The Utility of N-nitrosamines as Initiators for a 26-Week Rat Two-Stage Nasal Carcinogenesis Model

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Abstract: To examine the utility of N-nitrosamines as initiators for a 26-week two-stage rat nasal carcinogenesis model, male F344 rats were treated with 2,6-dimethylaniline (DMA), possessing promoting potential for the rat nasal cavity, after initiation with 3 subcutaneous injections of 1500 mg/kg of N-bis(2-hydroxypropyl)nitrosamine (DHPN), 30 mg/kg of N-nitrosobis(2-oxypropyl)amine (BOP), or 30 mg/kg of N-nitrosopiperidine (NPIP). Histopathologically diagnosed nasal epithelial hyperplasias and tumors, including carcinomas, were induced with all three, with significant increase in the DHPN+DMA, BOP+DMA, and NPIP+DMA groups compared with the DHPN alone, BOP alone, and NPIP alone groups, respectively. These results strongly suggested that repeated subcutaneous injections of DHPN, BOP, or NPIP are useful for initiation in the present 26-week nasal carcinogenesis model.

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Key words: N-bis(2-hydroxypropyl)nitrosamine, N-nitrosobis(2-oxypropyl)amine, N-nitrosopiperidine, 2,6-dimethylaniline, rat, two-stage nasal carcinogenesis model

Introduction

2,6-Dimethylaniline (DMA), one of the major metabolites of xylazine finding use as an α₂-adrenergic agonist in veterinary medicine, has been shown to cause a significant increase in the incidence of nasal tumors in both male and female CD rats when fed in the diet at 3000 ppm for 2 years¹. In our previous two-stage nasal carcinogenesis study in rats, the development of epithelial hyperplasias, dysplastic foci, and epithelial tumors, including adenomas and carcinomas, in the nasal cavity of male F344 rats receiving a single subcutaneous (s.c.) injection of 2400 mg/kg N-bis(2-hydroxypropyl)nitrosamine (DHPN), was also clearly enhanced by the dietary administration of 3000 ppm DMA for 52 weeks². The results strongly suggested that DHPN acts as an initiator of nasal carcinogenesis while DMA exerts tumor promoting effects. However, the experimental duration of this model is too long for general application to detect promoting potential of chemicals targeting the nasal tissues.

DHPN is well known to possess carcinogenic potential in the nasal cavity of rats³,⁴, Mohr et al. reporting very high incidences of epithelial tumors in this site in SD rats given s.c. injections of 178–1425 mg/kg DHPN once weekly for 20 weeks³. In addition, Ichinose et al. reported the incidence of nasal tumors in rats receiving a single intraperitoneal injection of DHPN at a dose of 5000 mg/kg to be 97–100% after 17 months⁵. Both N-nitrosobis(2-oxypropyl)amine (BOP) and N-nitrosopiperidine (NPIP) are also known to have carcinogenic potential in the rat nasal cavity⁶,⁷. Pour et al. reported the induction of nasal neoplastic lesions, including polyp, papillomas and squamous cell carcinomas, at total incidences of 75% and 67% in rats given weekly s.c. injections of 10 mg/kg BOP for the life span and 20 mg/kg BOP for 30 weeks, respectively⁶,⁷. Vollrath et al. and Sugihara reported nasal neuroblastoma development in 90% and 56% of rats receiving s.c. injections of 10 mg/kg NPIP twice weekly for 11 months and 35–38 weeks, respectively⁶,⁷. Therefore, repeated s.c. injections of DHPN, BOP, or NPIP may be appropriate as initiating treatments for a medium-term rat two-stage nasal carcinogenesis model.

The present study was performed to evaluate the utility of these initiators for a 26-week nasal carcinogenesis model in rats, using DMA as an established promoter.
Materials and Methods

Animal and chemicals

A total of 110 male F344 rats were obtained from Charles River Japan Inc., Kanagawa, Japan, at 4-weeks of age and housed 3 or 4 to a polycarbonate cage with wood chips for bedding under barrier system conditions (room temperature, 23 ± 2°C; relative humidity, 55 ± 5%; air changes, 18 times/hr; 12 hr-light/12 hr-dark cycle). They were quarantined for 1 week in the animal room, and given pulverized basal diet (Oriental CRF-1, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. All animals were handled in accordance with the Guideline for Animal Experimentation of the Japanese Association for Laboratory Animal Science.

