Promoting Effect of Snuff in Rats Initiated by 4-Nitroquinoline-N-Oxide or 7,12-Dimethylbenz(a)anthracene

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ABSTRACT

A canal was surgically created in the lower lip of male Sprague-Dawley rats and used as a reservoir for moist snuff. A total of 230 animals were randomized into six groups, five containing 40 rats and one containing 30 rats. After 2 wk of recuperation, the animals were treated as follows. Group I was initiated with 7,12-dimethylbenz(a)anthracene 3 times/wk for 4 wk followed by cotton pellet administration. Group II was initiated with 7,12-dimethylbenz(a)anthracene for 4 wk followed by snuff twice a day, 5 days/wk. Group III received snuff twice a day, 5 days/wk. Groups IV and V were initiated with 4-nitroquinoline N-oxide 3 days/wk for 4 wk. Thereafter Group IV received a cotton pellet, and Group V rats were treated with snuff twice a day, 5 days/wk. Group VI received a cotton pellet once a day, 5 days/wk. Treatment of all groups continued for a maximum of 104 wk. Group V rats had a significantly lower mean survival time than did the other groups because of the development of lip sarcomas in 66% of the rats as compared with 23% in Group II and 26% in Group III. One rat in each of Groups IV and VI developed lip sarcomas. The incidence of sarcomas in Group V as compared with the other groups is statistically significant (P < 0.05 to 0.001). Spindle cell proliferation, a possible precursor lesion of lip sarcoma, was found in five rats of Group II, seven of Group III, and four of Group V. These results show that snuff has strong promoting capability with regard to the development of lip sarcomas after 4-nitroquinoline N-oxide initiation, but not after 7,12-dimethylbenz(a)anthracene initiation. Snuff by itself caused three squamous carcinomas of the palate, two squamous cell papillomas of the lip, and ten lip sarcomas (in 38 rats as compared with one lip sarcoma in 30 control rats), showing snuff to be carcinogenic for the lip and oral cavity.

INTRODUCTION

Snuff dipping is prevalent among young males in western Europe and the United States (1). The use of moist snuff has increased during the last decade and is associated with serious health effects (2, 3). Snuff is a carcinogenic hazard in humans as emphasized by the International Agency on Research on Cancer and the NIH (4, 5). The risk of developing oral cancer in humans increases with increasing time of exposure and is approximately 50 times that of non-snuff users after four decades or longer of exposure (6). Most animal studies with snuff have been negative with regard to carcinogenesis. This is partly related to the absence of suitable animal models for snuff exposure. In a number of studies, a surgically created canal in the lower lip has been used as a snuff reservoir (7,9). This model, which was initially described by Hirsch and Thilander in 1981, mimics the human situation since snuff is mixed with saliva which presumably aids in the extraction of toxins from snuff as well as their distribution (10). This model has been related to the absence of suitable animal models for snuff exposure. In a number of studies, a surgically created canal in the lower lip has been used as a snuff reservoir (7, 9). This model, which was initially described by Hirsch and Thilander in 1981, mimics the human situation since snuff is mixed with saliva which presumably aids in the extraction of toxins from snuff. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.1 This study was supported by Smokeless Tobacco Research Foundation Grant 0144 and by NIH Research Grant CA36727.

3 The abbreviations used are: 4-NQO, 4-nitroquinoline N-oxide; DMBA, 7,12-dimethylbenz(a)anthracene; NAT, N'-nitrosanatabine; NAB, N'-nitrosobenamine; NNK, (N'-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone; GLC, gas-liquid chromatography; TCA, thermal energy analyzer; TSA, tobacco-specific N-nitrosamines; CRT, cathode ray tube; NN, N'-nitrosornicotine.
The mixture was applied to an Extrelut column (150-cm x 20-mm internal diameter), and after 15 min the column was eluted with dichloromethane (4 x 25 ml). The eluate was concentrated to about 1 ml in a water bath at 55°C, transferred to a vial, and diluted to 5.0 ml with chloroform.

Analyses were performed on an isothermal GLC (Model 2700; Varian, Palo Alto, CA) interfaced with a TEA (Model 502; Thermo Electron Corp., Waltham, MA). The furnace was removed from the TEA and connected to the GLC column via a 5.5-cm-long glass tube. For TSNA quantitation, aliquots of snuff extract (5.0 μl) were analyzed against external standards by injection onto a glass column (1.8-m x 1.9-mm internal diameter) containing 10% UCW-982 on Chromosorb W, AW-DMCS, 80/100 mesh. The GLC-TEA conditions were as follows: column temperature, 200°C; injector temperature, 250°C; helium carrier-gas flow, ~28 ml/min; furnace temperature, 475°C; oxygen flow, ~10 ml/min; and vacuum pressure, ~0.6 mm of Hg. A CRT gas stream filter (Thermo Electron Corp.) was used. The detection limits of the method were 0.01 to 0.02 mg of TSNA/kg of snuff (wet weight).

