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Cause and effect between concentration-dependent tissue damage and temporary cell proliferation in rat stomach mucosa by NaCl, a stomach tumor promoter

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This study was designed to test whether concentration or dose of NaCl was responsible for the initial tissue damage (after 1 min) and resulting temporary cell proliferation at 17 h in stomach mucosa of male F344 rats after gastric intubation of 0.65, 1.3, 2.6 and 3.7 M NaCl. Histological damage was studied by dual staining combining horseradish peroxidase-labeled *Griffonia simplicifolia* agglutinin-II staining (HRP-GSA-II) and periodic acid cold thionin-Schiff reaction (PATS). Cell proliferation was studied by measuring replicative DNA synthesis with liquid scintillation counting and by BrdU staining. NaCl at the same overall dose of 0.8 g/kg body weight induced different degrees of response depending on the concentration. For 4 ml of 0.65 M NaCl, there was no tissue damage after 1 min nor any increase in replicative DNA synthesis after 17 h in the pyloric mucosa. Administration of 1.3 M NaCl (2 ml), 2.6 M NaCl (1 ml) and 3.7 M NaCl (0.7 ml) induced concentration-dependent damage of the surface mucous cell layer after 1 min and increased replicative DNA synthesis after 17 h ($P < 0.05$). Concentration-dependent increase in replicative DNA synthesis at 17 h was also induced with the same volume (1 ml) of 1.3, 2.6 and 3.7 M NaCl, while a volume-dependent increase in replicative DNA synthesis at 17 h was induced with 0.4, 0.7 and 1 ml of 3.7 M NaCl. However, a greater increase in replicative DNA synthesis was always observed when using higher NaCl concentrations at the same dose. Liquid scintillation counting was well-correlated with BrdU staining. These results suggest that a high concentration of NaCl is responsible for the initial tissue damage and resulting temporary cell proliferation during stomach tumor promotion.

Introduction

Stomach cancer is one of the major cancers in various parts of the world (1). It is still a common cancer in Eastern Europe and in East Asian countries including Japan, although it is not a major cancer in Western Europe and the United States (2). It is important to identify possible carcinogens and tumor promoters of stomach carcinogenesis in our environment and to elucidate their mechanisms of action. NaCl is the most probable glandular stomach tumor promoter, and this has been studied epidemiologically (3,4) and experimentally (5–8). For example, intake of highly-salted foods is well correlated to stomach cancer mortality in Japan (9).

Cytotoxicity and cell proliferative activity of NaCl has been observed (6,10,11). Previously we reported cytotoxicity and induced cell proliferative activity of NaCl in rat stomach mucosa (12–14). Tissue damage was seen to occur rapidly (within 1 min) with 2.6 M NaCl (14). Although these studies examined dose-dependent (0.25–1.5 g/kg body weight) induction of mucosal damage and cell proliferation, they did not determine whether the concentration or the dose of NaCl was responsible for the effects. In order to better understand cause and effect in this system, the present study was designed to examine the concentration-dependent induction of initial mucosal damage and resulting cell proliferation. We employed a new dual staining method combining horseradish peroxidase-labeled *Griffonia simplicifolia* agglutinin-II staining (HRP-GSA-II*) and periodic acid cold thionin-Schiff reaction (PATS) to reveal subtle histological changes. This work demonstrated that high concentration of NaCl rather than high total dose was responsible for the induction of mucosal damage and cell proliferation, and that the initial mucosal damage resulted in a delayed and temporary cell proliferation as part of the tissue repair process.

Materials and methods

Chemicals

NaCl (reagent grade) was purchased from Wako Pure Chemical Industries, Ltd., Osaka. [CH_3-^{3}H]Thymidine ([^{3}H]dThd, 1.85–3.33 Tbq/m mol) was obtained from ICN Radiochemicals, Irvine, CA. 5-Bromo-2′-deoxyuridine (BrdU) was from Sigma Chemical Co., St Louis, MO.

Animals

Male Fischer rats (F344/Du Crj: Charles River Japan, Inc., Kanagawa), 8-weeks old and weighing 170–200 g, were kept in individual cages. They were given limited food (a piece of commercial pellet diet, ~4 g) overnight to reduce their stomach contents, and the following day were given NaCl solution by gastric intubation. At appropriate times, animals were sacrificed and stomachs surgically removed.

Histological examination

Each stomach was flushed with 5 ml of 10% phosphate-buffered formalin using a syringe and fixed in 10% phosphate-buffered formalin. Tissues were embedded in paraffin. Three rats per treatment group were prepared for histological examination. Sections were stained by hematoxylin and eosin (HE) for morphological study. To differentiate surface mucous cells and gland mucous cells (cardiac gland cells, mucous neck cells and pyloric gland cells), serial sections were stained by horseradish peroxidase-labeled HRP-GSA-II (15) and then stained by PATS (16). For BrdU staining, five rats per treatment group were given an i.p. injection of BrdU at a dose of 100 mg/kg body weight 1 h before sacrifice. The number of labeled cells per 100 cells was calculated (17).