DMA (purity, >99%) and NPIP (purity, >95%) were obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan) and Kanto Chemical Co. Inc. (Tokyo, Japan), respectively. DHPN and BOP were from Nacalai Tesque, Inc., (Kyoto, Japan).

Experimental design

The experimental design is summarized in Fig. 1. Ninety male F344 rats were divided into 3 groups, each composed of 30 rats, and given 3 subcutaneous injections of 1500 mg/kg of DHPN, 30 mg/kg of BOP, or 30 mg/kg of NPIP in isotonic sodium chloride solution at weekly intervals over the initial 3 weeks. On the basis of the median lethal dose (LD50) of DHPN, BOP or NPIP with single subcutaneous injection in rats, the injection dose levels in the present study were determined to be about 1/3 of the LD50 values. The remaining twenty animals were given isotonic sodium chloride solution alone in the same manner. Starting 1 week after the last injection of these initiators or the vehicle, the animals were subdivided into DHPN alone, DHPN+DMA, BOP alone, BOP+DMA, NPIP alone, and NPIP+DMA (15 each), Untreated and DMA alone (10 each) groups. The animals in the DMA-treated groups and the others were given pulverized diet containing 3000 ppm DMA and pulverized basal diet, respectively, for 26 weeks. DMA was first dissolved in corn oil (Sigma Aldrich Japan, Tokyo, Japan) and then directly admixed with the diet in 10 ml of corn oil per 1 kg. Test diets were prepared every week, stored in a refrigerator (temperature, 4°C) and changed twice a week. Stability data showed the DMA level in the diet stored at room temperature for 1 week to be 87.4% of that sampled at the preparation9. Individual body weights were measured every week, and food consumption for each cage was measured every week during the first 13 weeks and once every 2-week thereafter. The mean actual intakes of DMA for each group were calculated from the mean body weights and mean food consumption.

Necropsy and light microscopic examination

All animals were killed under ether anesthesia by exsanguination from the abdominal aorta at week 26, and subjected to a complete necropsy. Nasal cavities were fixed with 10% neutral buffered formalin after flushing the fixative into the posterior opening of the nasopharyngeal duct with a syringe. Following fixation, the heads were decalcified in 5% formic acid solution. Using the upper incisor teeth, incisive papilla, and first upper molar tooth as guides for trimming, six transverse sections including Levels I, II, and III of the nasal cavity were prepared based on the method of Nagano et al.10. The nasal sections were embedded in paraffin, sectioned at 4–5 µm and stained with hematoxylin and eosin (H & E) for light microscopic examination.

Statistical analysis

Raw data for body weights and food consumption were used to generate mean and standard deviation values, and intergroup differences were then analyzed by the Student’s *t*-test. In addition, the incidences and multiplicity of nasal proliferative lesions were analyzed by the Fisher’s exact test with permutation resampling11.

Results

No animals died or became moribund during the experimental period. There were no differences in body weights and food consumption between any of the treatment groups and the Untreated group. Mean actual intakes of DMA in the DMA-treated groups were 153–173 mg/kg/day. Histopathologically, epithelial hyperplasias and/or
tumors in the nasal cavity were observed in the olfactory, respiratory and/or squamous epithelium of all treated groups except the Untreated and DMA alone groups (Tables 1 and 2, Figs. 2–5). The hyperplastic lesions comprised simple, papillary, basal cell and glandular hyperplasias with/without squamous metaplasia (Tables 1 and 2). The tumors were squamous cell papillomas, adenomas, Bowman’s gland adenomas, squamous cell carcinomas, and undifferentiated carcinomas (Tables 1 and 2). Both epithelial hyperplasias and tumors were observed in all groups receiving DHPN, BOP, or NPIP initiation plus DMA treatment. Epithelial hyperplasias and tumors were observed in the DHPN + DMA group at incidences of 15/15, 2 (13.3) in the Untreated group and at incidences of 15/15, 2/15, and 1/15, respectively (Table 1), giving incidences of total tumors of 7/15, 9/15, and 6/15, respectively (Table 1). Although epithelial hyperplasias were observed in the DHPN, BOP, and NPIP alone groups at high incidences, actual tumors were only found with DHPN and NPIP and at an incidence of 2/15 in each case. Neither epithelial hyperplasias nor tumors were observed in the Untreated and DMA alone groups. The combined incidences of nasal tumors were significantly increased in the BOP + DMA group as compared with the BOP alone group (Table 1). The multiplicity of nasal epithelial hyperplasias was also significantly increased in the DHPN + DMA and NPIP + DMA groups as compared with the DHPN alone and NPIP alone groups, respectively (Table 2). Glandular hyperplasia was observed most frequently of all epithelial hyperplasias in the DHPN + DMA, NPIP alone, and NPIP + DMA groups (Table 2), multiplicities being significantly higher in the DHPN + DMA and NPIP + DMA groups than in the DHPN alone and NPIP alone groups, respectively (Table 2). In addition, the multiplicity of basal cell hyperplasias in the NPIP + DMA group was also significantly greater than that in the NPIP alone group (Table 2).

