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HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS DURING THE EARLY STAGES OF N-NITROSODIETHYLAMINE-INDUCED HEPATOCARCINOGENESIS IN TURKEYS

Branimir NIKOLOV[1], Vassil MANOV[1], Roman PEPOVICH[1], Tandzhu MEHMEDOV[1], Kalin HRISTOV[1], Krasimira GENOVA[1], Elena NIKOLOVA[2], Reneta PETROVA[2], Any GEORGIEVA[3], Anton KRIL[3]

[1] University of Forestry, 10 Kliment Ohridski Street, 1756, Sofia, Bulgaria
[2] National Diagnostic Veterinary Research Institute, 15 Pencho Slaveikov bul., Sofia, Bulgaria
[3] Institute of Experimental, Morpfology, Pathology and anthropology with museum, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., bl. 25, 1113, Sofia, Bulgaria

Corresponding author email: br_nikolov@abv.bg

Abstract

Some haematological and biochemical parameters in turkeys, hatched from embryonated eggs inoculated with the proven hepatocarcinogen N-nitrosodiethylamine were studied. Histopathology confirmed the presence of clear-cell and basophilic foci of altered hepatocytes and hyperplasia of cholangiocytes. The application of the chemical carcinogen affected both haematological and biochemical parameters. The established conditions such as thrombocytopenia and increased levels of the major liver enzymes were associated with the process of malignancy. In addition, leukogram abnormalities (leukocytosis, lymphocytosis and neutropenia) as well as hypoproteinaemia, hypoalbuminaemia and hypoglycemia were also observed.

Keywords*: in ovo tests, turkeys, hepatocarcinogenesis, N-nitrosodimethilamine, haematological and biochemical parameters.*

INTRODUCTION

Preneoplastic liver lesions have been widely used as a reliable indicator of the early stages of hepatocarcinogenesis. Sasaki and Yoshida (1935) first showed that the appearance of foci of altered hepatocytes (FAHs) preceded the onset of chemically-induced liver tumors. These preneoplastic alterations have the ability to progress to benign or malignant neoplasms and have been accepted as an early and reliable indicator for the development of the neoplastic process (Howe & Knox 2002). Data from rodent experiments suggest that the hepatocarcinogenesis is a multistage process, beginning 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 preneopastic lesion, directly preceding the appearance of the hepatocellular carcinomas (Bannasch et al., 1989). Preneoplastic foci of altered hepatocytes have been regularly detected in the livers of experimental rodents, as well as in the livers of people with an increased risk for the development of liver tumors (Fischer et al., 1986). It should be noted that the appearance of FAHs precede the development of liver tumors, irrespective of the mechanism by which the carcinogenic process is induced. It is generally accepted that focal liver lesions are a mandatory step in hepatocarcinogenesis and, thus, can be used as reliable endpoints in the carcinogenicity bioassays (Ito et al., 1989). There exist a variety of experimental models in laboratory rodents for the assessment of the carcinogenic, mutagenic and toxic effects of different substances potentially dangerous for both humans and animals. A large part of the experiments have been focused on the mechanisms of liver carcinogenesis (Weisburger, 1999; Iatropoulos et al., 2001; Pitot et al., 2007). The duration of the *in vivo* carcinogenicity tests in rodents is usually 18-24 months (long-term tests). The neoplastic

alterations induced by the test chemical in the laboratory animals are the endpoints measured by this experimental approach (Knight et al., 2006; Williams et al., 2008). In order to shorten the duration of the carcinogenicity assays and to reduce 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 and Ito, 1992; Tsuda et al., 2010). In addition, numerous short-term *in vitro* mutagenicity and genotoxicity tests have been implemented in an attempt to reduce and/or replace the animals needed for carcinogenicity assessment (Benigni, 2013; Anadón, 2014). The ethical aspects of biomedical research and the issues related to the welfare of experimental animals has 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 scientific interest as new and reliable alternative model systems (*in ovo* models) for studies on carcinogenesis. It has been shown that *in ovo* experiments can provide valuable information about the carcinogenic potential of chemical compouds and may fill the gap between the *in vivo* and *in vitro* experiments, combining some advantages of both approaches (Enzmann and Brunnemann, 1997).

