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In ovo induced hepatic preneoplasia by n-nitrosodimethylamine and n-nitrosodiethylamine in guinea fowl embryos

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Abstract: Avian embryos have been gaining 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. The toxic and carcinogenic effects induced *in ovo* by N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) in guinea fowl 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.

Keywords*: in ovo* tests; hepatic preneoplasia; avian embryos; guinea fowl; N-nitrosodimethylamine; N-nitrosodiethylamine

INTRODUCTION

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 the 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). 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 compounds 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 a system for studies on different pathological processes, including virus-induced 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., 2013). It has been found that the *in ovo* exposure to chemical carcinogens resulted in the appearance of eosinophilic and basophilic foci of altered hepatocytes (FAHs) 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 are most frequently used as experimental models (Enzmann et al., 1992; 1996; Nikolov et al., 2016; Nikolov, 2024). Here, we present results from a study of the ability of the known carcinogenic compounds N-nitrosodimethylamine and N-nitrosodiethylamine, to induce preneoplasia in guinea fowl embryos.

MATERIALS AND METHODS

Avian eggs

Fertilized guinea fowl (*Numida meleagris*) eggs were obtained from disease-free flock, bred in the animal housing facilities of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, BAS. 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 200µg and 300µg/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 208 embryos treated with the tested carcinogens, including the control ones, 115 remained alive up to the age required for the study. Pathoanatomical and histopathological examination was performed on these embryos, 27 of which were treated with NDEA 0,2 mg per egg; 22 of which were treated with NDEA 0,3 mg per egg; 24 of which were treated with NDMA 0,2 mg per egg; 19 of which were treated with NDMA 0,3 mg per egg and 23 vehicle-treated controls. Embryo incubation was terminated 4 days before hatching after refrigeration at temperature of 4°C for 2 hours. All embryos that survived up to 22 days of age (E 22) 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 the 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 study defined the effect of *in ovo* administration of NDEA and NDMA on guinea fowl embryos. The *in ovo* carcinogens applied in it had concentrations of 0.2 and 0.3 mg/egg. The results of the experiment regarding the embryotoxic and carcinogenic effect of the approved substances in respect of mortality, body weight, absolute and relative liver weight are displayed in tables 1 and 2. The table below shows that the mortality in embryos treated with NDEA 0.2 mg/egg was 40.0%. In the group inoculated with NDEA 0.3 mg/egg, the lethality was 51.1%. The mortality rate observed in embryos exposed to 0.2 mg/egg of NDMA was 46.6%, and in embryos exposed to 0.3 mg/egg of NDMA - 57.7%. The mortality rate seen in the control group was 17.8% (Table 1).

The data shown below (Table 2), ascertain a decrease in the bodily mass of embryos treated with carcinogen, as well as an increase in the absolute and relative liver weight in comparison to the control group. A statistically significant ($p\leq0.001$) embryo weight loss was found in the group injected with NDEA 0.2 mg/egg. Absolute liver weight differed from that of the control group, but with no significant statistical validity. Separately, a significant increase ($p\leq0.001$) in the relative liver weight was witnessed in the same embryos. Embryos treated with NDEA 0.3 mg/egg also presented a statistically significant ($p\leq0.001$) reduced embryo weight and a substan-

Table 1. Mortality rate of guinea fowl 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	0.2	45	27	18	40.0
	0.3	45	22	23	51.1
NDMA	0.2	45	24	21	46.6
	0.3	45	19	26	57.7
Control	0	28	23	5	17.8

Table 2. Effects of NDEA and NDMA on bodily mass, absolute and relative liver weight in guinea fowl 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	0,2	27	12.06±0.22***	0.24±0.02	2.03±0.04***
	0,3	22	11.22±0.20***	0.28±0.02**	2.69±0.07***
NDMA	0,2	24	12.33±0.20***	0.22±0.02	1.91±0.07***
	0,3	19	11.88±0.18***	0.26±0.02*	1.36±0.05***
Control	0	23	14.73±0.18	0.20±0.01	1.36±0.05

*Mean ± SE * p≤0.05; ** p≤0.01; *** p≤0.001*

tial increase (p≤0.01; p≤0.001) in the absolute and relative liver weight, compared to the values found in the control group. In those inoculated with NDMA 0.2 mg/egg, a distinctive reduction in embryo mass was also found with significant importance (p≤0.001). Absolute liver weight fluctuated from that of the control embryos, but statistical significance was not found. In the same embryos a significant increase (p≤0.001) in relative liver weight was ascertained. Embryos treated with 0.3 mg NDMA/egg also displayed statistically significant (p≤0.001) embryo weight loss and significant growth (p≤0.1; p≤0.001) in absolute and relative liver weight, compared to the values found in the control group.

Pathoanatomical examination of guinea fowl embryos

Macroscopic alterations

The conducted complete autopsy of guinea fowl embryos, treated with both chemical compounds revealed clearly pronounced macroscopic changes in the liver. They most often consisted of slightly protuberant sections in greenish-red color. Mostly localized in the two liver lobes, the space they took up to 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).

Histopatological alterations

Histopathological 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

Figure 1. A macroscopic find in a guinea fowl embryo liver, treated with NDEA 0.3 mg/egg (E 22)

Greenish red sections containing petechial hemorrhages. Blue arrow – a normal liver parenchyma with a yellow and brown color typical for bird embryos. Black arrows – an altered liver parenchyma with greenish-red color (bile imbibition).