**Analysis of Volatile N-Nitrosamines**

The 5-ml extract from the analysis of TSNA above was concentrated to about 0.5 μl in a water bath at 70°C. The final volume was measured with a 1000-μl Hamilton syringe.

Analyses were carried out by injection of 5.0-μl aliquots into a GLC-TEA equipped with a 1.8-m x 1.9-mm (internal diameter) glass column containing 20% Carbowax 20M and 2% KOH on Chromosorb W, AW-DMCS, 80/100 mesh. The GLC-TEA conditions were as follows: column temperature, 160°C; injector temperature, 200°C; helium carrier-gas flow, ~27 ml/min; furnace temperature, 475°C; oxygen flow, ~10 ml/min; and vacuum pressure, ~0.7 mm of Hg. A CRT gas stream filter was used.

The detection limits of the method were 0.5 to 1 μg of volatile nitrosamines/kg of snuff (wet weight).

Duplicate analyses were performed on every tenth box of snuff, and altogether 58 boxes were analyzed. The levels of tobacco-specific and volatile N-nitrosamines are given in Table 1. The values are based on wet weight (moisture content about 48%). The last 13 samples contained an unidentified N-nitrosamine, probably a tobacco-specific N-nitrosamine.

**Experimental Design**

The animals were allowed to recuperate for 2 wk after surgery before instillation of snuff or initiators. The end of this healing period and the beginning of treatment were considered to be the zero time of the experiment. In four rats (two in Group III and two in Group V), the creation of the surgical canals was not successful, and these rats were not used. The following groups were used:

- **Group I (40 Rats).** These rats were initiated with DMBA 3 times/wk for 4 wk. Thereafter they received a cotton pellet dipped in physiological saline once a day, 5 days/wk, for 104 wk.
- **Group II (40 Rats).** These rats were initiated with DMBA as in Group I for 4 wk, whereupon they received snuff twice a day, 5 days/wk for 104 wk.
- **Group III (38 Rats).** These rats received snuff twice a day, 5 days/wk from Day 0 of the experiment for 104 wk.

**Group IV (40 Rats).** These rats were initiated with 4-NQO 3 times/wk for 4 wk, whereupon they were treated with a cotton pellet dipped in physiological saline once a day, 5 days/wk for 100 wk.

- **Group V (38 Rats).** These rats were initiated with 4-NQO 3 times/wk for 4 wk, followed by snuff twice a day for 100 wk.
- **Group VI (30 Rats).** These rats received cotton pellets dipped in saline once a day, 5 days/wk, for 100 wk.

The rats were killed when moribund, when they developed lip tumors, or 104 wk after Day 0 of the experiment.

**Morphological Methods**

Animals were killed by CO₂ asphyxiation, and underwent a complete necropsy where the gross appearance of tumors and other pathological lesions was recorded. Specimens were routinely taken from the lip and lower jaw, lungs, heart, liver, esophagus, forestomach, glandular stomach, kidneys, urinary bladder, and other grossly abnormal tissues. The head was fixed separately for 48 h and divided longitudinally between the nostrils. After decalcification, three sections were taken from each half allowing optimal visualization of the palate and nasal cavity. The tissue specimens were fixed in 4% neutral buffered formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The lip lesions were also stained with antibodies against vimentin, desmin, S-100 protein, and low- and high-molecular-weight keratin by a conventional immunoperoxidase method.

**Statistical Methods**

Statistical significance was calculated by the Student t test and Fisher's exact test. A P value of < 0.05 was regarded as statistically significant (13).

**RESULTS**

The mean survival time as measured from Day 0 of the experiment is given in Table 2. There was a significant difference in the survival time between the rats in Group V (4-NQO followed by snuff) and all of the other groups (P < 0.05 to 0.01).

