Scientific Works. Series C. Veterinary Medicine. Vol. LX (1)
ISSN 2065-1295, ISSN Online 2067-3663, ISSN-L 2065-1295

IN OVO TESTS FOR CARCINOGENICITY, MUTAGENICITY AND EMBRYOTOXICITY

MINIREVIEW

Branimir NIKOLOV[1], Any GEORGIEVA[2], Vasil MANOV[1], Anton KRIL[2]

[1]University of Forestry, 10 Kliment Ohridski Street, 1756, Sofia, Bulgaria
[2]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

The significance of avian models for studying pathological processes including carcinogenesis, both from a chemical and from a biological viewpoint, has been already clearly demonstrated. The in ovo models appear to be the missing link between the in vitro and the in vivo experiments. This approach has considerable advantages: the tests are rapid, less expensive than animal experiments, less hazardous to the personnel, performing the experiments and they have reliable endpoints. Examples include preneoplastic liver lesions in embryonic avian livers in the In Ovo Carcinogenisity Assay (IOCA) and the induction of micronuclei in embryonic avian erythrocytes in hen's egg test for micronucleus induction (HET-MN). In addition, the use of avian embryos in embryotoxicity testing is discussed.

Key words*: embryonic avian, experimental carcinogencity, foci altered hepatocyte, rats liver foci.*

INTRODUCTION

Neoplastic diseases are one of the biggest problems of mankind. So far many of the causes leading to the development of malignancies have been explored. However, there are still many unproven factors responsible for their occurrence (Doll and Peto, 1981; Schmahl et al, 1989). The mechanisms through which these factors exert their effect are also not fully elucidated. The discovery of new and rapid methods for proving these causes is crucial. Nowadays a large number of experimental models in laboratory rodents for proving carcinogenic, mutagenic and toxic effects of various substances that are potentially hazardous to both people and animals (Weisburger, 1999; Iatropoulos et al., 2001; Pitot et al., 2007). The main carcinogenicity studies have been conducted mainly on rats and mice and are considered by most regulatory agencies worldwide as the" golden standard" (Enzmann et al., 1998a; 1998b). In relation to these studies long-term and short-term in vivo models using different rodents have been developed (Weisburger and Williams, 1984). The short-term tests are the most commonly used and have wide application. They are recognized by various organizations such as the "International Agency for Research on Cancer" (IARC, 1998) and the " International Conference on Harmonization" (ICH, 1997) as potential biological models for proving carcinogens. The two classic prototypes used for short-term carcinogenic tests in rodents are the skin of mice (MST) and the liver of rats with foci (RLF) of altered hepatocytes (FAH). However, experimentally carcinogenesis in other organs and systems as well. The essence of the models is based on early detection of pre-neoplastic lesions in the target organs since it is considered that they have the ability to progress to malignant tumors. In these experiments a number of substances and compounds have been tested in order to prove their potential to induce neoplastic alterations (Williams and Whysner, 1996).

For the welfare of laboratory animals, and for shortening the time for experimentation, in ovo tests on embryos from various birds have been developed. Avian embryos are one of the newest and most promising alternative models of short-term experiments on genotoxicity Tempel et al. (1992) and carcinogenicity

(Enzmann et al., 1995a; Enzmann and Brunnemann, 1997) by testing various chemicals. The conducted in ovo carcinogenicity tests (IOCA) leads to the development of preneoplastic liver lesions that comprise eosinophilic and basophilic foci of altered hepatocytes (FAH). The same authors state that at least one of these foci (basophilic FAHs) have the ability to develop into hepatocellular carcinomas. The significance of avian embryos as successful models for the study of chemical carcinogenesis is confirmed by (Williams et al., 2010). Other researchers, such as Wolf et al. (2007), use avian model to prove the mutagenic effect of various substances after detection of micronuclei in embryonic chicken erythrocytes (HET-MN test).

