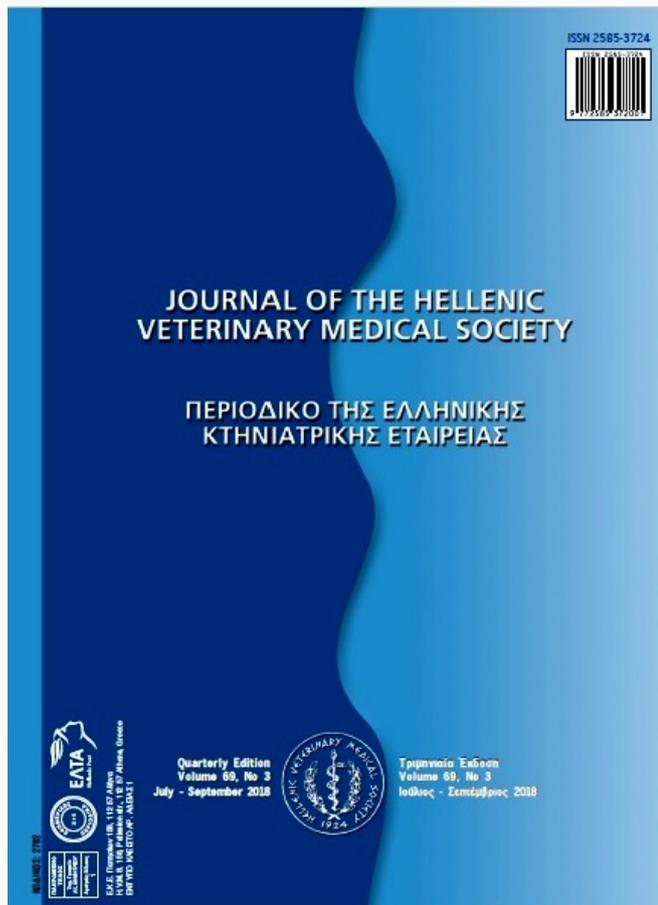


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In ovo hepatocarcinogenicity of N-nitrosodimethylamine and N-nitrosodimethylamine in White Leghorn chickens

**A. Kril¹, A. Georgieva¹, B. Nikolov², R. Pepovich²,
K. Hristov², G. Stoimenov^{2*}, E. Nikolova³, R. Petrova³,
J. Ananiev⁴, Vassil Manov²**

¹ *Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria*

² *University of Forestry, Faculty of Veterinary Medicine, Sofia, Bulgaria*

³ *National Diagnostic Veterinary Research Institute, Sofia, Bulgaria*

⁴ *Trakia University, Faculty of Medicine, Stara Zagora, Bulgaria*

ABSTRACT. Avian embryos have been gaining an increasing scientific interest as a valuable model system for the experimental cancer research that could contribute to a significant reduction of the number of laboratory animals. In the present study, the liver lesions induced by N-nitrosodimethylamine and N-nitrosodiethylamine in 15I line, White Leghorn embryos were identified and studied by routine histopathological methods. Foci of altered hepatocytes with basophilic and eosinophilic phenotype, well known as preneoplastic alterations were identified in the avian embryonal livers after *in ovo* exposure to both N-nitroso compounds. These studies were further extended by histopathological, haematological and biochemical examinations on the effects of N-nitrosodimethylamine in chickens hatched from carcinogen-inoculated eggs. In addition to the preneoplastic lesions observed in the avian livers, proliferations of oval and hepatocellular carcinoma cells, with clearly expressed signs of malignancy were found. The *in ovo* application of the chemical carcinogen was found to affect both hematological and blood biochemistry parameters measured in experimental birds. The established conditions such as thrombocytopenia and increased levels of liver enzymes, as an essential part of the paraneoplastic syndrome, were associated with the process of hepatocarcinogenesis. The results of this study confirm the preneoplastic nature of the focal lesions in embryonal avian liver and their progression to liver neoplastic alterations after a single *in ovo* application of known hepatocarcinogens. Moreover, the results indicate that 15I line, White Leghorn embryos are a new, valuable *in ovo* model for studies on hepatocarcinogenicity of chemical compounds and underline the importance of research on the development of different avian models of carcinogenicity.

