

HEPATIC PRENEOPLASIA INDUCED BY N-NITROSODIMETHYLAMINE AND N-NITROSODIETHYLAMINE IN JAPANESE QUAIL EMBRYOS

**Branimir Nikolov¹, Ani Georgieva¹, Roman Pepovich², Kalin Hristov²,
Tandzhu Mehmedov², Vasil Manov², Elena Nikolova³, Reneta Petrova³,
Ivelin Vladov¹, Anton Kril¹**

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 and Research Veterinary Medical Institute, Sofia, Bulgaria*

E-mail: br_nikolov@abv.bg

ABSTRACT

Toxic and carcinogenic effects induced *in ovo* by N-nitrosodimethylamine and N-nitrosodiethylamine in Japanese quail embryos were studied by histopathological methods. The obtained results indicate that both compounds induce preneoplastic hepatic alterations. The spectrum of macroscopic and microscopic lesions identified in carcinogen-treated embryos has been presented. The significance of avian embryos as an inexpensive and reliable model system for studies on hepatocarcinogenesis has been briefly discussed.

Key words: hepatocarcinogenesis, preneoplasia, avian embryos, japanese quail, N-nitrosodimethylamine, N-nitrosodiethylamine.

Introduction

Neoplastic diseases are a serious health problem with a great importance for both veterinary and human medicine. Experiments with laboratory rodents are still the main approach used in the scientific investigations on the factors and mechanisms responsible for the initiation and progression of cancer.

In recent years, issues related to the ethical aspects of biomedical research and the welfare of experimental animals have been gaining an increasing significance. There is a growing interest and a desire for implementation of more reliable, rapid and cost-effective alternative methods to supplement and/or replace animal experiments (Knight et al., 2006; Benigni et al., 2013; Anadón et al., 2014; Marone et al., 2014).

Avian embryos are a model system attracting the attention of experimental oncologists as an alternative to laboratory animals, which provides a multitude of possibilities for exploration of various processes related to carcinogenesis such as genotoxicity, mutagenicity, metastasis, angiogenesis, etc. as well as for assessment of carcinogenic/antineoplastic activity of various environmental factors (Enzmann et al., 1997; Wolf et al., 2008; Enzmann et al., 2013; El Hasasna et al., 2016).

Here, we present results from a study of the ability of the known carcinogenic compounds N-nitrosodimethylamine and N-nitrosodiethylamine to induce preneoplasia in the Japanese quail embryonal liver.

Materials and methods

Avian embryos. Fertilized Japanese quail (*Coturnix japonica*) eggs were obtained from pathogen-free flocks bred in a certified Bulgarian farm.

Chemical carcinogens and in ovo treatment. N-nitrosodimethylamine (NDMA; CAS № 62-75-9; Sigma-Aldrich) and N-nitrosodiethylamine (NDEA; CAS № 55-18-5; Sigma-Aldrich) were dissolved in sterile double distilled water and applied as a single dose of 100µg/egg with an injection volume of 100µL. The eggs were incubated at 37.8 ± 0.5 °C and 70 ± 10 % relative humidity in an automatic rotating incubator. Carcinogens were applied into the egg albumen during the first hours of incubation. Control eggs were inoculated with an equal volume of the vehicle. Four days before hatching, the incubation was terminated and the embryos were weighed and examined for gross lesions.

Histopathology. The livers of the control and of the treated embryos were dissected, weighed and immediately fixed in 10 % buffered formalin. The tissue samples were routinely dehydrated, paraffin embedded, sectioned at 5µm and stained with hematoxylin and eosin (H&E). Histopathological lesions were identified and documented with microscope Leica DM 5000 B.

Statistical analysis. The statistical significance of the differences between the control and the treatment groups was evaluated by GraphPad Prism software package, using one-way analysis of variance (ANOVA) followed by a Bonferroni's post hoc test. Values of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were considered statistically significant.

Results and discussion

Gross pathology revealed a significant reduction of body mass (Tabl. 1) and well-demarcated hepatic lesions in carcinogen-treated embryos. In both treatment groups, the livers were with diffuse reddish-green coloration, occupying in most cases 2/3 of hepatic parenchyma. In addition, multiple petechial hemorrhages were found in some experimental embryos. Gross pathological lesions were observed in 85.7 % and 63.30 % of the embryos treated with NDEA and NDMA, respectively (Fig. 1). 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 (Tabl. 1).

Table 1: Effects of the *in ovo* treatment with N-nitrosodimethylamine and N-nitrosodiethylamine on the embryo and liver weigh.

Experimental groups	Number of embryos	Embryo weight (g)	Liver weight (g)	Relative liver weight (%)
NDEA	14	$5.28 \pm 0.12^{***}$	0.14 ± 0.01	2.54 ± 0.21
NDMA	11	$4.86 \pm 0.09^{***}$	0.15 ± 0.02	2.97 ± 0.30
Control	12	5.76 ± 0.10	0.12 ± 0.01	2.01 ± 0.18

The *in ovo* treatment with both chemical compounds induced a statistically significant reduction of the embryo weight. The absolute and the relative liver weight were increased as compared to the control; however, the established differences were not statistically significant (Table 1).