CARCINOGENICITY MODELS

A large part of the experiments for proving the carcinogenic effect of various compounds have been conducted mainly on rodents. There exist statements that the use of well-defined preneoplastic lesions in various organs of rats and mice as end point in carcinogenicity testing can reduce the period of experimentation under 2 years (Enzmann et al., 1998b). The use of rodents in the experimental chemical carcinogenicity models is of great importance based on the similarity in the pathogenesis of different types of cancer in humans and used experimental animals (Bannasch 1986; 1986b). According to Weisburger and Williams (1984), there is an increasing interest and usage of preneoplastic lesions in rodents as an early indicator of the carcinogenic activity of various substances and similar approach was used by Jacobson-Kram (2010) for tested the effect of various pharmaceutical products on rodents. In experimental models with laboratory animals for proving mutagenicity and carcinogenicity a variety of chemicals have been tested, whereas the most commonly used were nitrosamines (Williams et al., 1993; Enzmann et al., 1995). The two most commonly used prototypes of short-term carcinogenesis in rodents are the skin of mice (MST) and the liver of rats with foci (RLF) of altered hepatocytes (FAH). Leading research on the carcinogens that cause skin tumors in mice was conducted by (Friedwald and Rous, 1944). A little later similar studies were also conducted by (Berenblum and Shubik, 1947). These same authors introduced the concept of the two stages of carcinogenesis - initiation and promotion. Initiation is the formation of neoplastic cells and promotion is the facilitation of their growth into a tumor formation (Berenblum, 1974). The models of chemical carcinogenesis in mouse skin were further developed by Slaga (1986) and (DiGiovanni, 1992). For induction of carcinogenic effects on the skin of the same animals species Yuspa (1986) used as chemical agents polycyclic aromatic hydrocarbons, alkylating agents and nitrosamines. The same author, after administration of these substances, ascertained a rapid and uncontrolled growth of epidermal cells in the test mice. The epithelial tumor cell progression, according to Kinzel et al. (1986) is associated with impaired DNA replication and synthesis, and according to Furstenberger et al. (1989) also with the genotoxic effects of the chemical carcinogen. Therefore, Yamasaki et al. (1992) proposed a classification of the carcinogens that defines them as genotoxic and non-genotoxic. The multivariant models of mouse skin carcinogenesis have necessitated the use of animals with and without fur for a greater reliability of the tests (Sundberg et al., 1997). In summary, the use of the skin of such laboratory animals as a multistage model for proving the effect of various carcinogens can also be successfully used as a screening system (Enzmann et al., 1998a).

The liver of rats and mice is also a commonly used model for testing the carcinogenic effects of chemical substances. The foci of altered hepatocytes in these species are widely used as a short-term model for determination of different substances, leading to hepatic neoplasias (Bannasch, 1986a; Ito et al., 1989). The positive results from these experiments are consequence of the greatest capality of the liver for bioactivation of carcinogens Weisburger and Williams (1982) and, hence, the successful formation of hepatic preneoplastic lesions. FAHs are very good are indicators for the effects of various chemical

carcinogens and can be readily observed using conventional techniques and methods (Williams, 1980; Moore and Kitagawa, 1986). Sasaki and Yoshida (1935) were the first who discovered and documented that the development FAHs preceded the appearance of chemically induced liver tumors.

Basophilic foci of hepatocytes were in the center of interest and were studied by (Bannasch et al., 1989a). The same team considered the development of eosinophilic foci of altered hepatocytes also important. They point out the fact that in comparison with the basophilic, the eosinophilic foci are less involved in the mechanism of liver carcinogenesis. It is proven that the basophilic cell foci are preneoplastic lesions that progress to hepatocellular carcinomas (Enzmann and Bannasch, 1987; Bannasch et al., 1989b). Preneoplastic foci of altered hepatocytes were often detected in the liver of experimental rodents as well as in the liver of people with increased risk of liver tumors (Fischer et al., 1986). It should be emphasized that the FAH precede the development of liver tumors, regardless of the mechanism of induction of the carcinogenic process, which according to various authors, indicates that these focal lesions are a mandatory step in hepatocarcinogenesis and can be used as end points when testing of chemical carcinogens (Bannasch, 1986a; Ito et al., 1989). Similar experimental models associated with chemical carcinogenesis in the liver were conducted in mice by Tokumo et al. (1991) and in hamsters by (Tanaka et al., 1987).

In experimental lung carcinogenesis in rodents Stoner et al. (1991) tested certain classes of chemical substances such as polycyclic aromatic hydrocarbons, nitrosamines, nitroso ureas, carbamates, hydrazines and certain metals.

In other experimental settings the lungs of mice were used for proving the effects of carcinogens such as 4-nitroquinoline-1-oxide Ymanaka et al. (1996), iron compounds Yano et al. (1994) and oral administration of glycerol (Inayama et al., 1986). The last established a high percentage of lung neoplasias in the test animals. In other experiments, benzo [a] pyrene (BaP), bound to iron oxide, was administered intratracheally for the induction of tumor lesions in the respiratory tract of Syrian golden hamsters (Wolterbeek et al., 1995b).

Hard (1986) conducts multiple studies in experimental renal carcinogenesis using various chemicals. He considers the emergence of atypical tubular hyperplasia or modified tubules - a lesion related to the development of renal cell carcinoma. According to Dietrich and Swenberg (1991) the increased accumulation of glycogen in the epithelial cells of the renal tubules plays a major role in the development of renal carcinomas. Bannasch et al. (1986) considered that positive histochemical reaction for detection of altered carbohydrate metabolism could be also an early indicator of neoplasias in these organs. For provoking chemical carcinogenesis in kidneys of rats Hiasa et al. (1991) used N-ethyl-N-hydroxyethyl nitrosamine. The same team claimed that proliferating basophilic tubular cells should be reported as preneoplastic conditions that have the potential to develop into renal cell carcinomas.

