

Dose- and Sex-related Carcinogenesis by N-Bis(2-hydroxypropyl)nitrosamine in Wistar Rats

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An initiation-promotion medium-term bioassay for detection of chemical carcinogens, developed in the male F344 rat, uses 0.1% N-bis(2-hydroxypropyl)nitrosamine (DHPN) among five genotoxic chemicals for the initiation of carcinogenesis in multiple organs. To establish this bioassay in the Wistar strain, the effects of two dose levels of DHPN were evaluated on the main DHPN rat target organs: lung, thyroid gland, kidneys and liver. Four groups of male and female animals were studied: Control—untreated group; Multi-organ initiated group (also referred to as DMBDD, based on the initials of the five initiators)—treated sequentially with N-diethylnitrosamine (DEN, i.p.), N-methyl-N-nitrosourea (MNU, i.p.), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN, drinking water), N, N'-dimethylhydrazine (DMH, s.c.) and DHPN (drinking water) for 4 weeks; a third group treated with 0.1% DHPN in drinking water for 2 weeks and the last group treated with 0.2% DHPN in drinking water for 4 weeks. The animals were sacrificed after 30 weeks. DHPN at 0.2% induced preneoplasia in the liver and kidneys of rats of both sexes, the number and area of the putative preneoplastic liver glutathione S-transferase-positive hepatocyte foci being significantly increased in these animals. It also induced benign and malignant tumors in female and in male rats. However, there was no relationship between the increased incidence of preneoplastic lesions and tumor development in the 0.2% DHPN-exposed groups of both sexes. DHPN at 0.1% induced only a few preneoplastic lesions in the liver and kidney and no tumors in both male and female rats. A clear dose and sex-related carcinogenic activity of DHPN was registered, although Wistar rats of both sexes showed a relative resistance to the carcinogenic activity of this compound.

Key words: N-Bis(2-hydroxypropyl)nitrosamine (DHPN) — Wistar rat — Multi-organ carcinogenesis — Preneoplasia and neoplasia — Chemical carcinogens

Currently, the most important *in vivo* experimental procedure to identify chemical carcinogens is the long-term assay with rodents.^{1,2} The extended duration, the complex operational procedures and the high cost per test substance make the use of the long-term bioassay very limited. These and other disadvantages of the long-term bioassay, e.g., the inconvenience of not considering the multistage character of chemical carcinogenesis, imply that more convenient and faster procedures for testing potential chemical carcinogens are necessary.³

A multi-organ medium-term system, the “initiated rat bioassay” (IRB), based on the initiation-promotion concept of chemical carcinogenesis,⁴ has been proposed as an alternative/complementary approach to the conventional long-term bioassay.^{5–9} Five genotoxic chemicals [N-diethylnitrosamine (DEN), N-methyl-N-nitrosourea (MNU), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), N,N'-dimethylhydrazine (DMH) and N-bis(2-hydroxypropyl)nitrosamine (DHPN)] are used to initiate carcinogenesis in

multiple organs, in what has been referred to as the DMBDD initiation protocol, based on the initials of these agents. Developed in the male F344 rat, the IRB has the advantages of shorter duration, the use of a single rodent species, and faster results at lower cost. The results of the IRB have been shown to be in line with those of the long-term conventional bioassay.^{5–8}

Since 1996, the IRB bioassay has been adopted, with some modifications, by the Brazilian Agency for the Environment (IBAMA) as a valid source of evidence of carcinogenicity of chemicals.¹⁰ Among the modifications established in the protocol is the use of both sexes of the Wistar strain of rats as subjects. Recently, a task-group gathered at the International Agency for Research on Cancer (IARC) recognized that, although the possibility of false-positive has not been rigorously tested, assay systems based on the initiation-promotion concept of carcinogenesis could be considered appropriate for identifying carcinogens in rodents.¹¹

Our laboratory has conducted studies to establish the IRB protocol in the Wistar strain of rats.^{12,13} Particular interest was focused on the pattern of specific lesions

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induced by each one of the chemicals used in the DMBDD initiation protocol. The present paper reports the findings obtained with DHPN, a potent chemical mutagen and wide-spectrum carcinogen, that induces tumors in the nasal cavities, lung, thyroid, liver, kidneys and urinary bladder of both sexes in various rat strains.^{14–20} In this study, DHPN was evaluated at a higher dose level (0.2%) and for a longer period of exposure than that adopted in the standard IRB protocol, in order to overcome the variable susceptibility of the Wistar rats to chemical carcinogenesis.

