitrates in drinking water and food suffers periodic changes, being revised down (10-100mg/L) [6]. In the present study, the possible toxic effect of nitrate magnesium ($Mg(NO_3)$) on avian embryo was investigated by taking into account the following considerations. Nitrate or magnesium nitrate is a magnesium salt with the nitric acid, of white color, soluble in water and in ethanol. It is manufactured and widely used in many fields: agriculture, cosmetics, petrochemical industry, construction materials, etc. In agriculture is a component of complex fertilizers, which are administered in solid ground or in the form of solutions used for leaf spraying crops. In the cosmetics industry, magnesium nitrate is used in the composition of creams (anti-aging), shampoos, hair conditioner and shower gels, as a color stabilizer. At the best of our knowledge, no studies were made about the effect of magnesium nitrate on embryonic or adult organism in any animal species (fish, birds, mammals).

Experimental part

Materials and methods

Different materials and working methods have been used in this study. 96 fertilized chicken eggs from the Bacau International Agricultural hatchery station were used as biological material. The birds, belonging to commercial hybrid for meat ROSS-308, were healthy and they aged 31 weeks. After their reception, they were stored for 3 days and were then exposed to ultraviolet rays, to achieve the sanitation process. On this occasion was performed the first mirage (egg candling), only fertile eggs being retained for the experiment. 3 groups were made up, one witness (control) and two experimental groups (EG1 and EG2). The number of eggs in these three groups varied, depending on the results of the first mirage, being of 8 in the control group and of 20 for each of the two experimental groups. The average weight of eggs from these three lots was close, so it can be admitted that these groups were homogeneous in this regard.

The incubator used in this experiment (MIP-96) has been set for the specific incubation parameters of Gallus domesticus species (at the end of the incubation period the air temperature was decreased from 37.7 to 37.2°C and the humidity ranged between 65 and 75 %), the return of the eggs being done automatically.

Before the starting of the incubation process, the eggs were subjected to a period of pre-incubation. This process is necessary in order to prevent the appearance of cracks in the mineral shell eggs which can lead in time to water loss and eventually to the death of the embryo [8]. The eggs of the experimental groups were injected with a solution of magnesium nitrate prepared in saline solution and having two different concentrations, as follows: for the first experimental group EG1, magnesium nitrate solution had a concentration of 0.016% (0.16 mg/L) and each egg was inoculated with an amount of 0.1 mL solution. To avoid the damaging of the eggs air space, the inoculation was done in the median area of the eggs. A solution of magnesium nitrate with a concentration of 0.61% was inoculated in the second experimental group of eggs (EG2). This concentration is 38.125 times higher than that used for the EG1. The inoculation of the solutions was made at different times of incubation period, namely after 2, 4, 6 and 8 days after the onset of incubation. After inoculation, the orifice was covered with a plug of molten paraffin in order to prevent water loss from eggs. On day 18 of incubation it was performed the second mirage when the degree of embryos development was verified with a candling lamp.

Eggs with dead or undeveloped embryos were removed from the incubator, loosened, and the embryos were studied: they were separated from embryonic annexes (allantois and amnion), were weighed and evaluated in terms of necropsy and after that the liver was extracted. This organ was weighed and tissue samples were taken and stored in a formaldehyde solution of 10%. Also, these embryos were evaluated by the Hamburger-Hamilton method [9] to establish the stage of development in which they were found at their removing from the eggs.

Then the histological samples were processed by using the paraffin sectioning technique and from the collected liver samples were obtained 20 histological slides having on them cross sections through the liver tissue. These sections were stained trichromic with hematoxylin, eosin and methylene blue (H.E.A.). The study of the blades was made using a Leica DM-750 binocular microscope, equipped with digital cameras.

The general appearance of liver tissue was took into account but also a series of tissular and cellular items (Remak cords, hepatic blood vessels, portal area, the condition of ducts bile, the workload of hepatocytes with lipids, the appearance of hepatocytes nuclei, etc.). The data obtained were processed statistical, calculating general indicators such as average, standard deviation, standard error of the mean variant and coefficient of variation. Moreover, percentage comparisons were made between the control group and the two experimental groups.

Results and discussions

In this research our results can be presented and discussed in two stages namely: the results regarding the weight of embryos and offspring and those related to comments on necropsy and the results on the pathological issues of the liver of these embryos.

Regarding the weight of embryos, the data presented in table 1 are very interesting and highlight the destructive effects of magnesium nitrate solution that was injected into them. Thus, the control group had offspring body weight (one hour of their removal from the incubator) of 39.407 g - 50 147 g, and the average statistics value was of 43.321 ± 1.305 g (v = 8.52 %).