**Table 2 Mean survival time in the different groups (measured from Day 0)**

<table>
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<tr>
<th>Group</th>
<th>n</th>
<th>Wk</th>
<th>Range</th>
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<tbody>
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<td>I</td>
<td>40</td>
<td>92</td>
<td>62–104</td>
</tr>
<tr>
<td>II</td>
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<td>III</td>
<td>38</td>
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<td>23–104</td>
</tr>
<tr>
<td>IV</td>
<td>40</td>
<td>88</td>
<td>36–104</td>
</tr>
<tr>
<td>V</td>
<td>38</td>
<td>75</td>
<td>29–104</td>
</tr>
<tr>
<td>VI</td>
<td>30</td>
<td>92</td>
<td>34–104</td>
</tr>
</tbody>
</table>

*Statistically significant compared with Groups I, II, III, IV, and IV (P < 0.05 to 0.01).
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**Table 3 Incidence of neoplasms in the head and neck region and in the gastrointestinal tract**

<table>
<thead>
<tr>
<th>Location</th>
<th>Type of tumor</th>
<th>Group I (n = 40)</th>
<th>Group II (n = 40)</th>
<th>Group III (n = 38)</th>
<th>Group IV (n = 40)</th>
<th>Group V (n = 38)</th>
<th>Group VI (n = 30)</th>
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<tbody>
<tr>
<td>Lip</td>
<td>Squamous cell papilloma</td>
<td>2</td>
<td>1</td>
<td>2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Squamous cell carcinoma</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Sarcoma</td>
<td>9</td>
<td>10</td>
<td>1</td>
<td>25</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spindle cell proliferation</td>
<td>5*</td>
<td>7*</td>
<td>1</td>
<td>4*</td>
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<td></td>
</tr>
<tr>
<td>Palate</td>
<td>Squamous cell carcinoma</td>
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<td>3</td>
<td>6</td>
<td>5</td>
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</tr>
<tr>
<td>Buccal mucosa</td>
<td>Squamous cell carcinoma</td>
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<td></td>
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</tr>
<tr>
<td>Nasal cavity</td>
<td>Squamous cell carcinoma</td>
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<td></td>
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<td>1</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
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<td></td>
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<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin of ear</td>
<td>Neurofibroma</td>
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<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malignant fibrous histiocytoma</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>3</td>
<td>17</td>
<td>19</td>
<td>13</td>
<td>36</td>
<td>2</td>
</tr>
</tbody>
</table>

*Spindle cell proliferations are not included in the total number of tumors.

A large number of the rats in Group V developed lip tumors, necessitating early termination. The initial weight of the rats in all groups varied between 331 and 349 g in the different groups. The mean weight of the rats during the experiment is given in Fig. 1. From wk 30 to a peak at wk 65, the differences in weight of the rats receiving snuff (Groups II, III, and V) were significantly lower than those of Groups I, IV, and VI (P < 0.01 to 0.001). The differences varied between 75 and 110 g, and this trend continued throughout the experiment. There was no statistically significant difference in food and water consumption among the different groups.

The incidences of tumors to the head and neck region and gastrointestinal tract are given in Table 3. Lip sarcomas were found in 66% of Group V rats initiated with 4-NQO followed by snuff. In Groups II and III, 23 and 27% of the rats, respectively, developed sarcomas, while only one rat of 40 in the 4-NQO only (Group IV) and one rat in the control group developed a sarcoma. The differences in incidences of sarcomas between Group V and Groups I, II, IV, and VI are statistically significant (P < 0.5 to 0.01). The difference is also significant between the rats in Groups II and III and Groups I, IV, and VI (P < 0.001). The sarcomas in Group V developed as early as 29 wk. The tumors were large (up to 4 cm in diameter), solid, polyoid masses, frequently replacing the whole lip and involving the surrounding tissue (Fig. 2). The majority of tumors were undifferentiated spindle cell sarcomas invading the striated muscle and surrounding subcutaneous tissue as well as the bone (Figs. 3 to 5). Some of the sarcomas showed features of malignant fibrohistiocytoma (Fig. 6). In one case (Group II), metastatic deposits were found in the lungs and another in the neck lymph nodes (Group V, Fig. 7). Immunohistochemical staining with antibodies against vimentin was positive in all sarcomas while keratin staining was negative, confirming the nonepithelial mesenchymal nature of the tumors. Three tumors stained positive with antibodies against S-100 protein: the nasal cavity-olfactory nerve sarcoma and 2 lip sarcomas indicating Schwann's cell origin. Two lip sarcomas stained positive with antibodies against muscle-specific actin and desmin, supporting muscle origin of these tumors. Five rats in Group II, 7 in Group III, and 4 in Group V had benign looking spindle cell proliferations (Fig. 8) in the lip which were not apparent upon gross inspection and were detected only during light microscopy. These lesions were composed of aggregates of spindle cells with bland looking nuclei (Fig. 9). For comparison, Fig. 10 shows normal lip histology for a control rat (Group VI). Some of the

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Fig. 2. Gross appearance of a lip sarcoma in a rat initiated with 4-NQO followed by snuff.
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Fig. 3. Lip sarcoma completely surrounding the lip canal of a Group III rat. H & E, x 30.