The importance of avian embryos as model system for studies on different pathological processes, including biological and chemical carcinogenesis has been growing. *In ovo* carcinogenicity tests have been described in detail by Enzmann et al., (1992; 1995a; 1995b); Enzmann and Brunnemann, 1997 and Enzmann et al., 2012. It has been found that the *in ovo* exposure to chemical carcinogens resulted in the appearance of eosinophilic and basophilic foci of altered hepatocytes in the embryonal avian liver. These lesions are morphologically identical to the FAHs observed in the liver of adult rats, after treatment with hepatocarcinogens. The *in ovo* experiments are more rapid, less expensive and safer for the personnel than *in vivo* experiments in rodents. In the *in ovo* carcinogenicity studies, turkey and quail embryos were most frequently used in the experiments (Enzmann et al., 1992; 1996).

The aim of the present study is to investigate the preneoplastic liver lesions and some haematological and biochemical parameters of turkeys during the early stages of hepatocarcinogenesis, induced after *in ovo* exposure to N-nitrosodimethilamine.

MATERIAL AND METHODS

Eggs

Fertilized turkey (*Meleagris gallopavo*) eggs were obtained from diseases-free flocks, bred in Stara Zagora, Bulgaria.

Carcinogen, treatment and incubation of experimental eggs

N-nitrosodimethilamine (NDMA; CAS № 62-75-9; Sigma-Aldrich) was provided by the Institute of Experimental Morphology, Pathology and Anthropology, BAS - Sofia. The carcinogen was diluted with sterile glass double distilled water and administered as single dose of 0.3 mg/per egg, with an injection volume of 0.1ml. Control eggs were injected with an equal volume of the vehicle. The eggs were inoculated during the first hours of incubation. To avoid cooling, only a few eggs were taken out of the incubator for the application of the test substance. After sterilization of the injection site with 70% ethanol, the shell was pierced at the pointed end of the egg, using a needle, making a hole with a size of 1-2mm. Test substance was inoculated into the egg white, with a syringe and then the opening was sealed with paraffin. The eggs were incubated at 37.8±0.5°C and 70±10% relative humidity in an automatic rotating incubator. At the end of the incubation, the eggs were transferred to hatcher at 37°C ± 0.2°C and 80-85% humidity.

Experimental birds

Eight turkeys hatched from the treated and control eggs were used in the experiments. The treatment and control group consisted of four birds each. Standard fodder mixtures for

turkeys were used for feeding. Food and water were given *ad libitum*.

Histopathology

All experimental birds were exsanguinated 18 weeks post hatching. Tissue samples were taken from the control and treated birds and immediately fixed in 10% buffered formalin for subsequent histopathological examination. Fixed 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 the original software.

Hematology

Venous blood was taken from the wing vein of the treated and control birds at the 14^{th} and 17^{th} week post hatching. Haematological parameters (WBC, 10^9/L; LYM, 10^9/L; MID, 10^9/L; GRA, 10^9/L; HGB, g/L; RBC, 10^{12}/L ; HCT,%; PLT, 10^9/L) were measured in whole blood by Veterinary automatic hematology analyzer Hema Screen 18 LIHD 170, (Hospitex diagnostics – Italy).

Biochemistry

Biochemical parameters (total protein, g/L; albumin, g/L; ALAT, U/L; ASAT, U/L; GGT, U/L, glucose, mmol/L) were measured in the blood serum at the 3^{rd}, 12^{th}, 14^{th} and 17^{th} week post hatching by a semi-automatic biochemical analyzer Screen Master LIHD 113, (Hospitex diagnostics – Italy) and reagent kits for biochemical analyses (Human – Germany).

RESULTS AND DISCUSSION

Histopathological examination revealed the presence of preneoplastic lesions in the liver of the turkeys treated *in ovo* with N-nitrosodimethilamine, during the early stages of the embryonal development. The observed lesions were classified as clear-cell and basophilic foci of altered hepatocytes (Fig. 1). In addition, a clearly expressed hyperplasia of cholangiocytes was regularly found (Fig. 2). Similar histopathological alterations were observed in turkey and quail embryos, after treatment with hepatocarcinogens (Enzmann et al., 1992; 1995a; 1996).

