Effect of Calcium on Rat Gastric Carcinogenesis Induced by \( N\)-Methyl-\( N'\)-Nitro-\( N\)-Nitrosoguanidine

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KOMATSU, S., MASUDA, T. and HISAMICHI, S. Effect of Calcium on Rat Gastric Carcinogenesis Induced by \( N\)-Methyl-\( N'\)-Nitro-\( N\)-Nitrosoguanidine. Tohoku J. Exp. Med., 1991, 165 (4), 291–297 —— The effect of calcium carbonate (\( \text{CaCO}_3 \)) on the initiation of gastroduodenal carcinogenesis induced by \( N\)-methyl-\( N'\)-nitro-\( N\)-nitrosoguanidine (MNNG) was examined under the conditions with and without sodium chloride. Male Wistar rats were given drinking water containing MNNG (100 mg/liter) and one of the following diets during the first 20 weeks ad libitum. Group 1 was given basal diet; group 2, diet with 10% \( \text{NaCl} \); group 3, diet with 10% \( \text{NaCl} \) and 2.5% \( \text{CaCO}_3 \); group 4, diet with 10% \( \text{NaCl} \) and 7.5% \( \text{CaCO}_3 \); group 5, diet with 7.5% \( \text{CaCO}_3 \). During the next 20 weeks, all groups were fed with the basal diet and tap water. The carcinogenic incidences of glandular stomach between the nonsalted diet groups, 1 and 5 (15% and 16% respectively), were not significantly different at the 40th week. The incidences in the salted diet groups 2, 3, and 4 were 59, 63, and 43%, respectively, indicating no statistical difference among them. Thus, \( \text{CaCO}_3 \) showed no anticarcinogenic effect on gastroduodenal carcinogenesis. In the groups 3 and 4, however, increased incidence of duodenal cancer was observed. —— calcium; gastric carcinogenesis; rat; sodium chloride; \( N\)-methyl-\( N'\)-nitro-\( N\)-nitrosoguanidine

Although the mortality of stomach cancer is now declining throughout the world, its mortality is still high in Japan, South America, and East-Europe (Kurihara et al. 1989). From the epidemiological point of view, one of the risk factors for stomach cancer is salty food (Haenszel et al. 1972; Joosens and Geboers 1983; Tajima and Tominaga 1985). In animal experiment, salt by itself does not show gastric carcinogenicity, but does enhance gastric carcinogenic effect of initiator (Takahashi et al. 1983) and acts as tumor promoter (Furihata et al. 1984). The mechanisms of the enhancing effect of salt on initiation stage are thought to be the damage to mucous barrier and the increased turnover of stomach mucous cells owing to the destruction of the stomach mucous epithelium. Thus, a carcinogen can easily get to the mucous epithelium and the tissues in the hyper-

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proliferative state have high sensitivity to a carcinogen, especially initiator (Takahashi et al. 1983; Takahashi 1986). Accordingly, avoiding salty food has been recommended as the way of preventing stomach cancer (Ministry of Health and Welfare 1983).

Daily salt intake of Japanese has been very high, taking an example 13.5 g in 1975 (Ministry of Health and Welfare 1990). Ministry of Health and Welfare has set the guideline of daily salt intake; under 10 g per capita (Ministry of Health and Welfare 1984). This recommended level is even higher than the recommended level of 5 g in the United States (U.S. Senate, Select Committee on Nutrition and Human Needs 1977). Although daily salt intake has been decreasing gradually in Japan, it was still 12.2 g per capita in 1988 (Ministry of Health and Welfare 1990) and has not reached the recommended levels in Japan yet. Because of the habitual high-intake of salt, a substitute way of preventing stomach cancer must be developed.

The epidemiology of stomach cancer demonstrated that in the geographical areas composed of limestone, which is rich in calcium carbonate (CaCO₃), stomach cancer mortality is significantly low (Minowa and Takahashi 1960; Segi and Kurihara 1960; Yonechi 1962; Takahashi 1974) and that the concentration of calcium in river water and the death rate of stomach cancer have a negative correlation (Minowa and Takahashi 1960). Furthermore, in short-term experiments with mice, calcium added to diet inhibited stomach mucous damage caused by high salt diet (Minowa et al. 1960a, b). The purposes of our long-term animal experiment were to examine whether calcium inhibits the carcinogenic effect of MNNG as well as the cocarcinogenic effect of salt.