MATERIALS AND METHODS

Animals A total of 90 male and female 6-week-old Wistar rats were obtained from the Paraná Institute of Technology (TECPAR, Curitiba, Brazil). They were housed five per polypropylene plastic cage with wood chips for bedding in an animal room with controlled conditions of temperature ($22 \pm 2^\circ\text{C}$), humidity ($55 \pm 10\%$) and lighting (12 h light/dark). They were fed commercial chow (NUVILAB-CR1, NUVITAL, Paraná, Brazil) and water *ad libitum*. The experiment was started after an acclimation period of 20 days, when the animals weighed about 200 g (male) and 150 g (female).

Chemical agents The chemicals used to initiate carcinogenesis were purchased from Sigma Chemical Co. (St. Louis, MO) [DEN, MNU, DMH], Tokyo Kasei Industries Co. (Tokyo) [BBN] and Nakalai Tesque, Inc. (Kyoto) [DHPN].

Experimental design The experimental design is presented in Fig. 1. Male and female rats were allocated respectively to four groups of 10–15 animals: Control group, untreated; DMBDD group, treated at subcarcinogenic doses with five initiating agents: DEN (100 mg/kg b.w., i.p., single dose) at the beginning of the experiment, MNU (20 mg/kg b.w., i.p., 4 times, two doses a week), BBN (0.05% in drinking water—d.w.) during the 1st and 2nd weeks, DMH (40 mg/kg b.w., s.c., 4 times, two doses a week) and 0.1% DHPN d.w. during the 3rd and 4th

weeks; 0.1% DHPN group, received 0.1% DHPN d.w., during the 3rd and 4th weeks; 0.2% DHPN group, was continuously treated with 0.2% DHPN d.w., for 4 weeks. BBN and DHPN solutions were stored in aluminium foil-wrapped bottles to avoid light decomposition.

Animal body weights, water and food consumptions were registered twice a week, from the 1st to the 4th week and every 15 days from the 6th to the 30th week. The average DHPN ingestion per mg/kg body weight/day was estimated from the average water ingestion and average body weight of each group at the end of each treatment period (2nd and 4th weeks). After carcinogen exposure, the animals were kept on basal diet and water *ad libitum* until the 30th week, when they were killed by exsanguination, under ether anesthesia. Complete necropsies were performed and the liver and kidneys were weighed immediately after having been removed and blotted dry.

Tissue processing Lungs, thyroid, kidneys, and liver—the main target organs of DHPN in the rat^{5, 18, 19}—were fixed in 10% buffered formalin during 48 h. The lungs were inflated via the trachea with the same fixative solution. Paraffin-embedded sections (5 μm thickness) were stained with hematoxylin and eosin (HE) for histopathological analysis. Liver sections were also prepared for quantitative assessment of immunohistochemically demonstrated glutathione S-transferase placental form (GST-P)-positive foci by the avidin-biotin-peroxidase method.²¹ This putative preneoplastic lesion has been utilized as a marker for rat liver carcinogenesis.²² GST-P-positive foci larger than 0.1 mm in diameter were measured with the aid of a Zeiss Axiophot microscope connected to a KS-300 apparatus (Kontron Elektronik, Hallbergmoof, Germany). Data were expressed as number and area (mm^2) of foci per liver section (cm^2).²²

Statistical analysis The differences among mean values of body weight, body weight gain, water and food consumption, and relative liver and kidney weights were evaluated by analysis of variance (ANOVA). The differences of incidence of lesions among groups were assessed by the use of Fisher's exact probability test and the χ^2 test. The number and area of GST-P-positive foci were analyzed by means of the Kruskal-Wallis test. Differences between groups were analyzed by using Tukey's test for both parametric and nonparametric tests. Differences were considered significant when $P < 0.05$.²³

RESULTS

Two male rats of the DMBDD group were found dead, one at week 11 and the other at week 23. One female rat of the DMBDD group was killed at week 23 because of extreme cachexia. These events were considered to have been caused by extended toxicity of the DMBDD treatment.