For first experimental group (EG1A) chicks were hatched with an average body weight of 36.953 grams; when compared with controls, the weight was reduced by 14.70 %. For the 1B experimental group (EG1B) chicks hatched from those eggs had body weights ranging from 33.842 g to 38.153 g, with an average statistic value of 36.233 ± 1.267 g. Note that the variability of data is reduced in this case because we have selected only the values that were closer (3 out of 5 of the lot/group). Compared to controls, the weight of chickens from EG1B is reduced by 16.36 %. Based on the weight of the eggs they hatched from, chicks have average weight of: 70.765 ± 1.39 % for the control group; 60.724 ± 5.762 % for EG1A; 60.608 ± 2.864 %, for EG1B. In this case the differences between

Specification		MU	n	Statistical indicators			Variation limits	
Studied character	Groups			$\bar{x} \pm s_{\bar{x}}$	s	V (%)	Min.	Max.
Embryos weight	Control group (CG)	gr.	8	43.321±1.305	3.691	8.52	39.407	50.147
	EG 1A*	gr.	5	36.953±3.481	7.784	21.06	24.446	44.880
	EG 1B**	gr.	3	36.233±1.267	2.194	6.05	33.842	38.153
	EG 1C***	gr.	3	1.8925±0.790	1.369	72.34	0.9908	3.4678
	EG 1D****	gr.	2	41.4553	-	-	39.9833	42.9272
	EG 2A*	gr.	2	40.5437	-	-	39.9274	41.160
	EG 2C***	gr.	4	0.357±0.068	0.1362	38.14	0.1564	0.4591
	EG 2D****	gr.	2	31.6562	-	-	25.9635	37.3490

Table 1
STATISTICAL INDICATORS REGARDING THE EMBRYOS WEIGHT

*the inoculation of magnesium nitrate solution was made after 2 days of incubation;
** the inoculation of magnesium nitrate solution was made after 4 days of incubation;
*** the inoculation of magnesium nitrate solution was made after 6 days of incubation;
**** the inoculation of magnesium nitrate solution was made after 8 days of incubation.

Specification		UM	n	Statistical indicators			Variation limits	
Studied character	Groups			$\bar{x} \pm s_{\bar{x}}$	s	V (%)	Min.	Max.
Embryos weight	Control group (CG)	%	8	70.765±1.390	3.9321	5.56	65.905	77.140
	EG. 1A	%	5	60.724±5.762	12.885	21.22	38.302	69.4575
	EG.1B	%	3	60.608±2.864	4.9613	8.19	56.0141	65.8692
	EG.1C	%	3	3.066±1.207	2.0906	68.19	1.587	5.4578
	EG.1D	%	2	70.182	-	-	66.4817	73.8822
	EG.2A	%	2	70.010	-	-	65.2977	74.723
	EG. 2B	%	3	0.951±0.284	0.4927	51.81	0.580	1.510
	EG.2C	%	4	0.574±0.111	0.2212	38.53	0.2543	0.7642
	EG.2D	%	2	51.40	-	-	39.9073	62.8936

Table 2
STATISTICAL INDICATORS REGARDING THE PROPORTION ON EMBRYOS WEIGHT TO THE WEIGHT OF THE EGGS FROM WHICH THEY HAVE HATCHED

the two experimental groups and the control group are of 10.041 % and 10.157 % respectively. For group EG1C, three of the five embryos included in the study stopped growing due to the effects that the magnesium nitrate solution had on embryonic development, so that their weight had very low values, with a statistic average of 1.8925 ± 0.79 g (table 1).

Based on the weight of the eggs from which they hatched, the weight of embryos from this group (EG1C), represents on average only 3.066 ± 1.207 % (table 2). Compared with controls, embryos weight from LE1C is reduced by 95.63%. The experimental group 1D (EG1D) had 5 embryos, but 3 of them stopped growing in 33 HH stage with a body weight between 1.3402 g and 1.4809 g, while the other embryos were developed almost normal, with an average body weight of 39.9833 g and respectively 42.9272 g (45 HH stage). Compared with the control group, EG1D embryos weights are inferior with 50.53 %. For the experimental groups in the second category, which received a solution of 0.61 % magnesium nitrate, the embryos development was severely affected, so that their weight at the end of the incubation process was very low (tables 1 and 2).

Regarding liver weight of chickens and embryos studied, the data in table 3, although disparate, are nevertheless very interesting. Thus, for the control group, this character has varied between 0.8551 g. and 1.6921 g with a statistic average value of 1.256 ± 0.095 g (table 3). From a macroscopic point of view, the liver had normal appearance for only 4 of the 8 chicks hatched. For the rest, the liver presented small areas where the color was changed and some microbleed areas and one of chicken had small areas of degeneration of the liver tissue. The liver's weight of the control group offspring represented 2.938 ± 0.273 % from the live weight of these offspring (table 3). The 1A experimental group chickens liver had an average weight of 1.170 ± 0.075 g. Based on the weight of body, the liver weight represents 3.318 ± 0.461 %. In terms of macroscopic view, the liver showed small areas of discoloration, microbleeds and degeneration of the tissue.