**Materials and Methods**

One hundred and four Wistar rats (Shizuoka Experimental Animal and Agriculture Co-operation, Shizuoka), 6-week-old male, were divided into 5 groups: 4 groups of 21 rats each and one group of 20 rats. Wistar rats were used to make a comparison with the incidence of gastroduodenal cancer in the previous long-term carcinogenesis experiment in which Wistar rats were also used (Takahashi et al. 1983). All rats were given N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Aldrich, WI, USA) in their drinking water as a carcinogen at a concentration of 100 mg/liter for 20 weeks. Rats in the group 1 were fed basal diet (Oriental MF; Oriental Yeast Co., Ltd., Tokyo); group 2, diet containing 10% sodium chloride (NaCl, Wako Pure Chemical Ind. Ltd., Osaka), that is 90% basal diet and 10% NaCl; group 3, diet containing 10% sodium chloride and 2.5% calcium carbonate (CaCO₃, Wako Pure Chemical Ind., Ltd.); group 4, diet with 10% sodium chloride and 7.5% calcium carbonate; and group 5, diet with 7.5% calcium carbonate. During the next 20 weeks, all groups were given diet for breeding (Clea CA-1; Clea Japan, Tokyo) and tap water ad libitum.

In this study, the amount of sodium chloride and calcium was decided according to the previous experiment in mice that showed the inhibitory effect of calcium on stomach mucous damage caused by high salt diet (Minowa et al. 1960a, b).

Under these conditions, groups 1 and 5 were used to determine the effect of calcium per se on gastroduodenal carcinogenesis by MNNG. To examine the effect of calcium on cocarcinogenic effect by high-salt diet, groups 2, 3 and 4 were compared.
The rats were housed in cages in an animal room with lighting cycles of 12 hr, at 24°C and 60% relative humidity. The volume of water intake was checked 3 times a week for the first 20 weeks. The body weight was measured weekly during the entire 40 weeks. The total consumption of diet was measured in each group during the first 20 weeks.

After 40 weeks, all the surviving rats were sacrificed and dissected. Animals lived for more than 30 weeks were also dissected and included in the effective number of rats. The stomach and intestine were fixed with 10% formalin to observe the existence of cancer macroscopically. Then, the suspicious lesions were dyed with hematoxylin and eosin, and examined microscopically.

The differences of the incidences of tumors were statistically analysed by chi-square test.

RESULTS

The changes in body weight are shown in Fig. 1. Groups 2, 3 and 4, with high-salt diet, showed inhibited weight gain during the first 20 weeks while being exposed to MNNG. Although the body weight of these groups increased markedly for a few weeks after the cessation of the treatment, their body weight were lower compared with the non-salted diet groups 1 and 5 throughout the last 20 weeks.

The daily consumption of water and MNNG are shown in Table 1. The

![Fig. 1. Changes of mean body weight of rats to which N-methyl-N'-nitro-N-nitrosoguanidine and experimental diets were given during the first 20 weeks. Group 1, basal diet; group 2, 10% NaCl in basal diet; group 3, 10% NaCl and 2.5% CaCO₃ in basal diet; group 4, 10% NaCl and 7.5% CaCO₃ in basal diet; group 5, 7.5% CaCO₃ in basal diet.](image-url)
high-salt diet groups showed larger amounts of MNNG intake than non-salted diet groups probably because of increased thirst.

Effective number of rats and the incidence of stomach and intestinal cancer are shown in Table 2. Stomach tumors were predominantly located in glandular stomach. Microscopic examination of glandular stomach showed well differentiated tubular adenocarcinoma. Between the non-salted diet groups 1 and 5, there was no significant difference in incidence of glandular stomach cancer \((p >0.75)\). Among the high-salt diet groups 2, 3 and 4, no significant difference was shown either \((p >0.5)\). Number of effective rats decreased in group 4 because of death.

In duodenum, the high-salt groups, especially groups 3 and 4, showed high incidence of cancer. There was no significant difference among groups 2, 3 and 4 in incidence of duodenal cancer \((p >0.1)\). Cancers of the small intestine were found in two out of 14 rats in group 4.