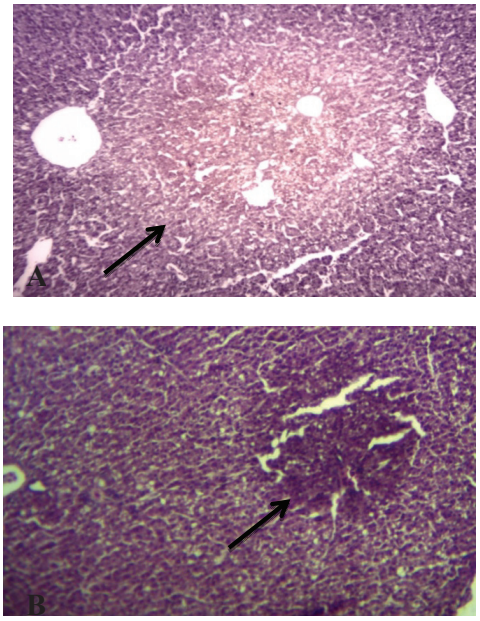


Figure 1. Foci of altered hepatocytes in the liver of turkey, after *in ovo* exposure to a single dose of 0.3 mg N-nitrosodimethilamine. **A** - Clear-cell focus of altered hepatocytes; H&E staining; Objective 20X. **B-** Basophilic focus of altered hepatocytes H&E staining; Objective 20X.

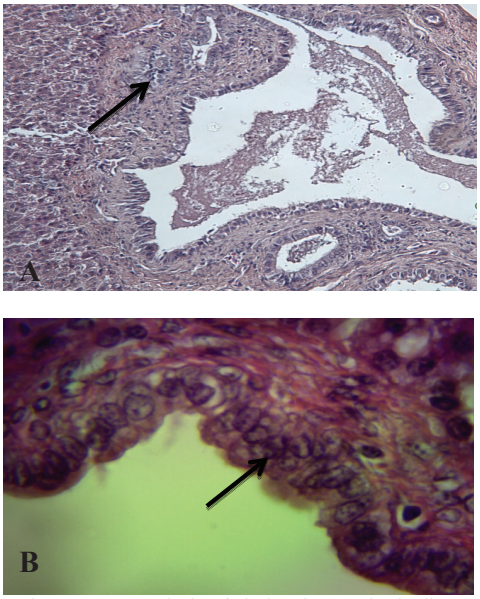
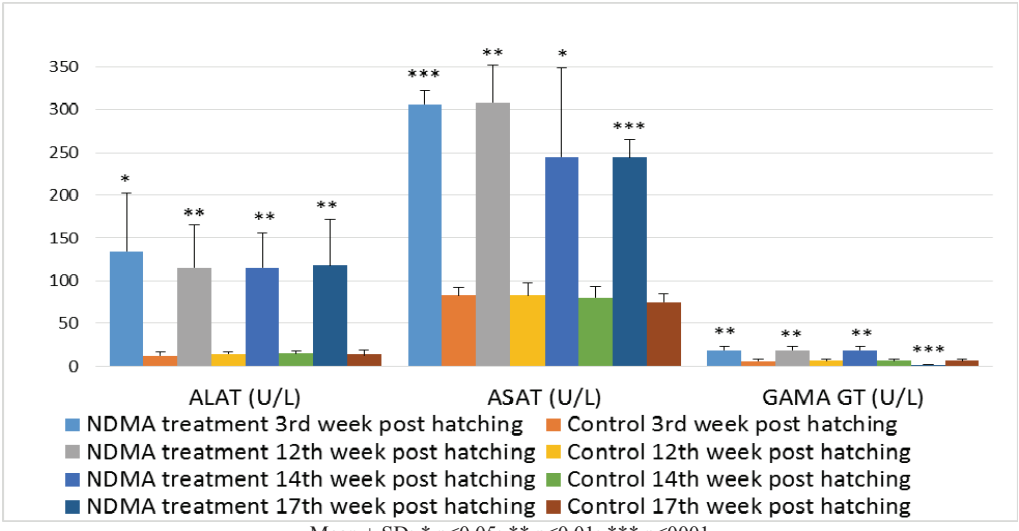


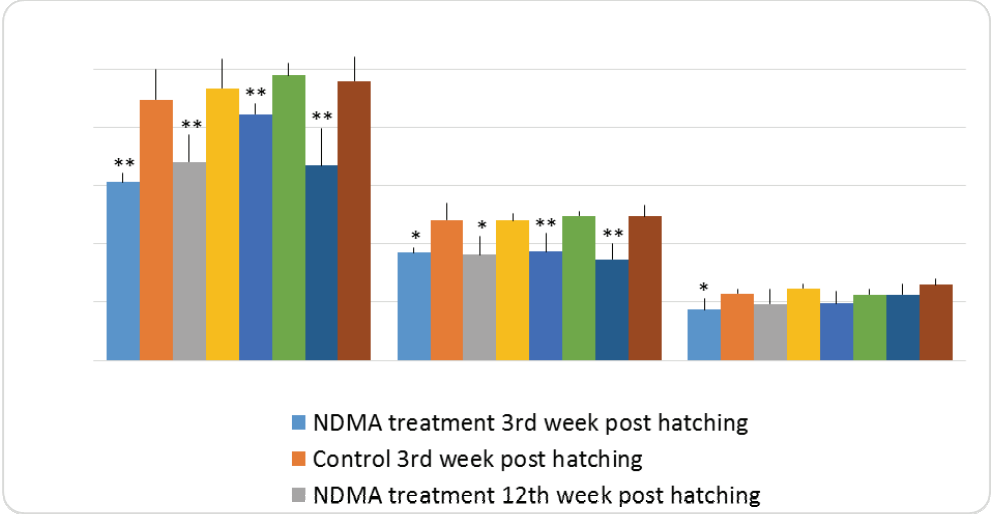
Figure 2. Hyperplasia of cholangiocytes in the liver of turkey, after *in ovo* exposure to a single dose of 0.3 mg N-nitrosodimethilamine. **A** – Hyperplasia of cholangiocytes; H&E staining; Objective 20X. **B** – Hyperplasia of cholangiocytes; H&E staining; Objective 100X.