For experimental verification of chemicals acting as carcinogens on the urinary bladder in rats Hicks and Chowaniec (1977) successfully used intravesically applicated N-methyl-N-nitrozourea. Fukushima et al. (1983) conducted an experiment by applying N-nitrozobutyl (4-hydroxybutyl) amine in the drinking water of rats for a period of four weeks. This experimental model can be further accelerated by the combined administration of the tested carcinogen with uracil (Masui et al., 1988). The effect of the tested substance is assessed by the presence of hyperplasia, papillomatous growths and cancerous muconasal alterations.

In experimental models of pancreatic carcinogenesis Longnecker and Curphey (1975) used azaserine. In other experimental settings Longnecker et al. (1985), applied N-nitroso (2-hydroxypropyl) (2-oxopropyl) amine. The same authors found preneoplastic lesions and cancerous formations in the pancreatic acini after exposure to these carcinogens. Others, such as Chu et al. (1997) used the same substance as the initiator of the

development of preneoplastic changes in the pancreas of Syrian golden hamsters.

Silva et al. (1995) used benzo [a] pyrene (BaP) for the induction of tumors in the stomach of rats, administered once or twice a week for four weeks, or until achieving effect. Takahashi et al. (1986) used N-methyl-N'-nitro-N-nitronitrosoguanidine for the same purpose establishing preneoplastic lesions in a 40-week period of application of the carcinogenic agent.

In the experimental models for proving small intestine carcinogens Liendenschmidt et al. (1987) achieved tumor growth in rats after a two-week application of 1,2-dimethylhydrazine. Jagadeesan et al. (1994) used the same substance, again in rats, but with exposure to the carcinogen for 9 weeks and found preneoplastic changes in the small intestine. In similar experimental models, when conducted in mice, Nakamura et al. (1974) successfully applied N-ethyl-N'-nitro-N-nitrosoguanidine.

Studies on chemical carcinogenesis in rodents showed that atypical crypt foci and increased proliferation of epithelial cells are critical for the development of colon carcinoma (Yamashita et al., 1994). A model for proving the carcinogenic effect of asbestos fibers on the colon in rodents was successfully applied (Corpet et al., 1993).

Various experimental models for induction of tumors in the oral cavity of experimental animals were developed (Fisker, 1990). Neoplastic growths were also induced by applying 4-nitroquinoline N-oxide on the hard palate for 4 weeks (Johansson et al., 1989). Other authors induced cancerous alteration of the tongue using the same substance, but applied in the drinking water over a period of 8 weeks (Tanaka et al., 1995).

Studies on the development of neoplastic processes in the salivary glands of hamsters and rats were conducted, using dimethylbenz [a] anthracene pellets implanted in the submandibular gland (Sheehan and Shklar, 1972). Authors reported that 8-10 weeks after the application macroscopically well-defined tumor changes in the salivary acini have been observed.

Enzamann et al. (1992) started using experimental models for proving chemical carcinogens other than rodents. In some of the experiments chicken embryos was used with great success. The main goal of the authors was shortening of the experimental period and replacing the widely used laboratory rodents. Nowadays, the alternative and much faster in ovo tests using the chick embryo as a model system exist. They can be used for demonstrating the mutagenic, toxic and carcinogenic effects of various chemicals in a very short period of time. The alternative in ovo tests can successfully fill the gap between in vivo and in vitro carcinogenisity testing.

IN OVO CARCINOGENICITY ASSAY (IOCA)

The in ovo carcinogenicity assays has been described in detail by Enzmann et al. (1992; 1995a; 1995b) and (Enzmann and Brunnemann, 1997). Compared to the experiments performed with rodents, they are faster and much cheaper. The experiments for analyzing various chemicals were most frequently conducted on turkey and quail eggs (Enzmann et al., 1992; 1996). The incubation was carried out at a temperature of 37,5 ± 0,5 ° C and a relative air humidity of 70% ± 10%. The tests included inoculation with the tested chemical carcinogen in the egg white of the experimental eggs during first two hours of incubation. The experiments are terminated 3-4 days before hatching and the lesions are examined using routine histological and histochemical methods (Enzmann et al., 1995a; 1995b). In ovo tests can be applied for the research of the action of chemical carcinogens on different target organs. Until now, most commonly the experiments have been focused on liver carcinogenesis. This is due to the fact that the liver is a target organ for the action of different chemical carcinogens (Ito et al., 1989). This organ had been the subject of detailed study and induction of preneoplastic liver lesions in avian embryos, resulting in the development of eosinophilic and basophilic foci of altered hepatocytes (FAHs) (Enzmann et al., 1992). In experiments performed on the liver of quail embryos Enzmann et al. (1996), hyperplastic adenomatous lesions (HAL) were demonstrated. In these experimental models,