**DISCUSSION**

In this study, effect of calcium carbonate \((\text{CaCO}_3)\) on chemical carcinogenesis

**TABLE 1. Water consumption in male Wistar rats during the exposure period to MNNG**

<table>
<thead>
<tr>
<th>Group</th>
<th>Drinking water</th>
<th>Diet</th>
<th>Water consumption* (ml/rat/day)</th>
<th>MNNG* intake (mg/rat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 mg/liter MNNG</td>
<td>NaCl</td>
<td>18.3±3.7</td>
<td>256.2±51.8</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>10%</td>
<td>46.9±10.3</td>
<td>656.6±144.2</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>10%</td>
<td>2.5%</td>
<td>36.6±12.1</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>10%</td>
<td>7.5%</td>
<td>52.0±14.0</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>7.5%</td>
<td>17.3±3.5</td>
<td>242.2±49.0</td>
</tr>
</tbody>
</table>

*Values are mean±s.d.

**TABLE 2. Effect of sodium chloride and calcium carbonate on gastrointestinal**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Number of animals at the start of experiment</th>
<th>Effective number of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MNNG</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>MNNG+NaCl</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>MNNG+NaCl+2.5% CaCO_3</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>MNNG+NaCl+7.5% CaCO_3</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>MNNG+7.5% CaCO_3</td>
<td>20</td>
<td>19</td>
</tr>
</tbody>
</table>

*aNumbers in parentheses indicate percent.
*bNot significantly different by chi-square test.
Calcium and Gastric Carcinogenesis

was examined in a long-term experiment. The incidences of gastroduodenal cancers in our experiment were similar to the results obtained with corresponding regimen in the experiment by Takahashi et al. (1983).

Excessive consumption of water owing to increased thirst were observed among the groups given high-salt diet. Thus, the intakes of MNNG in those groups were twice or three times larger than that in non-salted diet groups. The comparison of incidences of carcinogenesis among all groups, therefore, was unjustifiable. In our experiment effect of calcium were compared separately among the high-salt diet groups and between non-salted groups.

As for effects of calcium suplementation, the incidence of gastroduodenal cancer induced by MNNG was not affected, under the condition either salt was added or not.

Minowa et al. (1960a, b) reported protective effect of calcium on mucosal destruction of the stomach of mice given excessive salt. It is, thus, postulated that calcium inhibits gastric carcinogenesis by protecting mucosal cells. However, the inhibitory effect was not observed in this study, though the diet of the same regimen with Minowa's was used.

The explanation of the lack of the inhibitory effect of calcium on cocarcinogenic effect of salt in this study is difficult at this moment. However, the doses of calcium and length of administration may have influence the inhibitory effect on stomach mucous damage by salt.

Furihata et al. (1989a) showed that administration of one ml of 700 mM CaCl₂ alone increased replicative DNA synthesis (RDS) and induction of ornithine decarboxylase, indicating that CaCl₂ acted as tumor promoter. The same investigators suggested anti-tumor-promoting effect of calcium. In a short-term experiment pretreatment with one ml of 20-400 mM CaCl₂ remarkably inhibited the increase of RDS caused by sodium chloride in the pyloric mucosa of rats (Furihata et al. 1989b). This inhibition showed a clear dose-response relationship and one ml of 400 mM of CaCl₂ completely inhibited the increase. These results indicates

carcinogenesis in Wistar rats given MNNG in drinking water

<table>
<thead>
<tr>
<th>Fore-stomach</th>
<th>Glandular stomach</th>
<th>Duodenum</th>
<th>Small intestine</th>
<th>Large intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(5)*</td>
<td>3(15)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>10(59) n.s.</td>
<td>3(18) n.s.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>12(63) n.s. b</td>
<td>8(42) n.s.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>6(43)</td>
<td>6(43)</td>
<td>2(14)</td>
<td>0</td>
</tr>
<tr>
<td>1(5)</td>
<td>3(16)</td>
<td>1(5)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
that large amount of CaCl₂ affects on carcinogenesis differently from smaller amount of calcium.

The daily dose of calcium in our experiment roughly corresponds to 40 g for a person weighing 60 kg (in Group 3; 2.5% CaCO₃). This dose is extremely large when compared with the average intake of 524 mg/day of a Japanese (Ministry of Health and Welfare 1990). Moreover, it is even larger than the dose used in the experiment by Furihata et al. (1989a), which corresponds to 8.4 g/day for a person with the body weight of 60 kg.

Since epidemiological observations suggested inhibitory effect of calcium on gastric carcinogenesis, further experiments with lower doses of calcium and sodium chloride in both initiation and promotion stages will be meaningful.

Acknowledgment

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