The results of biochemical studies showed a statistically significant ($p \leq 0.01$; Fig. 3) increase in the levels of the major hepatic enzymes alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT), as well as significantly ($p \leq 0.01$) increased activity of gamma-glutamyl transferase (GGT) (Figure 3). The established hypoglycaemia in experimental birds was statistically significant ($p \leq 0.05$) as compared to the controls only at third week post hatching. Hypoproteinaemia and hypoalbuminaemia with statistical significance ($p \leq 0.01$; Fig. 4) were also registered. Data obtained from the biochemical analysis complement the observed morphological changes in the livers of experimental birds, showing a significant deterioration in the function of hepatocytes and confirms registered hyperplasia of cholangiocytes.



Mean ± SD; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0001$

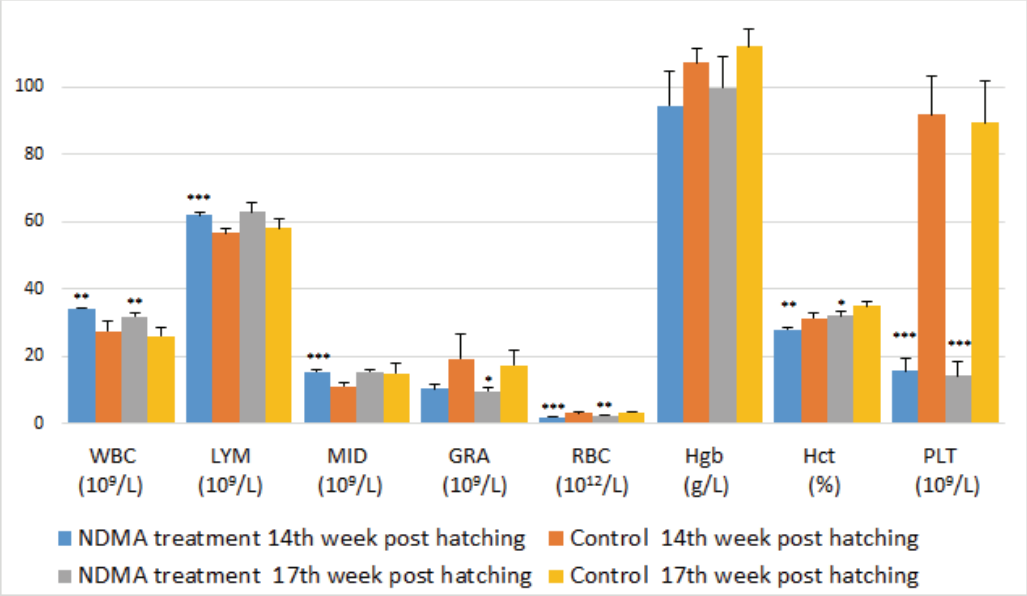
Figure 3. Some biochemical parameters in turkeys, treated *in ovo* with 0.3 mg N-nitrosodimethilamine.



Mean ± SD; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0001$

Figure 4. Some biochemical parameters in turkeys, treated *in ovo* with 0.3 mg N-nitrosodimethilamine.

Haematological investigations revealed a moderate leukocytosis with lymphocytosis, accompanied by neutropenia and prominent thrombocytopenia ($p \leq 0.001$; Fig. 5). In addition, the number of the red blood cells of the birds from the treatment group was significantly lower than those measured in the control group ($p \leq 0.001$; Fig. 5).



Mean ± SD; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0001$

Figure 5. Some haematological parameters in turkeys, treated *in ovo* with 0.3 mg N- nitrosodimethilamine.