the most commonly used chemical carcinogens were N - nitrosomorpholine, urethane and diethylnitrosamine (DEN). In these tests, Enzmann et al. (1992; 1995a) demonstrated the similarity between the FAHs induced in avian embryos and these in the in vivo experiments with rodents. Other authors (Jeffrey et al., 2011; Williams at al., 2011a) also used in their experiments turkey and chicken embryos for proving the genotoxic effect of different substances. The eosinophilic foci were composed of large hepatocytes that appeared with an optically empty cytoplasm and clearly separated from the intact tissue. The basophilic foci were represented by two types of cells - small hepatocytes with slight cytoplasmic basophilia and large hepatocytes with intense cytoplasmic basophilia. In some of the tests carried out in ovo with DEN small and large acidophilic and basophilic foci were often found. Similar foci have always been found in rodent after experiments on chemical liver carcinogenesis by (Bannasch et al., 1989).

The exist evidence that these focal lesions may progress to benign and malignant liver tumors. Studies have shown that the predominant sequence of cellular alterations during hepatocarcinogenesis consists of eosinophil and basophil cell populations. Some authors claim that the same changes have the ability to develop into hepatocellular carcinomas (HCC) (Libbrecht et al., 2005).

Enzmann and Brunnemann (1997) suggesed FAH to be used as endpoints in the in ovo carcinogenicity assay (IOCA). Another important preneoplastic lesion associated with the formation of HCC is the occurrence of trabecular structures constructed of basophilic and eosinophilic hepatocytes (Enzmann et al., 1992; Enzmann et al., 1995a). The same team found significantly enlarged nuclei of hepatocytes in avian embryos exposed to chemical carcinogens. The effect on the nuclear size depends on the dose and was observed at doses that do not induce preneoplastic lesions or toxic effects (Wiemann et al., 1999). The presence of large nuclei in combination with foci of altered hepatocytes is a valuable criteria for the assessment of chemically induced lesions in the in ovo models (Enzmannetal, 1995a).

For the induction of mutagenic effects in chicken embryos Wolf et al. (2010), used methanesulfonic acid methyl ester (MMS), cyclophosphamide (CP), ifosphamide (IF), mitomycin C (MMC), 7,12-dimethyl-benz [a] anthracene (DMBA) and Nnitrosodimethylamine (NDMA) and observed the presence of micronuclei in embryonic erythrocytes and/or binucleated erythrocytes.

The tests performed in ovo for liver testing of genotoxic carcinogens revealed impairment of mitochondrial DNA (mDNA) (Enzmann et al., 1995b). Thus, it can be successfully used as an endpoint in the genotoxicity testing of different substances. mDNA is much more sensitive to the effect of genotoxic chemicals, compared to nuclear DNA (Singh and Maniccia-Bozzo, 1990).

In order to compare the specificity of IOCA with the models for carcinogenicity in rodents it was necessary to test the effects of non-carcinogenic and non-mutagenic substances such as caprolactam, mannitol and nitrozoprolin on the liver of embryos of turkeys and quail. In experimental models with rats it has been found that these chemicals do not have tumorigenic properties. Mannitol is commonly used as a control substance and is applied for validation of short-term biological research (Bucher, 1998). It has been established that these substances did not induce FAHs in avian embryonic liver and did not cause enlargement of cell nuclei, although embryo mortality was high. This proves that IOCA can be used for testing of carcinogenic and non-carcinogenic substances (Brunnemann et al., 2002).

Some chemical compounds require metabolic activation to exert their carcinogenic effect. In this regard, Perrone et al. (2004) studied the biotransformation in embryonic turkey liver and the release of DNA adducts after treating the embryos with carcinogens. The results of this experiment showed that great importance of the following biotransformation enzymes: 7 - ethoxycoumarin de-ethylase (ECOD), 7 - ethoxyresorufin de-ethylase (EROD), aldrin epoxidase (ALD), epoxide hydrolase (EH), glutatione s-transferase (GST) and glucuronyltransferase (GLUT). It was found that the levels of enzyme activity in a poultry

fetal liver (EROD <ALD <ECOD <GLUT <EH <GST) was similar to that in the liver of an adult rat (EROD <ECOD <ALD <GLUT <EH <GST) (Perrone et al., 2004). The same author demonstrated a similarity between the DNA adducts and liver enzyme activity of avian embryos with those of rats that were inoculated with 2-acetylaminofluorene (2-AAF) and benzo [a] pyrene (BaP). These findings allow the application of the in ovo tests as models for proving carcinogens that require metabolic activation.