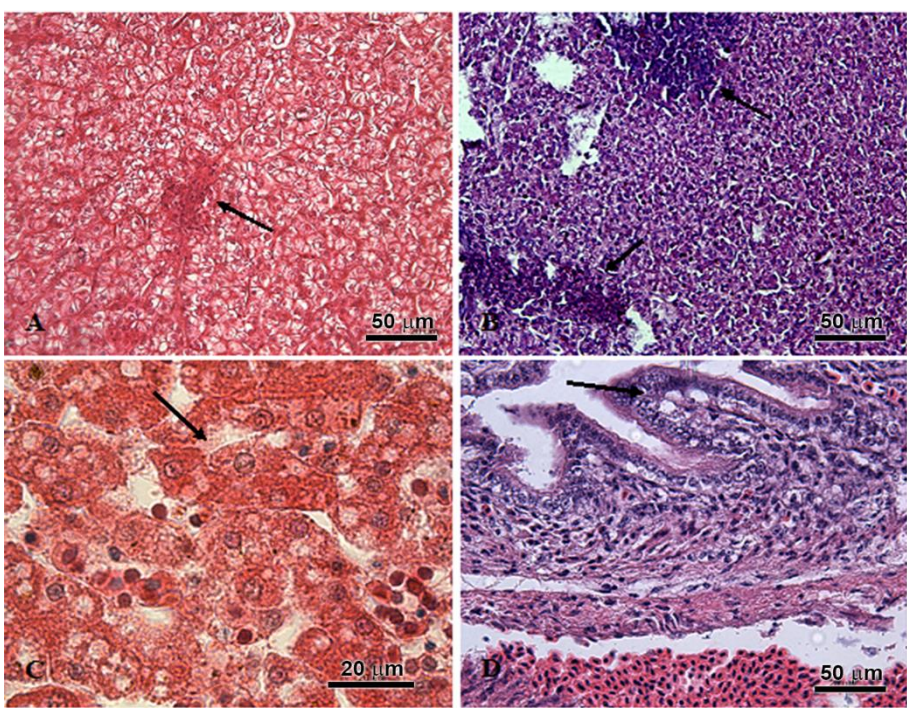

Figure 2. Light microscopy of liver lesions induced by N-nitrosodimethylamine and N-nitrosodiethylamine in guinea fowl embryos (E 22)

A) Eosinophilic focus of altered hepatocytes in an embryo in ovo treated with NDEA 0.3 mg/egg, H&E staining; bar = 50 µm
B) Basophilic focus of altered hepatocytes in an embryo in ovo treated with NDMA 0.3 mg/egg, H&E staining; bar = 50 µm
C) Megalocyte in liver hepatocytes in an embryo in ovo treated with NDEA 0.2 mg/egg, H&E staining; bar = 50 µm
D) Papillary hyperplasia of the bile ducts in an embryo in ovo treated with NDMA 0.2, H&E staining; bar = 50 µm

intense cytoplasmic basophilia (Figure 2 B). Eosinophilic foci 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 of both hepatocarcinogens caused separate megalocytes to appear, as well as papillary hyperplasia of the bile ducts (Figure 2 C, D).

DISCUSSION

The results of the experiments on guinea fowl embryos revealed higher sensitivity to the toxic and carcinogenic effects of NDMA compared to NDEA. The effect of the two chemical carcinogens was expressed in significant differences in the mass of the embryos and their absolute and relative liver weight. The histopathological liver lesion analysis of the treated embryos disclosed the existence of foci of altered hepatocytes with an eosinophilic and basophilic phenotype. Basophilic foci of altered hepatocytes were mainly found in embryos exposed to higher concentrations of the tested carcinogens. They were discovered relatively frequently in embryos exposed to NDMA. The cells of the altered foci were smaller than the intact hepatocytes and indicated intense cytoplasmic basophilia. The basophilic and eosinophilic foci of altered hepatocytes observed in the embryos of grasshoppers treated with NDEA and NDMA were similar to the preneoplastic lesions defined in turkey embryos (Enzmann et al., 1995a; Enzmann & Brunnemann, 1997; Williams et al., 2011; Nikolov, 2024). Light and mixed foci of altered hepatocytes were found in embryos treated with both carcinogens, but at lower concentrations. The results obtained in embryos exposed to NDEA also revealed a significant weight loss of the treated embryos and absolute and relative liver enlargement compared to those of the control ones. The macroscopic and histopathological lesions found in them were identical to those of the embryos treated with NDMA. The administration of both hepatocarcinogens caused the appearance of megalocytes, well-defined papillary hyperplasia of cholangiocytes, and bile duct obstruction resulting from bile thrombi. Similar investigations have been conducted on turkey embryos concerning the toxic and carcinogenic potential of NDEA (Enzmann et al., 1995a; 1995b; Williams et al., 2011; Enzmann et al., 2013). Scientists reported statistically significant differences in the body weight of the embryos and also in their absolute and relative liver weight. In similar fashion, they demonstrated characteristic preneoplastic lesions in the form of basophilic foci of altered hepatocytes and megalocytes, as well as cholangiocytic 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 guinea fowl liver. The fact that preneoplasic hepatic lesions develop within just 22 days highlights the significance of avian embryos as a valuable model system that could contribute to the reduction of animals used in experimental pathology.

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Conflicts of interest

The author declares no conflict of interest.

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