CONCLUSION

In ovo application of N-nitrosodimethilamine induced hyperplastic and preneoplastic lesions in the liver of turkeys hatched after treatment of embryos at the early stage of their development.

The results of haematological and biochemical studies are an essential complement to the observed morphological changes in the liver.

Established hypoalbuminaemia, relative anemia and hypoglycemia are not only indicators for the general changes in liver function, but they are also an essential part of paraneoplastic syndrome that accompanies the process of hepatocarcinogenesis.

ACKNOWLEDGEMENTS

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REFERENCES

Anadón A., Martínez M., Castellano V., Martínez-Larrañaga M., 2014. The role of in vitro methods as alternatives to animals in toxicity testing. Expert opinion on drug metabolism & toxicology, 10 (1):67-79.

Bannasch P, Enzmann H, Klimek F, Weber E, Zerban H., 1989. Significance of sequential cellular changes inside and outside foci of altered hepatocytes during hepatocarcinogenesis. Toxicologic Pathology, 17:617–29.

Benigni R., Bossa C., Tcheremenskaia O., 2013. Improving carcinogenicity assessment. Mutagenesis, 28 (1):107-116.

Enzmann H. G., C. Kuhlem, E. Löser, P. Bannasch., 1995b. Damage to mitochondrial DNA induced by the hepatocarcinogen diethylnitrosamine in ovo. Mutat Res., 329(2):113 - 20.

Enzmann H. G., Kuhlem C., Kaliner G., Löser E., Bannasch P., 1995a. Rapid induction of preneoplastic liver foci in embryonal turkey liver by diethylnitrosamine. Toxicologic pathology, 23(5):560 - 569.

Enzmann H. GBrunnemann K. D., 1997. The in ovo carcinogenicity assay (IOCA): A review of an experimental approach for research on carcinogenesis and carcinogenicity testing. Frontiers in Bioscience, 2:30 - 39.

Enzmann H., Brunnemann K. D., Iatropoulos M. and Williams G. M., 1996. Induction of hyperplastic lesions in embryonic quail liver in ovo. Proc. AACR Annual Meeting, 777:20 - 24.

Enzmann H.G., Kaliner B., Watta-Gebert &. Löser E., 1992. Foci of altered hepatocytes induced in embryonal turkey liver. Carcinogenesis, 13:943 - 946.

Fischer G., Hartmann H., Droese M., Schauer A and Bock KW., 1986. Histochemical and immunohistochemical detection of putative preneoplastic liver foci in women after long-term use of oral contraceptives. Virchows Archiv B, 50:321-337.

Hasegawa R., Ito N., 1992. Liver medium term bioassay in rats for screening of carcinogens and modifying factors in hepatocarcinogenesis. Food and Chemical Toxicology, 30 (11):979-992.

Howe M., Knox F., 2002. Pathology of incipient neoplasia (3rd edition). (Eds.Henson and Albores-Saavedra). Surgical Oncology, Oxford University Press, Oxford, 11(3):157.

Iatropoulos M. J., Jeffrey A. M., Enzmann H. G., von Keutz E., Schlueter G., Williams G. M.,2001. Assessment of chronic toxicity and carcinogenicity in an accelerated cancer bioassay in rats of moxifloxacin, a quinolone antibiotic. Exp Toxicol Pathol, 53:345 - 357.

Knight A., Bailey J., Balcombe J., 2006. Animal carcinogenicity studies: implications for the REACH system. Alternatives to Laboratory Animals, 34 (S 1):139–147.

Pitot H. C., 2007. Adventures in hepatocarcinogenesis. Annual Review of Pathology, 2:1 - 29.

Sasaki T, Yoshida T., 1935. Experimentelle Erzeugung des Lebercarcinoms durch Fütterung mit o-Amidoazotoluol. Virchows Archiv, 295:175–200.

Tsuda H., Futakuchi M., Fukamachi K., Shirai T., Imaida K., Fukushima S., Tatematsu M., Furukawa F., Tamano S., Ito N., 2010. A medium-term, rapid rat bioassay model for the detection of carcinogenic potential of chemicals. Toxicologic Pathology, 38:182–187.

Weisburger J. H.,1999. Carcinogenicity and mutagenicity testing, then and now. Mutat. Res., 437(2):105-112.

Williams G.M., Iatropoulos M.J., Enzmann H.G., 2008 Principles of testing for carcinogenic activity. (Ed. Hayes A.W.). In: Principles and methods of toxicology. Taylor and Francis, 1265–316.