Discussion

In our previous time-course study to examine DMA-induced nasal proliferative lesions using a 52-week rat two-stage nasal carcinogenesis model after initiation with a

| Table 1. Incidences of Nasal Proliferative Lesions in Rats Fed Diet Containing DMA for 26 Weeks Following DHPN, BOP or NPIP Initiation |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Groups                     | No. of animals examined     | Epithelial hyperplasia focus | Dysplastic squamous cell papilloma | Adenoma | Carcinoma | Total of tumors |
| Untreated                  | 10                          | 0                           | 0                              | 0                   | 0                   | 0                           |
| DMA alone                  | 10                          | 0                           | 0                              | 0                   | 0                   | 0                           |
| DHPN alone                 | 15                          | 12 *(80)                    | 2 (13.3)                      | 0                   | 0                   | 2 (13.3)                    |
| DHPN + DMA                 | 15                          | 15 (100)                    | 4 (26.7)                      | 2 (13.3)            | 1 (6.7)             | 7 (46.7)                    |
| BOP alone                  | 15                          | 8 (53.3)                    | 0                              | 0                   | 0                   | 0                           |
| BOP + DMA                  | 15                          | 11 (73.3)                   | 3 (20)                        | 4 (26.7)            | 2 (13.3)             | 9 *(60)                     |
| NPIP alone                 | 15                          | 14 (93.3)                   | 1 (6.7)                       | 1 (6.7)             | 0                   | 2 (13.3)                    |
| NPIP + DMA                 | 15                          | 15 (100)                    | 3 (20)                        | 2 (13.3)            | 1 (6.7)             | 6 (40)                      |

a: Number of animals with lesions (%).
b: Significantly different from the BOP alone group at P<0.01 (Fisher’s exact test with permutation resampling).

c: Significantly different from the NPIP alone group at P<0.01 (Fisher’s exact test with permutation resampling).

| Table 2. Multiplicity of Nasal Epithelial Hyperplasias in Rats Fed Diet Containing DMA for 26 Weeks Following DHPN, BOP or NPIP Initiation |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Groups                     | No. of animals examined     | Simple hyperplasia          | Papillary hyperplasia       | Basal cell hyperplasia      | Glandular hyperplasia       | Total |
| Untreated                  | 10                          | 0                           | 0                            | 0                            | 0                            | 0     |
| DMA alone                  | 10                          | 0                           | 0                            | 0                            | 0                            | 0     |
| DHPN alone                 | 15                          | 1.00 ± 1.00*                | 0.27 ± 0.46                  | 0.27 ± 0.46                  | 0.33 ± 0.62                  | 1.87 ± 1.70 |
| DHPN + DMA                 | 15                          | 0.60 ± 0.91                 | 0.60 ± 0.99                  | 0.33 ± 0.49                  | 3.20 ± 1.70                  | 4.73 ± 1.87  |
| BOP alone                  | 15                          | 0.13 ± 0.35                 | 0.27 ± 0.46                  | 0                            | 0.20 ± 0.41                  | 0.60 ± 0.63  |
| BOP + DMA                  | 15                          | 0.20 ± 0.56                 | 0.53 ± 0.52                  | 0.13 ± 0.35                  | 0.47 ± 0.74                  | 1.33 ± 1.11  |
| NPIP alone                 | 15                          | 0.20 ± 0.56                 | 0.07 ± 0.26                  | 0.20 ± 0.41                  | 2.80 ± 1.57                  | 3.27 ± 1.79  |
| NPIP + DMA                 | 15                          | 0.13 ± 0.35                 | 0.47 ± 1.06                  | 1.53 ± 1.30                  | 10.07 ± 3.65                 | 12.20 ± 3.90  |