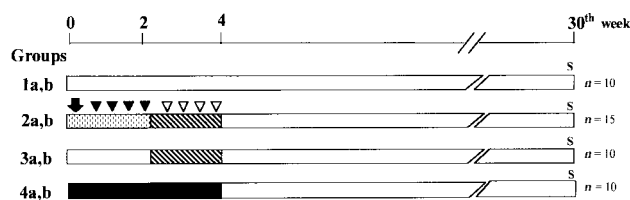


Fig. 1. Experimental design. \downarrow DEN 100 mg/kg, i.p., ∇ MNU 20 mg/kg, i.p., ∇ DMH 40 mg/kg, s.c., \square BBN 0.05% d.w., \square DHPN 0.1% d.w., \blacksquare DHPN 0.2% d.w., \square Control. S, animals killed; n, number of animals; a, male; b, female.

Table I. Incidence (%) of Wistar Rats with Preneoplastic and Neoplastic Lesions in the Main DHPN Target Organs at the End of the Experiment

Lesions /Groups	Control	DMBDD	0.1% DHPN	0.2% DHPN
Male				
Kidney	10 ^{a)}	13	10	10
Basophilic cell foci	3 (30) ^a	3 (23) ^a	2 (20) ^a	10 (100) ^b
Basophilic adenoma	0	1 (8)	0	0
Liver	10	13	10	9
Altered hepatocyte foci	0 ^a	11 (85) ^b	2 (20) ^a	6 (67) ^b
— Acidophilic cell foci	0 ^a	4 (31) ^{a,b}	1 (10) ^a	6 (67) ^b
— Clear cell foci	0 ^a	11 (85) ^b	1 (10) ^a	2 (22) ^a
— Basophilic cell foci	0	3 (23)	0	0
— Amphophilic cell foci	0	2 (15)	0	1 (11)
Thyroid gland	10	13	10	10
Follicular adenoma	0	0	0	1 (10)
Female				
Kidney	10	14	10	10
Basophilic cell foci	0	0	2 (20)	3 (30)
Clear cell adenoma	0	0	0	1 (10)
Basophilic cell carcinoma	0	1 (7)	0	0
Renal mesenchymal tumor	0 ^a	5 (36) ^b	0 ^a	2 (20) ^{a,b}
Liver	10	14	10	10
Altered hepatocyte foci	0 ^a	5 (36) ^c	5 (50) ^{b,c}	8 (80) ^b
— Acidophilic cell foci	0 ^a	3 (21) ^a	4 (40) ^{a,b}	7 (70) ^b
— Clear cell foci	0 ^a	5 (36) ^{a,b}	2 (20) ^a	5 (50) ^b
— Basophilic cell foci	0	3 (21)	0	1 (10)
— Amphophilic cell foci	0	2 (14)	0	2 (20)
Cholangioma	0	0	0	1 (10)
Lung	10	14	10	10
Bronchiolo-alveolar carcinoma	0	0	0	1 (10)
Thyroid gland	10	14	10	10
Follicular adenoma	0	1 (7)	0	1 (10)
Follicular carcinoma	0	1 (7)	0	0

a) Effective number of animals.

^{a, b, c} Different superscript letters indicate significant differences at $P < 0.05$ among the groups.

Body weights At the end of the experiment (30th week), no differences in mean final body weight and body weight gain were noted among the male groups. Among females, the DMBDD group presented a significant ($P < 0.05$) decrease of the final body weight and body weight gain when compared to the control or the 0.1% DHPN groups. The 0.2% DHPN group presented diminished final body weight and body weight gain, when compared to the Control or the 0.1% DHPN group (data not shown).

Water and DHPN intake At the end of the 4th week, the average water ingestion in the male DMBDD and 0.2% DHPN groups were significantly diminished ($P < 0.01$) when compared to the other two groups. Water ingestion in the female DMBDD and 0.2% DHPN groups was also significantly diminished ($P < 0.01$) at the 4th week, when compared to the Control group (data not shown). Because

the DMBDD and 0.2% DHPN groups of both sexes ingested less water than the other two groups, the planned levels of DHPN exposure were not attained: the male and female 0.2% DHPN groups ingested respectively 270 and 336 mg/kg/day (less than expected); the 0.1% DHPN groups of both sexes ingested more DHPN (150 mg/kg/day and 179 mg/kg/day for males and females, respectively) than the DMBDD groups (93 mg/kg/day and 121 mg/kg/day, males and females, respectively). Nevertheless, the 0.2% DHPN groups of both sexes ingested roughly 3 times more DHPN than the corresponding groups submitted to the DMBDD treatment.