Keywords: *in ovo* models, avian embryos, nitrosamines, hepatocarcinogenesis

Corresponding Author:

Department of Infectious Pathology and Food Hygiene, Faculty of Veterinary Medicine, University of Forestry, Kliment Ohridski Street 10, Sofia 1797, Bulgaria.
E-mail: georgi.stoimenov.vm@gmail.com

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INTRODUCTION

Foci of altered hepatocytes (FAH) represent the most prevalent form of hepatic preneoplasia observed in animals for a long time and more recently identified in human chronic liver diseases associated with, or predisposing to, hepatocellular carcinomas (Bannasch et al., 2003; Bannasch et al., 1997; Su et al., 2003). The lesions are composed of morphologically and functionally altered populations of cells that have no obvious neoplastic nature, but indicate an increased risk for the development of benign or malignant liver neoplasms (Bannasch et al., 1996; Bannasch et al., 2003). Sasaki and Yoshida (1935) were the first who described sequential series of cellular alterations, including the appearance of foci of cellular change, preceding the occurrence of rodent liver tumors in experimental studies on chemically-induced carcinogenesis in rats. Since then, the FAH have been the focus of numerous investigations on the early cellular events of hepatocarcinogenesis. The pathobiology of FAH and their relation to hepatic neoplasia have been studied most extensively in rats exposed to chemical carcinogens. Experimental data indicate, that the predominating sequence of cellular events in the hepatocarcinogenesis induced by DNA-reactive carcinogens begins with the appearance of clear cell and acidophilic foci, storing glycogen in excess, followed by their progression to mixed cell foci, composed of acidophilic and basophilic hepatocytes, and then to basophilic, glycogen-poor foci. The later are considered as the most advanced preneoplastic lesion, directly preceding the appearance of hepatocellular carcinomas (Bannasch et al., 1989). In addition to chemicals, other established hepatocarcinogenic agents, such as certain hormones, hepadnaviruses, transgenic oncogenes, *Helicobacter hepaticus* and radiation, have also been shown to induce FAH in appropriate animal models (Bannasch et al., 1996; Bannasch et al., 2003). Moreover, development of FAH has been found in all animal species studied, including primates (Bannasch et al., 1997). Morphological, biochemical, and molecular biological analysis revealed striking similarities in specific alterations of the cellular phenotype of preneoplastic FAH in experimental and human hepatocarcinogenesis, irrespective of the carcinogenic agents by which they was induced. The detection of phenotypically similar FAH in various animal models and in humans prone

to develop or bearing hepatocellular carcinomas favors the extrapolation of data obtained in animals to humans (Georgieva et al., 2012). Consequently, preneoplastic FAH have been widely used as endpoints in carcinogenicity testing, as well as in studies on the molecular mechanisms of early neoplasia (Bannasch et al., 2003; Iatropoulos et al., 2001; Ito et al., 1989; Pitot et al., 1935; Tsuda et al., 2010; Weisburger et al., 1999).

Long-term rodent bioassays have been the regulatory standard for carcinogenicity assessment of industrial and agro-chemicals, food additives, pharmaceuticals and environmental pollutants for over 50 years (Marone et al., 2014). The duration of the *in vivo* carcinogenicity tests in rodents is usually two years. The neoplastic alterations induced by the test chemical in the laboratory animals are the endpoints measured by this experimental approach (Knight et al., 2008; Williams et al., 2008). The obvious negative side of the standard bioassay in rodents is that it is extremely time-consuming and costly, and requires the sacrifice of large numbers of animals. For these reasons, the development of alternative predictive models remains a research priority. In order to shorten the experimental period of the *in vivo* carcinogenicity assays and to minimize the pain and suffering of the laboratory animals a large number of medium-term tests, with an average duration ranging from few weeks to few months, have been developed. These experiments are terminated before the appearance of solid tumors and metastases, and the induced preneoplastic lesions are used as endpoints (Hasegawa et al., 2009; Tsuda et al., 2010). In addition, numerous short-term *in vitro* mutagenicity and genotoxicity tests have been established in an attempt to reduce and/or replace the animals needed for carcinogenicity assessment (Anadón et al., 2014; Benigni et al., 2013). Investigations aimed at the development of alternative models and methods have been gaining an increasing importance since the adoption in 2010 and the implementation in 2013 of the new Directive 2010/63/EC of the European Parliament and the EU Council on the protection of animals used for scientific purposes.