Fig. 4. Another area of the same tumor in Fig. 3 showing a pleomorphic sarcoma invading striated muscle. H & E, x 240.

cells expressed muscle-specific actin and possibly represent myofibroblasts. These cell proliferations were not associated with ulceration or granular tissue formation.

The number of epithelial tumors, including squamous cell papilloma and carcinoma, are listed in Table 3, and there were no statistically significant differences among Groups II, III, IV, and V. Thus, 9 rats treated with 4-NQO alone developed carcinomas of the lip, palate, or buccal or nasal cavity in comparison with 6 rats treated with 4-NQO followed by snuff and 3 rats treated with DMBA followed by snuff or snuff only. The difference is not significant. It is interesting that 28 rats in Group V developed 36 tumors of the head and neck region or gastrointestinal tract as compared with 13 tumors in 10 rats in Group IV and 19 tumors in 14 rats in Group III. In Group II, 17 tumors were found in 14 rats. The incidence, location, and types of neoplasms outside of the head and neck or gastrointestinal tract are given in Table 4. Most of the tumors in these rats were found in those treated with DMBA with or without snuff (11 and 11, respectively). Only 3 tumors were found in the control group.

DISCUSSION

Chronic snuff exposure followed by 4-NQO (Group V) significantly reduced the mean survival time of the rats compared with all of the other groups ($P < 0.05$ to 0.01). In this study, initiation with 4-NQO but not DMBA, followed by promotion with snuff, resulted in a high (66%) incidence of sarcomas. This contrasts with an earlier study when 4-NQO followed by snuff did not result in increased tumor development and is related to the anatomical site of initiation and the amount of 4-NQO

Fig. 5. Spindle cell sarcoma invading bone in a Group V rat. H & E, x 120.

Fig. 6. Sarcoma showing features of malignant fibrohistiocytoma with a storiform pattern in a Group V rat. H & E, x 96.

Fig. 7. The same tumor as in Fig. 6 showing metastatic deposits in a neck lymph node. H & E, x 120.
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In addition, the inflammatory changes in the lip were associated with discomfort, preventing optimal food intake as evidenced by the lower food consumption and body weight (9).

We were unable to show significant promotion of epithelial tumors derived from the lip or oral squamous epithelium. However, the presence of 5 squamous lesions in Group V (2 papillomas of the lip and 3 squamous cell carcinomas of the palate) suggests that snuff has a weak carcinogenic potential with regard to squamous lesions, and these are the main lesions seen in humans. Chronic snuff exposure following initiation in the lip canal with 4-NQO was found to be a potent promoter. Thus, 66% of rats (28 of 38) exposed to this regimen developed sarcomas compared with one of 40 treated with 4-NQO only and one of 30 control rats ($P < 0.01$ to 0.001). In rats treated with DMBA followed by snuff, 23% developed sarcomas, and in the rats treated with snuff only, 26% developed sarcomas (not significantly different). The difference in tumor incidences between Group V and Groups I, II, and III, respectively, is

Fig. 8. A section of the lip canal in a Group III rat showing spindle cell proliferation. H & E, × 60.

administered (9, 14). In the first study, initiation took place on the hard palate, and the rats received only $\frac{1}{2}$ of the amount of 4-NQO used in the present study (800 mg during a 4-wk period). In another study (14), initiation took place in the lip, but the rats received only $\frac{1}{8}$ of the amount of 4-NQO compared with the present study. Initiation in the lip with DMBA followed by snuff did not result in an increased number of tumors as compared with snuff alone. 4-NQO was a more potent initiator than was DMBA. This may reflect the facile activation of 4-NQO by seryl tRNA synthetase (15). The shorter survival in the present study reflects the significant number (66%) of rats that developed lip tumors, necessitating early sacrifice.