IOCA can be used for research related to the molecular mechanisms of action of potential chemical carcinogens or drugs leading to the development of neoplasias. In support of this, chick embryos were used as an experimental model for proving the effect of phenobarbital (Frueh et al., 1997). According to the same author, the effect of the tested substance on the embryonic liver should be analyzed by DDRT-PCR (Differential Display of Reverse Transcribed mRNA amplified by the Polymerase Chain Reaction), which defines the expression or suppression of a large number of genes that represent the cellar response to phenobarbital.

A number of alternative in ovo tests exist, using the avian embryo as a model system. They can be used to demonstrate mutagenic, embryotoxic, and/or carcinogenic effects of various chemicals for a short period of time. In edition, it is apparent that in ovo tests can successfully fill the gap between the in vivo and in vitro experiments.

CONCLUSIONS

The in ovo carcinogenicity assay is a fast alternative method for proving the effect of various chemicals and compounds. Liver preneoplastic lesions can be induced within 24 days in turkey embryos and the damage to the mitochondrial DNA can be analyzed 4 days after exposure to the tested chemical. The performance of the test is much cheaper than the in vivo experimentation on laboratory animals and it doesn't require sophisticated methods and expensive equipment. Routine histological techniques are sufficient for detect of the induced foci of altered eosinophilic and basophilic hepatocytes, as well as nuclear atypia. The sensitivity of in ovo tests is comparable to the sensitivity of chemical carcinogenicity experiments in rodents (Enzmann et al., 1995a; 1995b). The in ovo testing has the potency to distinguish carcinogenic from non-carcinogenic substances (Brunnemann et al., 2002). Fertilized avian eggs and avian embryos provide a multi-organ model for testing of various carcinogens, mutagens and genotoxins. Moreover, the amount of avian embryonic tissues allow the performance of morphological, biochemical and molecular biological studies. The in ovo models can be used for rapid screening of chemical that are potentially dangerous to humans and animals. In addition to the lower coast of the in ovo experimentation, compared with in vivo tests, of great importance is the fact that many researchers preffer working with avian embryos because of the low exposure of the laboratory personnel to the tested chemicals and the minimal negative health impact.

ACKNOWLEDGEMENTS

This research work was carried out with the support of University of Forestry and project BG051PO001-3.3.06-0056 "Support for the development of young people in University of Forestry", Operational Programme "Human Resources Development" financed by the European Social Fund of the European Union.

REFERENCES

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, p. 617–29.

Bannasch P., 1986a. Preneoplastic lesions as end points in carcinogenicity testing. I. Hepatic preneoplasia. Carcinogenesis, 7, p. 689-695.

Bannasch P., 1986b. Preneoplastic lesions as end points in carcinogenicity testing. II. Preneoplasia in various non- hepatic tissues. Carcinogenesis, 7, p. 849-852.

Bannasch P., Enzmann H., Hacker H.J., Weber E. and Zerban H., 1989a. Comparative pathobiology of hepatic preneoplasia. In Liver Cell Carcinoma, ed. P. Bannasch, D. Keppler and G. Weber, p. 55-76.

Bannasch P., Enzmann H., Klimek F., Weber E. and Zerban H., 1989b. Significance of sequential

cellular changes inside and outside foci of altered hepatocytes during hepatocarcinogenesis. Toxicologic Pathology, 17, p. 617-629.

Bannasch P., Hacker H.J., Tsuda H. and Zerban H., 1986. Aberrant regulation of carbohydrate metabolism and metamorphosis during renal carcinogenesis. Advances in Enzyme Regulation, 25, p. 279-296.

Berenblum I., 1974. Carcinogenesis as a Biological Problem. In Frontiers of Biology, ed. A. Neuberger and E. L. Tatum. North Holland Publishing Company, Amsterdam, p. 97-112.

Berenblum J. and Shubik P., 1947. A new quantitative approach to the study of the stages of chemical carcinogenesis in the mouse's skin. British Journal of Cancer, 1, p. 389-391.

Brunnemann K., Enzmann H., Perrone C., Iatropoulos M., Williams G., 2002. In ovo carcinogenicity assay (IOCA): evaluation of mannitol, caprolactam and nitrosoproline. Arch Toxicol., 76(10), p. 606-612.

Bucher J.R., 1998. Update on National Toxicology Program (NTP) assays with genetically altered or "transgenic" mice. Environ Health Perspect, 106, p. 619-621.

Chu M., Rehfeld JF and Borch K., 1997. Chronic endogenous hypercholecystokininemia promotes pancreatic carcinogenesis in the hamster. Carcinogenesis, 18, p. 315-320.

Dietrich D.R. and Swenberg J.A., 1991. Preneoplastic lesions in rodent kidney induced spontaneously or by non-genotoxic agents: predictive nature and comparison to lesions induced by genotoxic carcinogens. Mutation Research, 248, p. 239-260.