During the last decades, avian embryos have attracted the interest of the scientific community as new and reliable alternative model systems (*in ovo* models) for studies on different pathological processes,

including viral and chemical carcinogenesis. It has been shown that *in ovo* experiments can provide valuable information about the carcinogenic potential of chemical compounds and may fill the gap between the *in vivo* and *in vitro* experiments, combining some advantages of both approaches (9). *In ovo* carcinogenicity assay (IOCA) has been described in detail by Enzmann and Brunnemann, (Enzmann et al., 1997) and the results revealed the appearance of eosinophilic (glycogen-rich, glycogenotic, glycogen-storing foci, basophilic foci of altered hepatocytes and mixed cell foci in the avian embryonal liver. These focal alterations are morphologically identical to those found in rodents and are considered as preneoplastic lesions able to progress to hepatocellular carcinomas, without further exposure to carcinogens. The *in ovo* experiments are more rapid, less expensive and safer for the personnel than the *in vivo* experiments in rodents. In the *in ovo* carcinogenicity studies, turkey and quail embryos were most frequently used as experimental models (Enzmann et al., 1998; Enzmann et al., 1997; Enzmann et al., 1996, Enzmann et al., 1992, Enzmann et al., 2013). The development of preneoplastic FAH was also found in the liver of chicken embryos after treatment with organic (Georgieva et al., 2012) and inorganic carcinogenic chemicals (Kril et al., 2011). Interestingly, identical hepatic lesions were identified in chicken embryos experimentally infected with oncogenic avian retroviruses (Georgieva et al., 2013).

In the present study, the preneoplastic liver lesions in 151 line, White Leghorn embryos, exposed *in ovo* to N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) were examined by histopathological methods. The progression of the neoplastic process, initiated *in ovo*, was studied in chickens, hatched from NDMA-treated eggs. In addition, some haematological and blood biochemical parameters in the experimental birds were followed eighteen weeks post hatching.

MATERIALS AND METHODS

Eggs. Fertilized eggs from 151 line, White Leghorn hens were obtained from diseases-free flock, bred in the animal housing facilities of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, BAS.

Carcinogens and treatment of embryonated eggs.

The tested carcinogens N-nitrosodimethylamine (CAS № 62-75-9; Sigma-Aldrich) and N-nitrosodiethylamine (CAS № 55-18-5; Sigma-Aldrich) were diluted with sterile glass double distilled water and administered as a single dose of 0.3 mg/per egg, with an injection volume of 0.1 mL. Control eggs were injected with an equal volume of the vehicle. The eggs were treated during the first hours of incubation. Briefly, after sterilization of the pointed site with 70% ethanol the test substances were inoculated into the egg albumen and the incubation continued in an automatic rotating incubator at $37.8 \pm 0.5^\circ\text{C}$ and $70 \pm 10\%$ relative humidity. At the end of the incubation, the eggs were transferred to hatcher at $37^\circ\text{C} \pm 0.2^\circ\text{C}$ and 80-85% humidity.

Avian embryos. A total of 93 avian embryos were examined at the 18th embryonic day- 36 treated with NDMA, 32 treated with NDEA and 25 vehicle-treated controls.

Experimental birds. Twelve birds hatched from NDMA-inoculated and control eggs were followed up to 18 weeks post hatching. The treatment and control group consisted of six birds each. Standard fodder mixtures and water were available *ad libitum*. This study was conducted in accordance with the European and National guidelines and regulations for animal welfare.

Histopathology. All experimental birds were exsanguinated 18 weeks post hatching. Tissue samples from control and treated embryos and birds were immediately fixed in 10% buffered formalin for subsequent histopathological examination. The tissues were routinely dehydrated, paraffin embedded, sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Histopathological lesions were observed and documented with microscope Leica DM 5000 B, equipped with a digital camera and original software.

Hematology and blood biochemistry. Venous blood was taken from the wing vein of the treated and control birds at the 13th and 18th week post hatching. Haematological parameters (WBC, $10^9/\text{L}$; LYM, $10^9/\text{L}$; GRA,

$10^9/L$; Hgb, g/L; RBC, $10^{12}/L$; Hct,%; Thr, $10^9/L$) were measured in whole blood by veterinary automatic hematological analyzer Hema Screen 18 LIHD 170, (Hospitex diagnostics – Italy). Biochemical parameters (total protein, g/L; albumin, g/L; ALT, U/L; AST, U/L; GGT, U/L and Glucose, mM) were measured in the blood serum by a semi-automatic biochemical analyzer Screen Master LIHD 113, (Hospitex diagnostics – Italy) and reagent kits for biochemical analyses (Human – Germany).

RESULTS

The alterations of the embryo weight, the absolute and relative liver weight, induced by NDEA and NDMA, were examined as important indicators for the toxic and carcinogenic potential of the tested compounds. The *in ovo* treatment with NDMA and NDEA, induced a statistically significant reduction of the embryo weight ($p \leq 0.001$) and significantly increased the absolute and the relative liver weight, compared to controls (Table 1).