For the EG1B chicks, the liver recorded an average weight of 1.035 ± 0.025 g and the from macroscopic point of view, the situation of this organ was similar to those from the first experimental group. Compared to control group of chickens, EG1B liver weight was by 17.6 % smaller. In the case of EG1C, the embryo development has stopped in early (30-33-37 HH) or very early (1 HH) stages so that their weight was very low (0.0311-3.4678 g.) and the embryos body has not been dissected to extract the liver, due to their small size. In the lot EG1D situation was similar, though recording liver weight average values of 1.2392 grams. The liver weight represented 4.616 % of chick alive body weight (table 3). Compared with control group, the liver weight of the offsprings in this group is higher by 52.35 %, so we can speak of a phenomenon of increase in volume and weight of this organ and we observed also small areas of discoloration and some portions of ulcerations on the liver tissue surface.

Specification		MU	n	Statistical indicators			Variation limits	
Studied character	Groups			$\bar{x} \pm s_{\bar{x}}$	s	V (%)	Min.	Max.
Liver weight	Control group (CG)	gr.	8	1.256±0.095	0.2678	21.32	0.8551	1.6921
		gr from ABV*		2.938±0.273	0.7718	26.27	2.024	4.2940
	EG. 1A	gr.	5	1.170±0.075	0.1677	14.34	0.8942	1.3472
		gr from ABV*		3.318±0.461	1.0319	31.10	2.3129	5.0127
	EG. 1B	gr.	3	1.035±0.025	0.0432	4.17	0.9878	1.0725
		gr from ABV*		2.866±0.146	0.2535	8.85	2.5890	3.0867
	EG. 1D	gr.	2	1.9135	-	-	1.852	1.975
		gr from ABV*		4.61635	-	-	4.6008	4.6319
	EG. 2A	gr.	2	1.2392	-	-	1.2258	1.2526
		gr from ABV*		3.0576	-	-	2.9780	3.1372
	EG. 2D	gr.	2	1.1162	-	-	-	-
		gr from ABV*		2.9886	-	-	-	-

*ABV=Alive Body Weight.

Table 3
STATISTICAL INDICATORS REGARDING LIVER WEIGHT AND ITS PROPORTION FROM CHICKS ALIVE BODY WEIGHT

For all four experimental groups in the second category (inoculation with a magnesium nitrate solution of 0.61 %) we have studied the situation of this organ (liver) is as follows. In EG2A, some embryos had very low body weights, their development being stopped in stages such as 14, 21 and 26HH, but other embryos developed normally, recording values of 1.2258-1.2526 g for liver weight, which represents 3.058 % from total body weight (table 3). Also, we observed microbleeds, especially on the right cranial lobe. EG2B and EG2C embryos have stopped development in stages 18-33 HH and 14-33HH, so body weight was too small to be able to perform dissections. The 2D experimental group (magnesium nitrate solution 0.61 % was administered after 8 days of incubation) had liver weights with 11.13 % lower compared to control group.

Regarding anatomical and histopathological aspects found in the liver, the situation was as follows. The control group had a normal liver tissue histological appearance. The hepatocytes were intact, but we observed a temporary physiological process of steatosis (fig. 1), determined by the fact that intercitoplasmatic vacuoles were full of lipids mobilized from egg yolks. The cellular and nuclear membranes were intact; blood capillaries bile tubules were not harmed (fig. 1). For the experimental groups in the first category (EG1A - EG1D) (0.016 % magnesium nitrate), the aspects of the liver tissue were very different.

Thus, in 1A experimental group, we observed that hepatocytes appeared in the microscopic field with a broken plasmalema and an intracytoplasmic protein clotting phenomenom, which is an aspect of severe hepatotoxicity (fig. 2). We also observed protein filaments and granules inside the tissue due to the fact that hepatocytes had a damaged cell wall. There are also steatosis cases (multiple lipid vacuoles) and some hepatocyte nuclei are pyknotic.

Eosinophils, after they passed outside blood vessels (diapedesis process), were crowded in the perivascular area forming sleeves (or nests), which shows an allergic reaction (fig. 7). Liver tissue was hyperhydrated so that the cells retained a lot of water, and a lot of hemosiderin (a pigment resulting from the degradation of hemoglobin). Besides this, we observed a process of liver congestion (fig. 6).

In group EG1D the liver tissue is severely damaged, dystrophic processes are similar to those seen in the lot LE1A. Thus, we see here the liver congestion (sinus expansion). Fatty liver disease – steatosis is present (fig. 3), carioliza (fig. 5), Kupfer cell destruction, which shows hemosiderin pygment in the cytoplasm. We can also notice lysis to hepatocytes cellular membranes and intracytoplasmic protein coagulation (formation of protein filaments and granules).

