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# N-NITROSODIMETHYLAMINE AND N-NITROSODIETHYLAMINE INDUCED HEPATIC PRENEOPLASIA IN TURKEY EMBRYOS

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**ABSTRACT**

The toxic and carcinogenic effects induced *in ovo* by N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) in turkey embryos have been examined by means of pathoanatomical and 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 and the potential use of avian embryos as an inexpensive and reliable model system for studies on the hepatocarcinogenesis has been discussed.

**Key words:** *in ovo* tests, hepatocarcinogenesis, preneoplasia, avian embryos, 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 turkey embryos.

## Materials and methods

### *Avian eggs.*

Fertilized turkey (Meleagris gallopavo) eggs were obtained from pathogen-free flocks bred in a certified Bulgarian farm. The eggs were incubated at 37.8±0.5°C and 70±10% relative humidity in an automatic rotating incubator.

### *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 300μg; 500μg; 800μg/egg with an injection volume of 100μL. Carcinogens were applied into the egg albumen during the first hours of incubation. Control eggs were inoculated with an equal volume of the vehicle.

***Tested embryos.***

Out of the initial 156 embryos treated with the tested carcinogens, including the control ones, 85 remained alive up to the age required for the study. Pathoanatomical and histopathological examination was performed on these embryos, 13 of which were treated with NDEA 0,3 mg per egg; 12 of which were treated with 0,5 mg per egg; 9 of which were treated with NDEA 0,8 mg per egg; 16 of which were treated with NDMA 0,3 mg per egg; 14 of which were treated with NDMA 0,5 per egg; 11 of which were treated with NDMA 0,8 per egg and 10 of which were control eggs. Embryo incubation was terminated 4 days before hatching after refrigeration at temperature 4°C for 2 hours. All embryos that survived up to 24 days of age were examined.

***Histopathology.***

The livers of the control and 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 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

***Effects of the in ovo treatment with NDEA and NDMA on mortality, bodily mass, absolute and relative liver weight.***

The results of the study reveal that in the NDEA group mortality varies between 45.8 and 62.5%, based on the amount of NDEA used per egg. In the embryos inoculated with NDMA, the ratio also impacts the lethality rate. Groups treated with different concentrations had a mortality rate varying between 33.3 and 54.2%. The mortality rate of the control group was 16.6% (Table 1).

**Table 1: Mortality rate of turkey embryos subjected to NDEA and NDMA *in ovo* treatment**

| Carcinogen used | Dose (mg/egg) | Number of treated eggs | Number of living embryos | Number of dead embryos | Mortality rate (%) |
|---|---|---|---|---|---|
| NDEA | 0.3 | 24 | 13 | 11 | 45.8 |
| | 0.5 | 24 | 12 | 12 | 50.0 |
| | 0.8 | 24 | 9 | 15 | 62.5 |
| NDMA | 0.3 | 24 | 16 | 8 | 33.3 |
| | 0.5 | 24 | 14 | 10 | 41.6 |
| | 0.8 | 24 | 11 | 13 | 54.2 |
| Control | 0 | 12 | 10 | 2 | 16.6 |

The data shown below (Table 2) ascertains a decrease in the bodily mass of embryos treated with either carcinogen, as well as an increase in the absolute and relative liver weight in comparison to the control group. In the NDEA group, a decrease in the weight of the embryos was statistically determined ($p \leq 0.001$) compared to that of the control group. The absolute liver weight in embryos inoculated with NDEA 0.3 and 0.5 mg/egg differs from that of the control group, although that information is statistically unreliable. In the NDEA 0.8 mg/egg group, the absolute liver weight was reliably ($p \leq 0.001$) higher than that of the untreated embryos. An increase in the relative liver weight ($p \leq 0.01$; $p \leq 0.001$) was observed in the groups treated with NDEA 0.3, 0.5 and 0.8 mg/egg. In embryos inoculated with NDMA 0.3, 0.5 and 0.8 mg/egg, a statistically reliable ($p \leq 0.001$) decrease in the weight of the embryos, as well as a reliable increase ($p \leq 0.001$) of the relative and absolute weight of the liver has been determined when compared to the control group.