The reduction of the embryo weight was more pronounced in NDEA-treated group and an increase of the absolute liver weight was more prominent in NDMA-treated group. The relative liver weight values for both carcinogen-treated groups were similar.

The gross pathology revealed the presence of well-demarcated reddish-green areas in the livers of carcinogen-treated White Leghorn embryos (Fig. 1a). In the livers of chickens *in ovo*-treated with NDMA grayish-white nodular proliferations (2-4 mm) were found. In addition, bile imbibition and petechial hemorrhages were also present (Fig. 1b).



Fig. 1. Gross pathology of lesions in embryonic liver, induced by N-nitrosodiethylamine (a) and in the livers of 18 weeks old White Leghorn chickens, treated *in ovo* with N-nitrosodimethylamine (b)

The histopathological examination of liver sections from NDMA and NDEA-treated embryos revealed the presence of morphologically distinct foci of altered hepatocytes (Fig. 2). Basophilic FAH were found in embryonic livers after exposure to NDEA. The cells of the altered foci were smaller than the surrounding unaffected hepatocytes and showed an intense cytoplasmic basophilia (Fig. 2 A). Clear and acidophilic foci were the hepatic lesions most frequently found in NDMA-treated embryos (Fig. 2 B). In addition, small, mixed cell foci (composed of basophilic and acidophilic hepatocytes) and small groups of basophilic hepatocytes were detected in sections from embryonic livers of this experimental group. After treatment with both hepatocarcinogens, megalocytes and obstruction of bile ductules by bile plugs were also found (Fig. 2 C, D). Neither of the described lesions were detected in samples from vehicle-treated controls

Histopathology of liver samples from 18 weeks old chickens, *in ovo* treated with NDMA, showed neoplas-

Table 1: Effect of N-nitrosodimethylamine and N-nitrosodiethylamine-treatment on the body weight, absolute and relative liver weights of 15I line, White Leghorn embryos

Treatment groups	Dose (mg/egg)	Number of embryos	Embryo weight (g)	Liver weight (g)	Relative liver weight (%)
Control	0	25	20.31±0.41	0.31±0.01	1.51±0.06
NDEA	0.3	32	13.93±0.37***	0.35±0.01**	2.53±0.12***
NDMA	0.3	36	17.64±0.46***	0.42±0.01***	2.38±0.10***

Values are means ± SD; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ compared to control

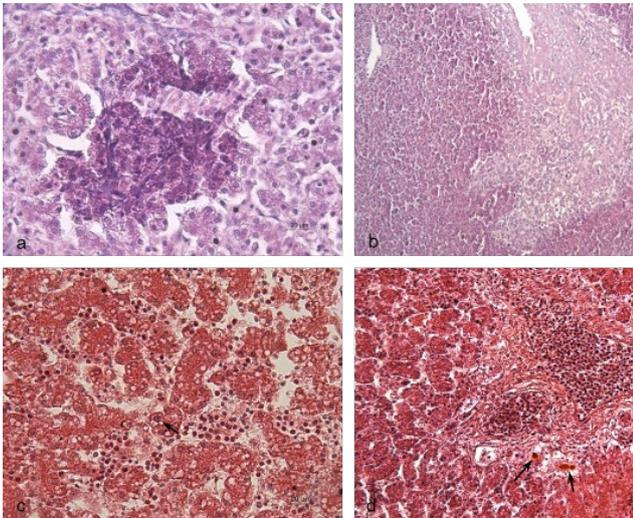


Fig. 2. Histopathology of liver lesions in White Leghorn embryos after *in ovo* exposure to N-nitrosodimethylamine and N-nitrosodimethylamine. A. Basophilic focus of altered hepatocytes; B. Eosinophilic focus of altered hepatocytes; C. Liver megalocytes; D. Bile plugs; NDEA treatment (A, C); NDMA treatment (B, D); H&E; bar=20 μ m.