For the experimental groups in the second category (EG2A - EG2D) - 0.61 % $Mg(NO_3)_2$, the situation is as follows: besides tissue and cellular toxic aspects (severe hypoxia), which were observed in liver samples from the first series of experiments, we observed some cases of chronic steatosis, meaning the agglomeration of several microvacuole in the cytoplasm of hepatocytes. Besides this pathology aspect we observed the emergence of bile pigments (biliverdin) in the cytoplasm of hepatocytes, pathological aspect known as hepatocellular jaundice (fig. 4).

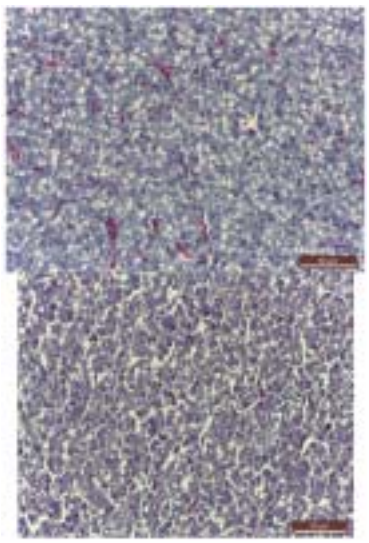

Fig. 1. Physiologic hepatic steatosis (normal aspect) - hepatocytes lipid vacuoles

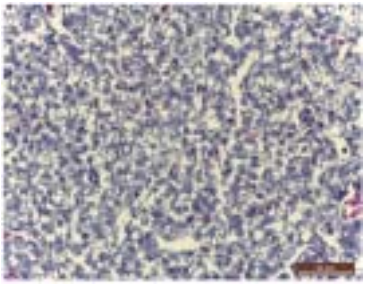

Fig. 2. Severe hepatotoxicity - rupture of cell membranes; intracytoplasmic protein coagulation resulting protein filaments – cellular hypoxia

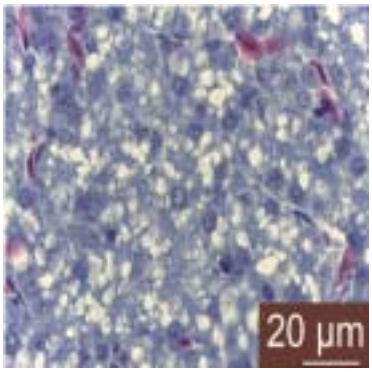

Fig. 3. Chronic steatosis – hepatocytes

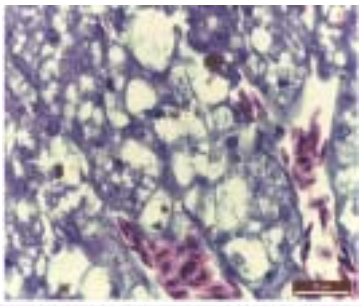

Fig. 4. Bile pigments (biliverdine) (1,2,3,4) with lipidic microvacuoles

Fig. 5. Damage of hepatocytes membranes

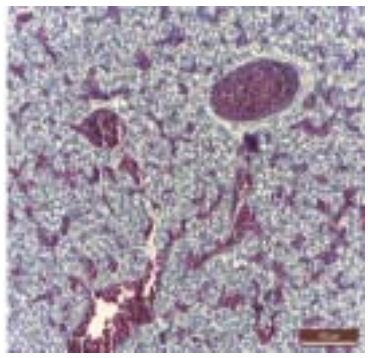

Fig. 6. Hepatic congestion

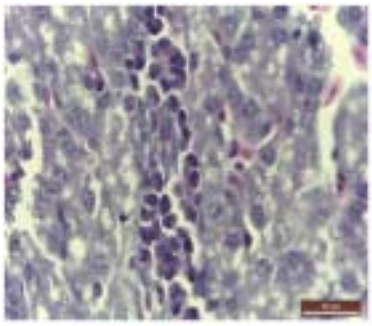

Fig. 7. Eozinocites in perivascular cuffs - allergic reaction

Conclusions

Body weight of embryos and hatched chicks from the eggs studied was drastically reduced after injection of magnesium nitrate solutions.

The most serious adverse effects of magnesium nitrate solutions on the development of body and health of embryos and chickens were observed when solutions were injected at 4 and respectively 6 days after the beginning of the incubation period.

In liver tissue the toxic effect of nitrate magnesium resulted in: chronic steatosis, lysis of hepatocytes membranes, intracytoplasmic proteins coagulation, pyknosis of nucleus of hepatocytes, hemosiderin presence in hepatocytes and between the intracellular spaces, Kupfer cell destruction, hepatocellular jaundice, allergic processes and liver congestion.

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