The initial body weight between groups was virtually identical after a few weeks; however, it was found that the rats exposed to snuff (Groups II, III, and IV) with or without initiation had significantly lower weight gains. These peaked at 35 wk and remained relatively constant throughout the remainder of the experiment (Fig. 1). At 35 wk, rats weighed approximately 100 g less in groups treated with snuff as compared with the other groups. In the present study, there was no significant difference in the food consumption between the snuff-treated rats and the other groups of rats. The snuff-exposed rats have a high serum concentration of nicotine (9), which results in an increased level of activity and restlessness, and this may have contributed to the lower weight gain among the snuff-treated rats. The water consumption did not significantly differ among the different groups of rats. All groups had, however, a somewhat increased water consumption toward the end of the experiment, possibly as a result of the development of rat nephrosis, an age-associated lesion commonly seen in male Sprague-Dawley rats. This was present in the majority of rats at the time of sacrifice (16). These results are similar to those observed previously (7, 9).

In our earlier study, the weight difference could be explained to a large degree by the severe inflammatory changes in the lip of snuff-treated rats. This was associated with foreign body giant cell reaction (9). The inflammatory changes and the foreign body giant cell reaction were much less pronounced in the present study and could be related to the use of generic snuff compared with the earlier study where a commercially available brand was used.

Fig. 9. Higher power of Fig. 8 showing the bland appearing spindle cells. Note the absence of inflammatory changes. H & E, × 120.

Fig. 10. A section of the lip canal from a control rat (Group VI) showing a normal appearance. H & E, × 96.
factors for the promotional effect of snuff since 4-NQO fol-
as TSNA (which are present in mg/kg levels), are important
tumor development. It is also possible that carcinogens, such
over following snuff treatment, which may be a prerequisite for
bromodeoxyuridine into the DNA of subepithelial tissue in the
ated with spindle cell proliferation. It is possible that these
were not included since granulation tissue is frequently associ-
blasts (Figs. 8 and 9). For comparison, see Fig. 10. Spindle cell
GLC-TEA method which is specific for nitrosamines. TSNA
values of NNK, NNN, and NAT may be related to storage of
This difference may be related to the use of a commercially
available snuff that was purchased from a local supermarket,
such as NNN and NNK, were higher in the earlier study (9).
Statistically significant (P < 0.05 to 0.001).
Snuff by itself can cause development of sarcomas, since 26% 
of the rats developed this type of tumor. Furthermore, 3 rats 
treated with snuff developed squamous cell carcinomas of the 
palate and 2 lip papillomas compared with one lip sarcoma in 
the control group. The difference (13 of 38 versus 1 of 30) is 
statistically significant (P < 0.01) and shows that snuff by itself 
is a carcinogen for the lip and oral cavity. The incidence of 
tumors is higher than observed previously (9), where the chronic 
inflammatory changes were more severe and amounts of TSNA, 
such as NNN and NNK, were higher in the earlier study (9). 
This difference may be related to the use of a commercially 
available snuff that was purchased from a local supermarket, 
and we had no control over storage and shelf life. The higher 
values of NNK, NNN, and NAT may be related to storage of 
moist snuff. In a study by Andersen et al. (17), storage of snuff 
1S3 (24°C for up to 1 yr) resulted in a significantly increased 
level of NNN, NNK, and NAT. The baseline values of NNK and 
NNN were 5 to 7 times higher than ours, and this may be 
related to differences in analytical methods, since we used the 
GLC-TEA method which is specific for nitrosamines. TSNA 
levels similar to the ones in this paper were reported (18) for 
moot Kentucky 1S3 snuff analyzed by the GLC-TEA method.

We also identified spindle cell proliferation in Groups II, III, 
and IV as a new lesion. This lip lesion was composed of 
aggregates of spindle cells, probably fibroblasts or myofibro-
and IV as a new lesion. This lip lesion was composed of

**Table 4. Incidence, location, and types of neoplasms outside of head and neck and gastrointestinal region**

<table>
<thead>
<tr>
<th>Location</th>
<th>Group I (n = 40)</th>
<th>Group II (n = 1)</th>
<th>Group III (n = 38)</th>
<th>Group IV (n = 40)</th>
<th>Group V (n = 38)</th>
<th>Group VI (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Adenoma</td>
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<td>Mediastinum</td>
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<tr>
<td>Skin-subcutaneous tissue</td>
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<td>Abdomen</td>
<td>Desmoplastic fibroma</td>
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<td></td>
<td>Fibroadenoma</td>
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<td>Malignant</td>
<td>Lymphoma</td>
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<td>2</td>
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* NOS, not otherwise specified.

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REFERENCES

PROMOTION BY SNUFF IN RATS INITIATED BY 4-NQO OR DMBA


Promoting Effect of Snuff in Rats Initiated by 4-Nitroquinoline-N-Oxide or 7,12-Dimethylbenz(a)anthracene

Sonny L. Johansson, Johnaqa Saidi, Bengt-Göran Österdahl, et al.


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