DiGiovanni J., 1992. Multistage carcinogenesis in mouse skin. Pharmacology and Therapeutics 54, p. 63-128.

Doll R., Peto R., 1981. The causes of cancer. J. Natl. Cancer Inst., 66, p. 1191-1308.

Enzmann H. and Bannasch P., 1987. Potential significance of phenotypic heterogeneity of focal lesions at diferent stages in hepatocarcinogenesis. Carcinogenesis, 8, p. 1607-1612.

Enzmann H., Bomhard E., Iatropoulos M., Ahr H.J., Schlueter G. and Williams G.M., 1998a. Short and Intermediate-term Carcinogenicity Testing-A Review. Part 1: The Prototypes Mouse Skin Tumour Assay and Rat Liver Focus Assay. Food and Chemical Toxicology, 36, p. 979-995.

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. Abstr. 777, Washington, DC, April, p. 20-24.

Enzmann H., Iatropoulos M., Brunnemann K.D., Bomhard E., Ahr H.J., Schlueter G., Williams G.M., 1998b. Short- and intermediate-term carcinogenicity testing—a review. Part 2: available experimental models. Food Chem Toxicol, 36, p. 997–1013.

Enzmann H., Zerban H., Kopp-Schneider A., LoÈser E. and Bannasch P., 1995. Efects of low doses of N-nitroso- morpholine on the development of early stages of hepatocarcinogesis. Carcinogenesis, 16, p. 1513-1518.

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

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

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), p. 560-569.

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

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

Fisker A.V., 1990. Experimental oral carcinogenesis. Danish Medical Bulletin, 37, p. 433-442.

Friedwald W.F. and Rous P., 1944. Initiating and promoting elements in tumor production; analysis of efects of tar, benzpyrene and methylcholanthrene on rabbitskin. Journal of Experimental Medicine, 80, p. 101-126.

Frueh F., Zanger U., Meyer U., 1997. Extent and Character of Phenobarbital-Mediated Changes in Gene Expression in the Liver. Molecular Pharmacology, 51, p. 363-369.

Fukushima S., Hagiwara A., Ogiso T., Shibata M. and Ito N., 1983. Promoting effect of various chemicals in rat urinary bladder carcinogenesis initiated by N-nitrosobutyl(4-hydroxybutyl)amine. Food and Chemical Toxicology, 21, p. 59-68.

Furstenberger G., Schurich B., Kaina B., Petrusevska R.T., Fusenig N.E. and Marks F., 1989. Tumor induc- tion in initiated mouse skin by phorbol esters and methyl methanesulfonate, correlation between chromo- somal damage and conversion in vivo. Carcinogenesis, 10, p. 719-723.

Hard G.C., 1986. Experimental models for the sequential analysis of chemically-induced renal carcinogenesis. Toxicologic Pathology, 14, p. 112-122.

Hiasa Y., Konishi N., Nakaoka S., Nakamura M., Nishii S., Kitahori Y. and Ohshima M., 1991. Possible application to medium-term organ

bioassays for renal carcinogenesis modifiers in rats treated with N-ethyl-N hydroxyethylnitrosamine and unilateral nephrectomy. Japanese Journal of Cancer Research, 82, p. 1385-1390.

Hicks R.M. and Chowaniec J., 1977. The importance of synergy between weak carcinogens in the induction of bladder cancer in experimental animals and humans. Cancer Research, 37, p. 2943-2949.

IARC, 1998. Chemical-induced rodent preneoplastic lesions as indicators of carcinogenic activity. IARC Scientific Publications. In press. International Agency for Research on Cancer, Lyon.

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, p. 345–357.

ICH, 1997. International Conference on Harmonisation; Draft Guideline on dose selection for carcinogenicity studies of pharmaceuticals; addendum on the limit dose. Federal Register, 62, p. 15715-15716.

Inayama Y., Kitamura H., Ito T. and Kanisawa M., 1986. Efects of glycerol on 4-nitroquinoline 1-oxide induced pulmonary tumorigenesis in nddY mice. Japanese Journal of Cancer Research, 77, p. 103-105.

Ito N., Imaida K., Hasegawa R. and Tsuda H., 1989. Rapid bioassay methods for carcinogens and modifiers of hepatocarcinogenesis. Critical Reviews in Toxicology, 19, p. 386-415.

Jacobson-Kram D., 2010. Cancer risk assessment approaches at the FDA/CDER: Is the era of the 2-year bioassay drawing to a close? Toxicologic Pathology, 38, p. 169–70.

Jagadeesan V., Rao N.J. and Sesikeran B., 1994. Effect of iron- deficiency on DMH-induced gastrointestinal tract tumors and occurrence of hepatocyte abnormalities in Fischer rats. Nutrition and Cancer, 22, p. 285-291.