a: Mean ± S.D.
b: Significantly different from the DHPN alone group at P<0.01 (Fisher’s exact test with permutation resampling).
c: Significantly different from the NPIP alone group at P<0.01 (Fisher’s exact test with permutation resampling).
single s.c. injection of 2400 mg/kg DHPN, adenomas were observed at 26-week interim kill, and carcinomas at 52-week terminal kill\(^9\). In the present study, nasal tumors, including carcinomas, were induced within 26 weeks in the DHPN+DMA, BOP+DMA, and NPIP+DMA groups, with clearer higher incidences than the respective carcinogen alone groups. Thus the promoting potential of DMA on the nasal olfactory mucosa proved readily detectable in the present medium-term study, with repeated s.c. injections of DHPN, BOP, or NPIP evidently appropriate for initiation.

The olfactory epithelium of the nasal cavity in rodents is made up of sustentacular, sensory, and basal cells\(^12,13\). The latter are generally considered as stem cells for regeneration of the olfactory epithelium\(^12,13\). In the rat nasal cavity, papillomas, adenomas, neuroepitheliomas, neuroblastosomas, adeno-squamous cell carcinomas, squamous cell carcinomas, transitional cell carcinomas, and/or undifferentiated carcinomas have all been induced with s.c. or intraperitoneal injection of DHPN, s.c. injection of BOP, and/or s.c. injection or oral administration (in the drinking water) of NPIP\(^3–7,14\). In addition, nasal polyp, papillomas, adenomas, squamous cell carcinomas, adenoacarcinomas, neuroendocrine carcinomas, neuroepitheliomas, and neuroblastomas result from intraperitoneal/s.c. injection or oral administration (in the drinking water) of other \(N\)-nitrosamines, including \(N\)-nitrosomorpholine, \(N\)-nitrosomethylpiperazine and \(N\)-nitrosonornicotine, in laboratory rodents\(^3,5,15,16\). In a review of nasal cavity carcinogenesis due to \(N\)-nitrosamines, Schüller reported epithelial tumors to arise from basal cell hyperplasias following cellular injury in the squamous, respiratory and olfactory epithelium in rodents\(^15\). In addition, we have reported that Bowman’s glands are a target of DMA, adenomas developing from the cells initiated by DHPN in the epithelium of Bowman’s glands.
via glandular hyperplasias in a rat-two stage nasal carcinogenesis model after initiation with DHPN\textsuperscript{9,17}. Considering these reports, we can conclude that basal cell hyperplasias and/or glandular hyperplasias of the nasal epithelium are morphological lesions indicating the magnitude of initiating effects of \(N\)-nitrosamines. In the present study, their incidences were much higher in the NPIP+DMA group than in the DHPN+DMA and BOP+DMA groups. The findings thus suggest that initiating effects of NPIP on the rat nasal olfactory mucosa were stronger than those of DHPN or BOP under the experimental conditions in our study.

We here identified squamous metaplasias in glandular hyperplasias as a conspicuous modification. However, such metaplasia was not evident in our previous investigations using a single s.c. injection of 2400 mg/kg DHPN as the initiator treatment\textsuperscript{9,17}. Therefore, squamous metaplasias are apparently dependent of the level of carcinogen exposure, arising only with repeated dosing of DHPN, BOP, and NPIP.

Recently, the number of pharmaceutical companies intending to change the clinical route of application for their medicines from intravenous injection to intranasal administration has been increasing. Clearly, medium-term nasal carcinogenesis models need to be established to detect possible carcinogenic and/or promoting potential of medicines on the nasal cavity. The present model may evidently have utility for evaluating promoting effects on nasal tissues. Further studies are now underway to test its capacity to detect modifying effects of medicines or other chemicals.

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References