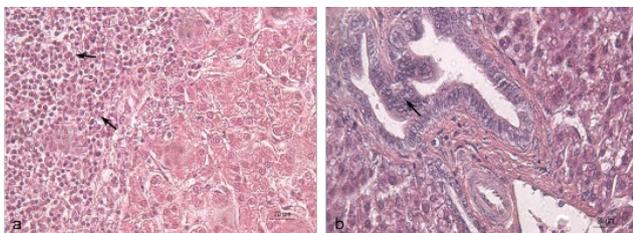
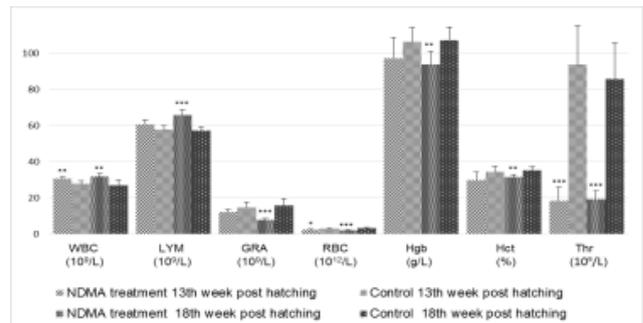


Fig. 3. Hyperplastic and neoplastic lesions in the liver of chickens, *in ovo* treated with N-nitrosodimethylamine. A. Proliferation of oval and hepatocyte-like carcinoma cells; B. Hyperplasia of cholangiocytes; H&E; bar=20 μ m.

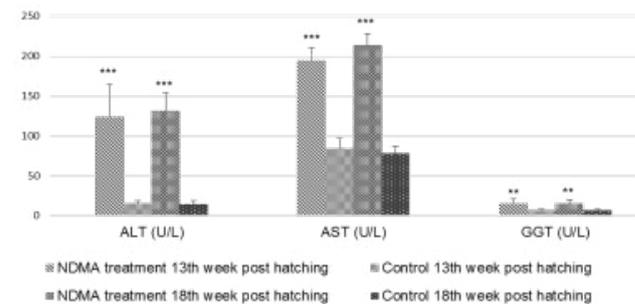
tic alterations, represented by the proliferations of oval cells and hepatocyte-like cells with clearly expressed signs of malignancy (Fig. 3 A). Moreover, hyperplasia of cholangiocytes with pseudopapillary intraluminal projections was frequently found (Fig. 3 B). In addition, preneoplastic changes such as basophilic, eosinophilic and mixed foci of altered hepatocytes were found in the livers of experimental birds. No such lesions were detected in the liver samples from the control birds.

The investigation of the blood samples from experimental birds 13 weeks after hatching revealed significant elevation ($p \leq 0.01$) of the number of the white blood cells, compared to control. An increase of lym-



Values are means \pm SD; * $p \leq 0.05$; ** $p \leq 0.01$;
*** $p \leq 0.001$ compared to control

Fig. 4. Hematological parameters of 151 line, White Leghorn chickens, treated *in ovo* with N-nitrosodimethylamine

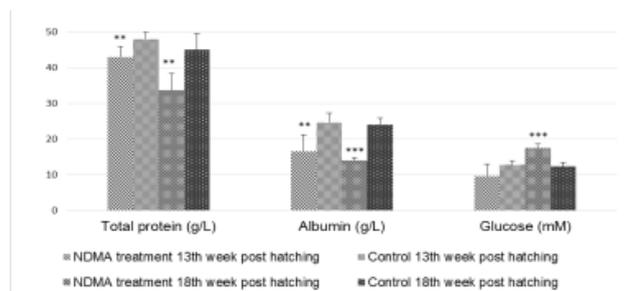


Values are means \pm SD; * $p \leq 0.05$; ** $p \leq 0.01$;
*** $p \leq 0.001$ compared to control

Fig. 5. Blood serum activities of alanine aminotransferase, aspartate aminotransferase and gamma-glutamyl transferase in 151 line, White Leghorn chickens treated *in ovo* with N-nitrosodimethylamine

phocyte count was also noted, without statistical significance. All other tested hematological parameters were lower than the those measured in the control group of birds. However, only the decrease of the values of red blood cells and thrombocytes reached statistical significance (Fig. 4). Eighteen weeks post hatching, the WBC and the LYM values were significantly elevated ($p < 0.01$ and $p < 0.001$, respectively), compared to controls. The other tested parameters were significantly lower, as compared to the controls (Fig. 4).

The results from the biochemical studies showed a statistically significant ($p \leq 0.001$) increase in the levels of the major hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as



Values are means \pm SD; * $p \leq 0.05$; ** $p \leq 0.01$;
*** $p \leq 0.001$ compared to control

Fig. 6. Blood serum concentrations of total protein, albumin and glucose in 151 line, White Leghorn chickens treated *in ovo* with N-nitrosodimethylamine

well as significantly ($p \leq 0.01$) increased activity of gamma-glutamyl transferase (GGT) (Fig. 5).