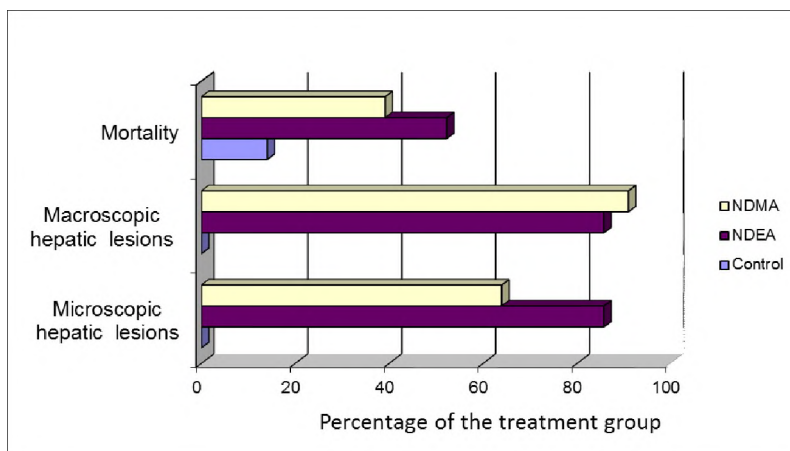


Figure 1: Mortality and hepatic lesions induced by N-nitrosodimethylamine and N-nitrosodiethylamine in Japanese quail embryos

Histopathological examination of the liver sections from NDMA- and NDEA-treated embryos showed the presence of foci of altered hepatocytes (FAHs) with a clear cell and basophilic phenotype (Fig. 2a). The application of both hepatocarcinogens induced spongiosis (Fig. 2b) of liver parenchyma and obstruction of bile ductules by bile plugs (Fig. 2c). In addition, megalocytes and a clearly pronounced hyperplasia of cholangiocytes (Fig. 2d) were found in some carcinogen-treated embryos.

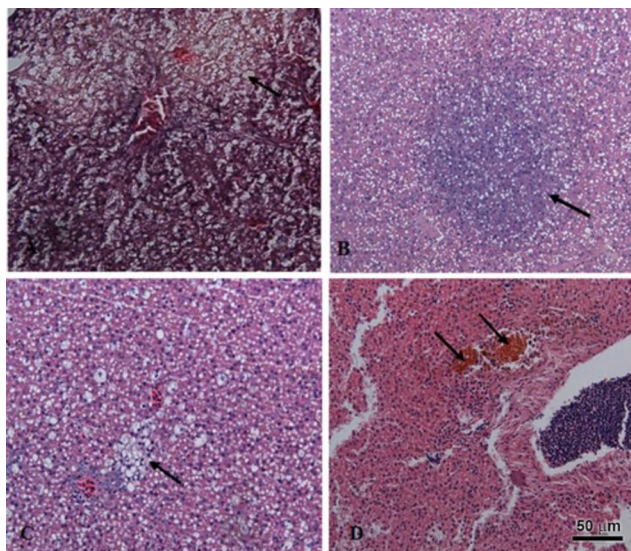


Figure 2: Light microscopy of liver lesions induced by N-nitrosodimethylamine and N-nitrosodiethylamine in Japanese quail embryos

A – Basophilic focus of altered hepatocytes; B – Spongiosis hepatis; C – Bile thrombi; D – Hyperplasia of cholangiocytes; a-,b- in ovo treatment with 100 µg NDMA/ egg; c-,d- in ovo treatment with 100 µg NDEA/ egg; H&E staining; bar = 50 µm.

Our results are in accordance with previous findings (Enzmann et al., 1997), which demonstrate that the application of NDEA induce preneoplastic lesions in Japanese quail embryonal liver. In addition, we were able to demonstrate formation of bile thrombi and marked hyperplasia of cholangiocytes in the livers of NDEA-treated embryos. Identical focal hepatic alterations were identified after the *in ovo* application of NDMA, which confirms the previously published data indicating that the FAHs show similar cellular phenotype irrespective of the carcinogenic agents by which they was induced (Bannasch et al., 2003, Su and Bannasch, 2003).

Foci of altered hepatocytes 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). These preneoplastic lesions 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; Pitot et al., 2007; Tsuda et al., 2010; Enzmann et al., 2013).

Conclusion

The results of the present study indicate that the hepatocarcinogens NDEA and NDMA initiate carcinogenesis in embryonal Japanese quail liver. The fact that preneoplastic hepatic lesions develop within just 14 days highlights the significance of avian embryos as a valuable model system that could contribute for the reduction of animals used in experimental oncology.

Acknowledgement

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