Preneoplastic lesions and neoplasia The incidences of male and female Wistar rats with preneoplastic lesions and tumors are presented in Table I. The 0.2% DHPN male group presented a significantly higher ($P < 0.05$) incidence

Table II. Incidence and Frequency of Preneoplasia and Neoplasia in the Main Rat DHPN Target Organs (Lung, Thyroid Gland, Kidney and Liver) of Wistar Rats

Groups	Effective No. of animals	No. of animals with preneoplasia (%)	No. of animals with neoplasia (%)	Benign tumors		Malignant tumors	
				No. of tumor-bearing rats (%)	No. of tumors/group	No. of tumor-bearing rats (%)	No. of tumors/group
Male							
Control	10	3 (30) ^a	0	0	0	0	0
DMBDD	13	11 (85) ^{b,*}	1 (8)	1 (8)	1	0	0
0.1% DHPN	10	4 (40) ^a	0	0	0	0	0
0.2% DHPN	10 ^{a)}	10 (100) ^{b,*}	1 (10)	1 (10)	3	0	0
Female							
Control	10	0 ^a	0 ^a	0	0	0 ^a	0
DMBDD	14	5 (36) ^{c,*}	6 (43) ^{b,*}	1 (7)	1	6 (43) ^{b,*}	8
0.1% DHPN	10	5 (50) ^{b,c,**}	0 ^a	0	0	0 ^a	0
0.2% DHPN	10	8 (80) ^{b,*}	5 (50) ^{b,*}	3 (30)	3	3 (30) ^{a,b}	4

a) Considered preneoplastic lesions in the kidney.

^{a,b,c} Different superscript letters indicate significant differences among the groups at * $P < 0.05$, ** $P < 0.01$.

of animals with basophilic cell foci in the kidneys than the other three groups. The DMBDD and 0.2% DHPN groups presented higher ($P < 0.05$) incidences of altered hepatocyte foci than the Control or 0.1% DHPN group. Considering altered hepatocyte foci subtypes, the incidence of 0.2% DHPN male rats with acidophilic cell foci was significantly higher than in the Control or 0.1% DHPN group. The incidence of male rats with clear cell foci was significantly higher in the DMBDD group, when compared to the other groups. Neoplasias were seen only in the DMBDD and 0.2% DHPN male groups, which developed adenomas in the kidney and in the thyroid gland, respectively. No malignant tumors were registered in male rats.

Liver preneoplastic lesions were observed in all three female groups treated with carcinogens, mostly in the 0.2% DHPN group. This group presented a higher incidence ($P < 0.05$) of females with altered hepatocyte (acidophilic and clear cell) foci than the Control group. The DMBDD and 0.1% DHPN groups presented an intermediate incidence of animals with clear and acidophilic cell foci, when compared to the 0.2% DHPN (higher incidence) and the Control (lower incidence) groups. Benign and malignant tumors were seen only in DMBDD- and 0.2% DHPN-treated animals. The incidence of female rats with renal mesenchymal tumors (RMT) was increased in the latter two groups, mainly in the DMBDD group ($P < 0.05$).

The incidence and frequency of preneoplasia and neoplasia in male and female rats are summarized in Table II. The DMBDD and 0.2% DHPN male groups presented a

Table III. Quantitative Data for Liver GST-P-positive Cell Foci in Wistar Rats

Groups	Effective No. of animals	GST-P-positive liver foci	
		Number (No./cm ²)	Area (mm ² /cm ²)
Male			
Control	9	0 ^a	0 ^a
DMBDD	11	6.08±2.88 ^b	0.23±0.15 ^b
0.1% DHPN	10	0.94±1.29 ^a	0.01±0.01 ^a
0.2% DHPN	10	5.88±5.11 ^b	0.27±0.38 ^b
Female			
Control	10	0 ^a	0 ^a
DMBDD	12	5.47±2.35 ^b	0.29±0.22 ^b
0.1% DHPN	10	0.93±2.11 ^a	0.01±0.03 ^a
0.2% DHPN	10	5.67±7.93 ^b	0.43±0.90 ^b