**Table 2: Effects of NDEA and NDMA on bodily mass, absolute and relative liver weight in turkey embryos subjected to *in ovo* treatment**

| Carcinogen used | Dose (mg/egg) | Number of embryos | Embryo weight (g) Mean±SE | Liver weight (g) Mean±SE | Relative liver weight (%) Mean±SE |
|---|---|---|---|---|---|
| NDEA | 0.3 | 13 | 37.44±0.40*** | 0.72±0.08 | 1.91±0.03** |
| | 0.5 | 12 | 35.98±0.48*** | 0.70±0.01 | 1.93±0.04*** |
| | 0.8 | 9 | 32.22±0.83*** | 0.78±0.01*** | 2.44±0.08*** |
| NDMA | 0.3 | 16 | 35.44±0.49*** | 0.76±0.01** | 2.13±0.05*** |
| | 0.5 | 14 | 33.50±0.41*** | 0.78±0.01*** | 2.34±0.04*** |
| | 0.8 | 11 | 31.92±0.38*** | 0.81±0.01*** | 2.54±0.04*** |
| Control | 0 | 10 | 40.69±0.38 | 0.73±0.01 | 1.77±0.03 |

***Pathoanatomical examination of turkey embryos***

*Macroscopic alterations*

The conducted complete autopsy of turkey embryos, treated with both chemical compounds revealed clearly pronounced macroscopic changes in the liver. They most often consisted of slightly protuberant sections in a greenish red color. Mostly localized in the two liver lobes, the space they took up varied between 1/3 and 2/3 of the liver parenchyma. In most embryos the liver lesions had multiple petechial hemorrhages. In some livers, a clearly pronounced diffuse bile imbibition was found (Figure 1).

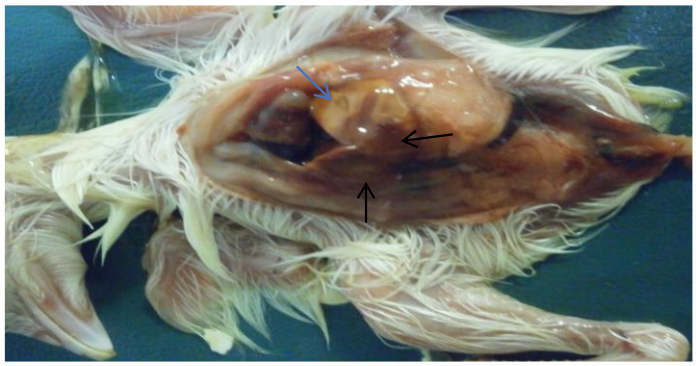

**Figure 1: A macroscopic find in a turkey embryo liver, treated with NDEA 0.5 mg/egg – Greenish red sections containing petechiae, blue arrow – a normal liver parenchyma with a yellow and brown color typical for bird embryos. Black arrows – an altered liver parenchyma with a greenish red color (bile imbibition).**

*Histopatological alterations*

Histopatological examination of the alterations in the NDMA and NDEA inoculated embryos livers showed the presence of foci altered hepatocytes (FAHs) with eosinophilic and basophilic phenotype (Figure 2 A, B). Basophilic foci of altered hepatocytes (BaFAHs) were mostly found in embryos exposed to larger concentrations of the aforementioned carcinogens. The cells of the altered foci were smaller than those of the unchanged hepatocytes and revealed an intense cytoplasmic basophilia (Figure 2 B). Eosinophilic focus of altered hepatocytes (EoFAHs) and mixed foci of altered hepatocytes (MiFAHs) were found in embryos, treated with lower doses of NDEA and NDMA. Moreover, the application both hepatocarcinogens caused separate megalocytes to appear, as well an obstruction of bile ductules by bile plug (Figure 2 C, D).