The total serum protein content was significantly reduced ($p < 0.01$) at both test points. The albumin values measured at 13th and 18th week post hatching were also lower, compared to the controls ($p < 0.01$ and $p < 0.001$, respectively). At the 13th week after hatching the blood sugar values were lower than the control, but the differences were not significant. In contrast, significant elevation ($p \leq 0.001$) of blood glucose was observed 18 weeks post hatching (Fig. 6).

DISCUSSION

The results indicate that the treatment of chicken embryos with NDMA and NDEA induces a pronounced hepatotoxic effect, evidenced by the substantial and statistically significant alterations in the embryo weight and the absolute and relative liver weights as compared to the control. The results obtained revealed that chicken embryos show higher sensitivity to the toxic effects of NDEA, compared to those of the NDMA. A number of studies on the effects of NDEA on turkey and quail embryos have been carried out (Brunnemann et al., 2002; Enzmann et al., 1995; Enzmann et al., 1997; Enzmann et al., 2013; Williams et al., 2011). These *in ovo* experiments showed a significant increase of the absolute and relative liver weights of the carcinogen-treated avian embryos. The changes of the same parameters, established in the present study correspond well with the previously published data.

The identified basophilic and eosinophilic foci of

altered hepatocytes in White Leghorn embryos treated with NDEA are quite similar to the preneoplastic lesions described in embryos of other avian species after treatment with the same carcinogen. The basophilic FAH reported in NDEA-treated turkey embryos, were often composed of intensely basophilic small hepatocytes or large hepatocytes with a diffuse basophilia of the cytoplasm (Enzmann et al., 1995). The results from our study showed exclusively the presence of basophilic foci of smaller altered hepatocytes. Histopathological examination of the liver of chickens, treated with N-nitrosodimethylamine during early stages of embryonic development showed the presence of preneoplastic lesions in all birds studied. These lesions were classified as clear/acidophilic, mixed and basophilic foci of altered hepatocytes. In addition, clearly expressed hyperplasia of cholangiocytes was regularly observed in the liver of experimental birds. Similar histopathological alterations were found previously in quail embryos, *in ovo* treated with hepatocarcinogens (Brunnemann et al., 2002; Enzmann et al., 1996). In addition to the preneoplastic and hyperplastic alterations affecting hepatocytes and cholangiocytes, respectively, the development of neoplastic processes evidenced by the appearance of proliferations composed of oval and hepatocyte-like carcinoma cells were identified in the experimental birds. Similar results were obtained in experiments with line LM, Leghorn chickens exposed to NDEA, at doses ranging from 20 to 100 mg, applied through a drinking water or injection, at the 20th day post hatching. After 24 weeks, macroscopically visible neoplastic lesions were identified and classified histopathologically as hepatocellular carcinomas (Kawaguchi et al., 1987).

The results from the biochemical studies showed substantial and statistically significant increase in the levels of the major hepatic enzymes ALT and AST, as well as significantly increased activity of GGT. Marked hypoproteinemia and hypoalbuminemia, were also registered. Hematological investigations revealed a moderate leukocytosis with lymphocytosis, accompanied by prominent neutropenia and thrombocytopenia. In addition, anemia demonstrated by reduced erythrocytes count, decreased hemoglobin and hematocrit values was found in the experimental group. The blood biochemistry results complement

the observed morphological changes in the liver of experimental birds, showing a significant deterioration of the hepatocytes function and confirm the registered hyperplasia of cholangiocytes. The established elevated values of the key liver enzymes, the hypoproteinaemia, hypoalbuminaemia, the relative anemia and marked thrombocytopenia are not only indicators for general changes in liver function, but they are also an important part of the paraneoplastic syndrome, that accompanies the process of hepatocarcinogenesis.

CONCLUSION

This study shows that *in ovo* exposure to N-nitrosodimethylamine and N-nitrosodiethylamine induces preneoplastic liver lesions in 15I line, White Leghorn chicken embryos, morphologically identical to preneoplastic alterations found after similar treatment in embryonic livers of other avian species. To the best of our knowledge, we are the first to describe the development of neoplastic liver lesions in 15I line, White Leghorn chickens, as a result of the single *in ovo* treat-

ment with hepatocarcinogens. These findings strongly support the statement about the progression of different types of FAH to hepatocellular carcinoma.

We also conclude that 15I line, White Leghorn embryos are valuable model that allows reliable, rapid and inexpensive assessment of the carcinogenic potential of chemical compounds and are strictly in line with the animal protection regulations and ethical aspects of the scientific investigations.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest. ■

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