^{a,b} Different superscript letters indicate significant differences among the groups at $P < 0.05$.

higher incidence of animals with preneoplastic lesions ($P < 0.05$) than the other two groups. Lesions were mostly observed in the liver and in the kidneys, respectively (Table I). The incidence of male rats with neoplasia was very low in these groups, with no differences between them.

The 0.2% DHPN female group presented a significantly higher ($P < 0.05$) incidence of animals with preneoplastic lesions compared to the Control and DMBDD groups, but did not differ from the 0.1% DHPN group. The DMBDD and 0.2% DHPN female groups presented a significantly higher ($P < 0.05$) incidence of animals with neoplasia. The

tumors were more frequent in the kidneys (Table I). The female DMBDD group presented a significantly increased ($P < 0.05$) incidence and the highest frequency of malignant tumors in this study (Table II).

Significantly increased number and area of GST-P-positive foci of hepatocytes were registered in male and female animals treated with 0.2% DHPN or with the DMBDD protocol, when compared to the other two groups (Table III).

DISCUSSION

In the present study, the incidence of preneoplastic lesions and tumors induced by two dose levels of DHPN in the lung, thyroid gland, kidney and liver—the main target organs of the carcinogen DHPN in the rat^{5, 18, 19)}—was evaluated in both sexes of the non-isogenic Wistar rat strain. This was done during the process of standardization of an alternative medium-term multi-organ bioassay for carcinogenesis previously developed in the male F344 rat.⁵⁻⁹⁾

The incidence of animals developing preneoplastic lesions (altered cell foci)^{24, 25)} in the selected target organs was relatively high in the 0.2% DHPN and DMBDD groups. The lesions occurred in the kidney and liver, but not in the lung and thyroid gland (Table I). In the liver, the number and area of GST-P-positive foci were significantly enhanced in both sexes exposed to 0.2% DHPN or to the DMBDD treatment, when compared to the animals exposed to 0.1% DHPN or to the Control group (Table III). Since the administration of 0.1% DHPN d.w. for 6 weeks, after initiation with DEN and partial hepatectomy²⁶⁾ or for 2 weeks, but with no initiation with DEN and partial hepatectomy,²⁷⁾ induced a significant increase of the number and area of GST-P-positive foci in male F344 rats after 8 and 28 weeks, respectively, the absence of enhanced GST-P-positive foci development in the 0.1% DHPN-treated groups indicates a resistance of the local Wistar rats to the induction of GST-P-positive foci by that relatively low dose of DHPN. The significant increase in the number and area of these altered positive foci in Wistar rats of both sexes exposed to the DMBDD protocol—which uses 0.1% DHPN among a total of five initiators of carcinogenesis—a finding described in the male F344 rats,^{7, 28, 29)} is probably dependent on synergism among the five genotoxic carcinogens used for the multi-organ initiation.

Although the tumors registered in this study were relatively scarce and not associated with the most frequently observed preneoplastic lesions, they were assumed to be dependent on the treatments because they are not known as spontaneously developing tumors in the Wistar strain and occurred only in 39-week-old animals. The thyroid gland was the only site of tumor development in the local

male Wistar rat, while the kidneys were the most commonly affected organ in females. In these organs, preneoplastic lesions were not registered or were not related to tumor development—in the kidneys the registered preneoplastic lesion is a marker for renal cell adenocarcinoma,²⁵⁾ and not for RMT. Sequential analysis of chemically induced carcinogenesis in rodents indicated that the number of preneoplastic lesions generally exceeds that of neoplasia, indicating that not all putative preneoplastic lesions are committed to progression towards neoplasia, and some probably regress.³⁰⁾ The possibility also exists that the time frame of 30 weeks adopted in this study was not long enough for the development of neoplasia in the organs that developed preneoplastic lesions.