Jeffrey A.M., Brunnemann K.D., Duan JD, Schlatter J., Williams G.M., 2011. Furan induction of DNA cross-linking and strand breaks in turkey fetal liver in comparison to 1,3-propanediol. Food and Chemical Toxicology, 50(3-4), p. 675-678.

Johansson S.L., Hirsch J.M., Larsson P.A., Saidi J. and Osterdahl B.G., 1989. Snuff-induced carcinogenesis:effect of snuff in rats initiated with 4 nitroquinoline N- oxide. Cancer Research, 49, p. 3063-3069.

Kinzel V., Furstenberger G., Loehrke H. and Marks F., 1986. Three-stage tumorigenesis in mouse skin, DNA synthesis as a prerequisite for the conversion stage induced by TPA prior to initiation. Carcinogenesis, 7, p. 779-782.

Lindenschmidt R.C., Tryka A.F. and Witschi H.P., 1987. Modification of gastrointestinal tumor development in rats by dietary butylated hadroxytoluene. Fundamental and Applied Toxicology, 8, p. 474-481.

Longnecker D.S. and Curphey T.J., 1975. Adenocarcinoma of the pancreas in azaserine-treated rats. Cancer Research, 35, p. 2249-2258.

Longnecker D.S., Roebuck B.D., Kuhlmann E.T. and Curphey T.J., 1985. Induction of pancreatic carcinomas in rats with N-nitroso(2 hydroxypropyl)(2-oxo-propyl)amine: histopathology. Journal of the National Cancer Institute, 76, p. 1101-1112.

Masui T., Shirai T., Takahashi S., Mutai M. and Fukushima S., 1988. Summation efect of uracil on the two stage and multistage models of urinary bladder carcinogenesis in F344 rats initiated with N-butyl-N-(4-hydroxy butyl)nitrosamine. Carcinogenesis, 9, p. 1981-1985.

Moore M.A. and Kitagawa T., 1986. Hepatocarcinogenesis in the rat: the efect of promoters and carcinogens in vivo and in vitro. International Review of Cytology, 101, 125-173. Naito M. and DiGiovanni J. (1989) Genetic background and development of skin tumors. Carcinogenesis, 11, p. 187-212.

Nakamura T., Masuyama M. and Kishimoto H., 1974. Tumors of the esophagus and duodenum induced in mice by oral administration of N-ethyl-N'-nitro-N-nitro-soguanidine. Journal of the National Cancer Institute, 52, p. 519-522.

Perrone C., Ahr H., Duan J., Jeffrey A., Schmidt U., Williams G., Enzmann H., 2004. Embryonic turkey liver: activities of biotransformation enzymes and activation of DNA-reactive carcinogens Archives of Toxicology Arch Toxicol., 78(10), p. 589-598.

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

Sasaki T. and Yoshida T., 1935. Experimentelle Erzeugung des Lebercarcinoms durch Futterung mit o-Amidoazotoluol. Virchows Archiv, Pathologische Anatomie, 295, p. 175-200.

Schmahl D., Preussmann R., Berger MR., 1989. Causes of cancer: an alternative

Sheehan R. and Shklar G. (1972) The effect of cyclophosphamide on experimental salivary gland neoplasia. Cancer Research, 32, p. 420-425.

Silva R.A., Monoz S.E., Guzman C.A .and Evnard AR., 1995. Effects of dietary N-3, N-6 and N-9 polyunsaturated fatty-acids on benzo(a)pyrene-induced forestomach tumorigenesis in C57BL6J mice. Prostaglandins, Leukotrienes and Essential Fatty Acids, 53, p. 273-277.

Singh G., Maniccia-BozzoE., 1990. Evidence for lack of mitochondrial DNA repair following cis-dichlorodiammineplatinum treatment. Cancer Chemother. Pharmacol., 26, p. 97-100.

Slaga T.J., 1986. SENCAR mouse skin tumorigenesis model versus other strains and stocks of mice. Environmental Health Perspectives, 68, p. 27-32.

Stoner G.D., 1991. Lung tumors in strain A mice as a bioassay for carcinogenicity of environmental chemicals. Experimental Lung Research, 17, p. 405-423.

Sundberg J.P., Sundberg B.A. and Beamer W.G., 1997. Comparison of chemical carcinogen skin tumor induction e cacy in inbred, mutant, and hybrid strains of mice: morphologic variations of induced tumors and absence of a papilloma virus. Molecular Carcinogenesis, 20, p. 19-32.

Takahashi M., Hasegawa R., Furukawa F., Toyody K., Sato H. and Hayashi Y., 1986. Effects of ethanol, potassium metabisulfite, formaldehyde and hydrogen peroxide on gastric carcinogenesis in rats after initiation with N-methyl-N'-nitro-N-nitrosoguanidine. Japanese Journal of Cancer Research, 77, p. 118-124.

