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Inter-laboratory comparison of turkey in ovo carcinogenicity assessment (IOCA) of hepatocarcinogens

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ABSTRACT

In three independent laboratories carcinogens (diethylnitrosamine, DEN, 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, NNK) and non-carcinogens (N-nitrosoproline, nicotine) were evaluated in turkey eggs for in ovo carcinogenicity assessment (IOCA). Compounds were injected into aseptic fertilized eggs. After incubation for 24 days, foci of altered hepatocytes (FAH), some with a pseudoglandular structure and/or signs of compression of the surrounding tissue were observed in the fetal liver. All laboratories were able to distinguish unequivocally the hepatocarcinogen-exposed groups from those exposed to non-carcinogens or the vehicle controls, based on the pre-specified evaluation parameters: tumor-like lesions, pseudoglandular areas and FAH. In addition to focal changes, only the carcinogens induced hepatocellular karyomegaly. Lower doses of the carcinogens, which did not induce FAH, were sufficient to induce hepatocellular karyomegaly. After exposure to 4 mg DEN, gall bladder agenesis was observed in all fetuses. The IOCA may be a valuable tool for early investigative studies on carcinogenicity and since it does not use rodents may complement chronic rat or mouse bioassays. Test substances that are positive in both rodents and fertilized turkey eggs are most probably trans-species carcinogens with particular significance for humans. The good concordance observed among the three laboratories demonstrates that the IOCA is a reliable and robust method.

1. Introduction

Histologically defined, preneoplastic liver lesions are widely used as indicators of hepatocarcinogenesis. Sasaki and Yoshida (1935) were the first to notice that foci of altered hepatocytes (FAH) precede the occurrence of chemically induced liver tumors. They described clear and basophilic cell foci, which have been centers of interest ever since. FAH are regarded as preneoplastic lesions which progress to benign and malignant liver cell tumors through an ordered sequence of phenotypically distinct lesions, leading from clear to basophilic cell foci and finally to hepatocellular nodules and carcinomas (Bannasch et al., 1989). In rodents, FAH have been used widely to study mechanistic aspects of carcinogenesis (Williams and Iatropoulos, 2002; Groos et al., 2007; Pitot, 2007) and/or as endpoints in carcinogenicity testing (for reviews see Enzmann et al., 1998; Tsuda et al., 2003; Bannasch et al., 2003; Tsuda et al., 2010). Since the role of the chronic (2-year) rodent bioassay as the standard in carcinogenicity testing of chemicals (Knight et al., 2006) and pharmaceuticals (Jacobson-Kram, 2010) has been questioned, alternative test strategies and assays are needed (Williams, 2008).

The use of birds in experimental cancer research is even older than the experimental induction of tumors by chemicals. Four years before the first chemical induction of an experimental tumor was described in rabbits (Yamagiwa and Ichikawa, 1915), viral induction of chicken sarcomas was published (Rous, 1911). Subsequently, birds have predominately been used for studies of viral carcinogenesis, but their sensitivity to chemical carcinogens became obvious with the aflatoxin-induced turkey X disease (Lancaster et al., 1961) that resulted in the discovery of the strongest known carcinogens, the aflatoxins.

The *in ovo* carcinogenicity assessment (IOCA) assay using fertilized avian eggs has been proposed as an experimental approach to study chemically induced hepatocarcinogenesis or to detect carcinogenic potential (Enzmann et al., 1992, 1998). Dose dependent effects of genotoxic hepatocarcinogens on hepatocyte nucleus

Abbreviations: FAH, foci of altered hepatocytes; IOCA, in ovo carcinogenicity assessment; DEN, diethylnitrosamine; NNK, 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone.

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morphology (Wiemann et al., 1999) and mitochondrial DNA (Enzmann et al., 1995) have been described after in ovo exposure of embryonic turkey livers. In addition, induction of liver DNA damage by genotoxic agents could be shown by various methods using turkey and chicken eggs (Jeffrey et al., 2011; Williams et al., 2011a). After injection of DEN into turkey eggs, tumor-like lesions, pseudoglandular areas and FAH have been described in the embryo-fetal liver, these histologically detectable changes being proposed to reflect hepatocarcinogenesis (Enzmann et al., 1992).

A prerequisite for a broader use of this experimental approach is the demonstration of the reproducibility in different laboratories. To that end, the IOCA assay was conducted with standard carcinogens and non-carcinogens in different laboratories on different continents.

2. Materials and methods

2.1. Test chemicals

DEN and nicotine were purchased from Sigma–Aldrich (St. Louis, MO, USA). N-nitrosoproline and NNK were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repositories, Midwest Research Institute (Kansas City, MO, USA) for experiments in the USA and from Toronto Research Chemicals, Canada for experiments in the other laboratories.