The site distribution and frequency of DHPN-induced tumors in the local Wistar strain differ from previous reports on other rat strains, that indicate the lung as the main target organ for tumor development, followed by the thyroid, kidney and liver.^{15, 16, 18, 19)} One male rat (10%) exposed to 0.2% DHPN developed follicular adenoma in the thyroid, and no other tumor was registered (Tables I and II). Although Konishi *et al.*¹⁵⁾ and Hiasa *et al.*¹⁶⁾ found no tumor development in male Wistar rats after a 39-week daily administration of 500 ppm of DHPN in d.w. and after s.c. injections of 0.7 g/kg of DHPN once a week for 4 weeks, respectively, the present incidence is similar to the 8% reported in male Wistar animals 20 weeks after a single i.p. injection of 2.1 g/kg of DHPN.¹⁷⁾ The incidence of thyroid adenomas is also lower than that of 35% reported 50 weeks after 0.2% DHPN d.w. administration to male F344 rats for 7 days¹⁹⁾ and those registered 48 weeks after DHPN exposure for 14 and 21 days, which were, respectively, 78% and 100%.¹⁸⁾ Therefore, the present results indicate that the local male Wistar rats are relatively more resistant than the male F344 rats to induction of neoplasia in the thyroid gland by a high dose of DHPN.

Among male rats exposed to the DMBDD protocol, only one rat (8%) developed one adenoma in the kidney, an incidence similar to the 5% observed by Yamamoto *et al.*²⁸⁾ in male F344/DuGrj rats, but different from those of Takahashi *et al.*⁷⁾ and Kimura *et al.*,²⁹⁾ who respectively reported renal adenoma incidences of 33% and 21% in the F344 strain. Among female rats, a striking finding was the development of RMTs in the group exposed to 0.2% DHPN (incidence of 20%) or to the DMBDD protocol (incidence of 36%). The RMT is an exclusively mesenchymal neoplasm³¹⁾ that was previously observed in this laboratory with an incidence of 33% in female Wistar rats submitted to the DMBDD protocol (Rocha *et al.*, unpublished results).

The reduced water intake during the first 4 weeks of 0.2% DHPN treatment, with consequent decreased intake of the carcinogen, could partially explain the relatively

low incidence of neoplasia in the local Wistar rats, compared with the observed incidence in the F344 strain.^{18, 19)} However, Shirai *et al.*¹⁸⁾ also registered diminished levels of DHPN ingestion, and observed larger number of neoplasia in F344 rats exposed to DHPN at the same dose level as in this study. Therefore, strain differences could explain the relative low incidence of neoplasia in the local Wistar rats. Differences in quantitative and/or qualitative metabolic degradation, route of administration, variations in strain-related enzymatic activation and other intrinsic factors might be involved in determining target organ specificity and susceptibility.³²⁾ In line with the low incidence of preneoplastic lesions, no tumors were found in the selected organs of male or female 0.1% DHPN-treated Wistar rats. Hasegawa *et al.*²⁰⁾ and Yoshida *et al.*²⁷⁾ respectively reported incidences of 100% and 20% of neoplasias in the lung and of 5% and 7% in the thyroid gland in F344 male rats exposed to 0.1% DHPN. These observations again indicate the relative resistance of both sexes of the local Wistar rat strain to the carcinogenic influence of a relatively low dose of DHPN.

Apparently, hormonal status at the time of the treatment and during the carcinogenic process, together with the type of carcinogen and the animal's age, play important roles in the differences among sexes regarding chemical induction of cancer.³³⁾ Thus, female Wistar rats were more sensitive than males to the carcinogenic activity of 0.2%

DHPN or DMBDD treatments, developing more neoplasias in more target organs than the males. These animals seemed to be particularly prone to the development of renal mesenchymal tumors.

In conclusion, the present findings indicate that the Wistar strain of rats, although relatively resistant to the carcinogenic activity of DHPN, develops preneoplastic lesions and tumors in the main target organs of this compound. A dose- and sex-related carcinogenic activity was registered, since animals treated with 0.1% DHPN presented low incidence of preneoplasia and no tumor development after 30 weeks. Despite the relative resistance of the Wistar strain of rats, we consider that DHPN can be used as an initiating agent in alternative medium-term assays for carcinogenesis which adopt that strain as the test system.

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