Tanaka T., Makita H., Ohnishi M., Mori H., Satoh K. and Hara A., 1995. Chemoprevention of rat oral carcinogenesis by naturally-occuring xanthophylls, astaxanthin and canthaxarathin. Cancer Research, 55, p. 4059-4064.

Tanaka T., Mori H. and Williams G.M., 1987. Enhancement of dimethylnitrosamine-initiated hepatocarcinogenesis in hamsters by subsequent administration of carbon tetrachloride but not phenobarbital or p,p' dichlorodiphenyltrichloroethane. Carcinogenesis, 8, p. 1171-1178.

Tempel K., Ignatius A., Stammberger I., 1992. A short-term test for nucleotoxicity that uses chick embryo cells treated *in vitro* and *in vivo* - physico-chemical and biochemical investigations. Comp Biochem Physiol., 103C, p. 73-78.

Tokumo K., Iatropoulos M.J. and Williams G.M., 1991. Butylated hydroxytoluene lacks the activity of phenobarbital in enhancing diethylnitrosamine-induced mouse liver carcinogenesis. Cancer Letters 59, p. 193-199.

Doll and Peto. Klin. Wochenschr., 67, 1989, p. 1169-1173.

Weisburger J.H. and Williams G.M., 1982. Classification of carcinogens as genotoxic and epigenetic as basis for improved toxicologic bioassay methods. In Environmental Mutagens and Carcinogens, ed. T. Sugimura, S. Kondo and T. Takebe, p. 283-294.

Weisburger J.H. and Williams G.M., 1984. Bioassay of carcinogens: in vitro and in vivo tests. In ACS Monograph 182, Chemical Carcinogens, Vol. 2, ed. C. E. Searle, 2nd Ed. p. 1323-1373.

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

Wiemann C., Enzmann H., Löser E., Schlüter G., 1999. Nonlinearity of nuclear enlargement in hepatocytes induced by the carcinogen N-nitrosomorpholine in ovo. Cancer Detection and Prevention, 23(6), p. 485-495.

Williams G.M. and Whysner J., 1996. Epigenetic carcinogenesis, Evaluation and risk assessment. Experimental and Toxicologic Pathology, 48, p. 189-195.

Williams G.M., 1980. The pathogenesis of rat liver cancer caused by chemical carcinogens. Biochimica et Biophysica Acta, 605, p. 167-189.

Williams G.M., Brunnemann K.D., Iatraopoulos M.J., Smart D.J., Enzmann H.G., 2010. Production of liver preneoplasia and gallbladder agenesis in turkey fetuses administered diethylnitrosamine. Arch Toxicol, 85, p. 681–687.

Williams G.M., Gebhardt R., Sirma H. and Stenback F., 1993. Non-linearity of neoplastic conversion induced in rat liver by low exposures to diethylnitrosamine. Carcinogenesis, 14, p. 2149-2156.

Wolf T., Niehaus-Rolf C., Banduhn N., Eschrich D., Scheel J., Luepke N., 2007. The hen's egg test for micronucleus induction (HET-MN): Novel analyses with a series of well-characterized substances support the further evaluation of the test system. Mutation Research, 650, p. 150–164.

Wolterbeek A.P., Schoevers E.J., Rutten A.A. and Feron V.J., 1995b. A critical appraisal of intratracheal instillation of benzo(a)pyrene to Syrian golden hamsters as a model in respiratory tract carcinogenesis. Cancer Letters, 89, p. 107-116.

Yamanaka K., Ohtsubo K., Hasegawa A., Hayashi H., Ohji H., Kanisawa M. and Okada S., 1996. Exposure to dimethylarsenic acid, a main metabolite of inorganic arsenics, strongly promotes tumorigenesis initiated by 4 Nitroquinoline 1-oxide in the lungs of mice. Carcinogenesis, 17, p. 767-770.

Yamasaki H., Mesnil M. and Nakazawa H., 1992. Interaction and distinction of genotoxic and non-genotoxic events in carcinogenesis. Toxicology Letters, 64-65, p. 597-604.

Yamashita N., Minamoto T., Onda M. and Esumi H., 1994. Increased cell proliferation of azoxymethaneinduced aberrant crypt foci of rat colon. Japanese Journal of Cancer Research, 85, p.692-698.

Yano T., Obata Y., Ishikawa G. and Ishikawa T., 1994. Enhancing efect of high dietary iron on lung tumorigenesis in mice. Cancer Letters, 76, p. 57-62.

Yuspa S.H., 1986. Cutaneous chemical carcinogenesis. Journal of the American Academy for Dermatology, 15, p. 1031-1044.