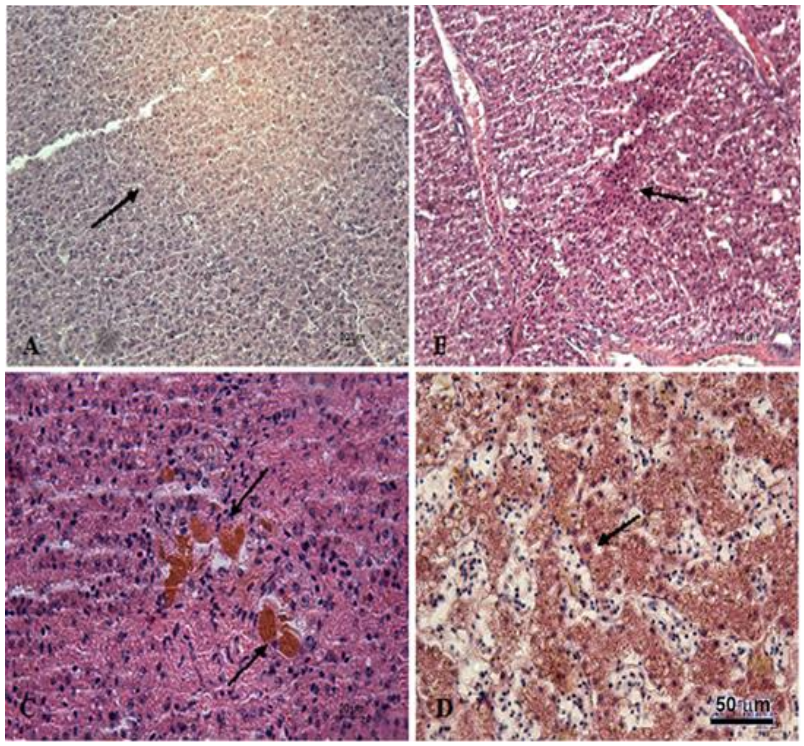


**Figure 2: Light microscopy of liver lesions induced by N-nitrosodimethylamine and N-nitrosodiethylamine in turkey embryos.** ***A. Eosinophilic focus of altered hepatocytes in an embryo in ovo treated with NDMA 0.8 mg/egg, H&E staining; bar = 50 µm; B. Small basophilic focus of altered hepatocytes in an embryo in ovo treated with NDEA 0.8 mg/egg, H&E staining; bar = 50 µm; C. Bile thrombi in an embryo in ovo treated with NDMA 0.5 mg/egg, H&E staining; bar = 50 µm; D. Megalocyte in liver hepatocytes in an embryo in ovo treated with NDEA 0.5 mg/egg, H&E staining; bar = 50 µm***

## Discussion

The study shows that the hepatocarcinogens NDMA and NDEA induce a clearly pronounced embryotoxicity, as well as a hepatotoxic effect, which impacts the mortality rate, the absolute and relative liver weight of the embryos, as well as the weight of the embryos themselves as opposed to the control group. The data reveals that the turkey embryos are more sensitive to the toxic and carcinogenic effects of the NDEA carcinogen. Similar studies regarding the effects of NDEA on

turkey embryos also show statistically significant differences in embryo weight and in absolute and relative liver weight (Williams *et al.*, 2011; Enzmann *et al.*, 2013). Histopatological examination of the liver alterations in turkey embryos inoculated with NDMA and NDEA reveals a dominant presence of altered hepatocytes with an eosinophilic and a basophilic phenotype. Basophilic foci of altered hepatocytes are found mostly in embryos exposed to higher concentrations of the tested carcinogens. BaFAHs were found considerably more often in embryos exposed to NDEA. The cells of altered foci were smaller than those of the intact hepatocytes and revealed an intense cytoplasmic basophilia. The observed basophilic and eosinophilic foci of altered hepatocytes in turkey embryos treated with NDEA are similar to the preneoplastic lesions found in bird embryos after the use of the same carcinogen (Enzmann *et al.*, 1995a; Enzmann and Brunnemann 1997). Eosinophilic and mixed foci of altered hepatocytes were found in embryos treated with NDEA and NDMA in lower dosages. The revealed results in embryos inoculated with NDMA also showed a statistically reliable decrease in the bodily mass of treated embryos and an increase in the absolute and relative liver weight as opposed to the control group. Their macroscopic and histopathological lesions are identical to the ones found in embryos treated with NDEA. In addition, the use of both hepatocarcinogens caused megalocytes, cholangiocyte hyperplasia and an obstruction of bile ductules by bile plug to appear. A number of other similar studies on the effect of NDEA on turkey embryos have been conducted (Enzmann *et al.*, 1995a; 1995b; Williams *et al.*, 2011). In the conducted experiments, the researchers similarly noticed the distinctive decrease in the bodily mass of the embryos and the increase of the absolute and relative liver weight. Williams *et al.* (2011) find not only changes in the weight of the turkey embryos, but the presence of preneoplastic lesions in the form of basophilic foci of altered hepatocytes, megalocytes and cholangiocyte hyperplasia. These preneoplastic lesions have been widely used as endponds 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). The results of our study are considerably similar to those of the aforementioned authors.

## Conclusion

The results of the present study indicate that the hepatocarcinogens NDEA and NDMA initiate carcinogenesis in embryonal turkey liver. The fact that preneoplasic hepatic lesions develop within just 24 days highlights the significance of avian embryos as a valuable model system that could contribute for the reduction of animals used in experimental oncology.

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