2.2. Participating research groups

The experiments were conducted in parallel (not necessarily at the same time) by research groups in three different geographic locations: 1. New York: Gary M. Williams, New York Medical College, Valhalla, New York, USA. 2. Kiev: Vasyl F. Chekhun, R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, Kiev, Ukraine. 3. Malcolm A. Moore, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan. As turkey hatching eggs became unavailable in Japan after the first pilot studies, the in ovo part for the Nagoya arm of the study was conducted at BfArM, Bonn, Germany and the paraffin embedded tissue samples were shipped to Japan for processing and histological evaluation.

2.3. Origin of eggs

Fertilized eggs of different turkey strains were used, according to the local availability. For experiments in the USA, hatching eggs of the breeding strain, medium white, were purchased from Nicholas Turkey Breeding Farms (Lewisburg, WV, USA). For experiments in the Ukraine, hatching eggs of the White broad-chested hard cross Beauty-8 strain were purchased from State Enterprise Research Farm "Agroimpex" of the Institute of Poultry Keeping of the National Agricultural Academy of Sciences (NAAS) of Ukraine, Borki village, Zmiivskyy area, Kharkov region, Ukraine. For experiments in Japan/Germany hatching eggs of the strain BIG 6 were purchased from Putenbrüterei Schabrockerhof GmbH, Wachtendonk, Germany.

2.4. Dose selection

There was no mandatory design for the dose finding studies. The justification for the selection of the high dose was based on decreased survival versus the concurrent control group, or decreased embryo-fetal weight versus the concurrent control group, or detection of pseudoglandular areas, tumor-like lesions and FAH (positive result) or severely changed liver histology. Historical control data were used as supportive evidence. In cases of a very steep dose effect curve, half of the dose that resulted in excessive mortality was used as a back-up definition for the high dose. The low dose was ¼ of the high dose. Additional dose groups were at the discretion of the participating laboratories. Calculation of group size was estimated to compensate for decreased survival. For each dose and vehicle control group, no less than 10 samples were planned to be available.

2.5. Administration of test chemicals and incubation

All eggs were numbered on the egg shell. Injection sites 1–2 cm from the pointed end were marked, disinfected using alcohol swabs and pierced with a pointed instrument (e.g. small scissors or truncated injection needle). All test chemicals were dissolved in water, except in the Ukraine where 8% dimethylsulfoxide was employed. Injection volume was 0.2 ml in the USA and Ukraine and 0.5 ml in Germany and Japan. After injection of the test substance or vehicle with a sterile hypodermic needle, the injection sites were sealed with adhesive tape. Eggs were incubated for 24 days at $37.5 \pm 0.5\,^{\circ}C$ and in a 60% relative humidity atmosphere (USA) or at $37.7 \pm 0.3\,^{\circ}C$ in a 65% relative humidity atmosphere (Ukraine) or at $37.8 \pm 0.5\,^{\circ}C$ in a 65% relative humidity atmosphere (Germany/Japan).

2.6. Tissue sampling and histological evaluation

On day 24, the eggshells were opened and the fetuses were removed and decapitated. The abdominal cavity was opened and the liver was removed, weighed and fixed in 10% phosphate buffered formalin (containing 3.6–4.0% formaldehyde). The tissues were processed, embedded, and cut, and slides stained with haematoxylin and eosin (H&E) were prepared for histological evaluation. Microscopic evaluation was performed in a "blinded" fashion, the investigator being unaware of the treatment of the sample using a standardized evaluation sheet. The templates prespecified the following mandatory evaluation endpoints: FAH were described according to cytoplasmic tinctorial changes with the distinction of an increased basophilia (basophilic cell foci), increased eosinophilia (eosinophilic cell foci) or both an increased basophilia and eosinophilia (amphophilic cell foci); FAH exceeding several liver lobules in size with loss of the regular lobular architecture within the FAH and with compression of the surrounding tissue were evaluated as tumor-like lesions. When a tubular or pseudoglandular histological structure of the liver was observed outside well-demarcated FAH or tumor-like lesion these were evaluated as pseudoglandular areas. Single hepatocytes or groups of hepatocytes with strikingly enlarged nuclei and frequently with prominent nucleoli were evaluated as karyomegaly. The templates listed additional findings including hepatocellular pleiomorphism and could be used to report unexpected findings e.g. of the hepatobiliary system in free text.

3. Results

3.1. Interlaboratory concordance

All participating research groups were able to distinguish unequivocally the hepatocarcinogen-exposed groups from those exposed to non-carcinogens or the vehicle controls. Histological correlates of hepatocarcinogenesis, such as tumor-like lesions, FAH and pseudoglandular areas were found in the high dose groups of the carcinogens but were never observed in the non-carcinogen or vehicle control groups in any of the laboratories. Karyomegaly of hepatocytes was found after exposure to DEN or NNK only. This complete concordance between the different laboratories was achieved despite differences in the experimental details (e.g. turkey strains, incubation conditions, doses) and thus clearly demonstrated the robustness of the experimental design (Table 1).