Replicative DNA synthesis

Replicative DNA synthesis was determined by a liquid scintillation counting method (18). Five to ten rats per group were examined. After stomach removal, samples of pyloric mucosa weighing 20 mg were cut into small pieces (~1×1×1 mm), transferred into L-shaped glass tubes, and cultured individually in 3 ml of L-15 medium at 37°C for 2 h with gentle shaking. The medium contained 370 Kbq [^{3}H]dThd/ml, 100 μg streptomycin/ml, and 100 U of penicillin G/ml. DNA was extracted with 10% trichloroacetic acid and hydrolyzed at 80°C for 30 min in 5% trichloroacetic acid. An aliquot of supernatant was dissolved in ACS II scintillator and counted in a liquid

*Abbreviations: HRP-GSA-II, *Griffonia simplicifolia* agglutinin-II; PATS, periodic acid cold thionin-Schiff reaction; HE, hematoxylin and eosin.

scintillation counter. The DNA content of the supernatant was determined fluorometrically with 3,5-diaminobenzoic acid. Incorporation of [^{3}H]dThd/μg DNA was calculated.

Statistics

The results were analyzed statistically by Student's *t*-test or Welch's test.

Results

Concentration-dependent mucosal damage, using different volumes to achieve constant NaCl dose

In the gastric pyloric mucosa, surface mucous cells stained blue with PATS, whereas pyloric gland cells stained brown with HRP-GSA-II (Figure 1a). NaCl at the constant dose of 0.8 g/kg body weight induced different degrees of damage according to the concentration of the test solutions. Administration of 0.65 M NaCl (4 ml) did not induce morphological changes in the pyloric mucosa after 1 min (Figure 1b). However, 1.3 M NaCl (2 ml) induced slight damage to the surface mucous cells; some of these cells were desquamated (Figure 1c, arrow). At the next two concentrations, 2.6 M (1 ml) and 3.7 M (0.7 ml), NaCl induced increasingly greater damage where disruption of foveolar epithelium was evident within 1 min (Figure 1d and 1e). Similar mucosal damage was observed in the fundic mucosa (not shown). These results clearly show that the high concentration of NaCl is responsible for the tissue damage.

Concentration-dependent increase in replicative DNA synthesis at the same NaCl dose with different volumes

Figure 2 illustrates the measurements of replicative DNA synthesis in the pyloric mucosa 17 h (maximal time, see Figure 5) after NaCl administration. NaCl at the same dose of 0.8 g/kg body weight induced different results according to the concentration. Administration of 4 ml of 0.65 M NaCl did not induce an increase in replicative DNA synthesis. Thereafter a concentration-dependent increase in replicative DNA synthesis was induced by 2 ml of 1.3 M NaCl (4-fold, $P < 0.05$), 1 ml of 2.6 M NaCl (7-fold, $P < 0.05$) and 0.7 ml of 3.7 M NaCl (8-fold, $P < 0.01$) 17 h after administration. These data demonstrate that the high concentration of NaCl is responsible for induction of replicative DNA synthesis. The results in Figure 1 and Figure 2 therefore show cause and effect for NaCl-induced tissue damage and cell proliferation.

Concentration-dependent increase in replicative DNA synthesis with the same volume at different doses

Figure 3 shows a concentration and dose-dependent increase in replicative DNA synthesis with 1.3 M ($P < 0.05$), 2.6 M ($P < 0.05$) and 3.7 M ($P < 0.05$) NaCl with the same volume of 1 ml in the pyloric mucosa 17 h after administration. This is an ordinary dose–response study. Administration of 1 ml of 1.3 M NaCl (0.4 g/kg body weight) induced a significant increase in replicative DNA synthesis; this can be compared with the 4 ml of 0.65 M NaCl (0.8 g/kg body weight) in Figure 2 that did not increase replicative DNA synthesis at all. The administration of the 1 ml aliquots of 2.6 M and 3.7 M NaCl appeared to exponentially induce replicative DNA synthesis (Figure 3). These results further suggest that high concentration of NaCl is responsible for induction of replicative DNA synthesis in the pyloric mucosa.

Increase in replicative DNA synthesis by 3.7 M NaCl with increasing volumes

We then examined the effect of increasing the administered volume of a single stock NaCl solution. Figure 4 shows that 3.7 M NaCl (the highest concentration in the present study) in volumes of 0.4, 0.7 and 1 ml increased significantly ($P < 0.05$) the replicative DNA synthesis in the pyloric mucosa 17 h after administration. The greater the volume, the greater the induced increase in replicative DNA synthesis. This may be because it requires at least 1 ml of solution to fill up the rat stomach. When a small volume of NaCl solution was administered, the solution was probably diluted quickly with gastric juice that was secreted rapidly after NaCl dosing. However, a higher increase in replicative DNA synthesis was observed after giving 0.4 ml of 3.7 M NaCl (0.46 g/kg body weight) than with 1 ml of 1.3 M NaCl (0.4 g/kg body weight) in Figure 3 ($P < 0.05$) or with 4 ml of 0.65 M NaCl (0.8 g/kg body weight) in Figure 2 ($P < 0.01$). These results thus suggest that high concentration of NaCl is responsible for induction of replicative DNA synthesis in the pyloric mucosa.

Comparison of liquid scintillation counting and BrdU method

In order to compare the methods of measuring DNA and cell proliferation, namely [^{3}H]dThd incorporation and BrdU labeling, we conducted a time-course after administration of 1 ml of 2.6 M NaCl. Figure 5 shows for pyloric mucosa the results of the study with [^{3}H]dThd (replicative DNA synthesis), revealing marked response over 6–24 h, maximal effect at 17 h, and a return to baseline levels by 48 h. Figure 6 and Figure 7 show the time course of changes in proportion of S-phase cells in the pyloric and fundic mucosa, respectively, after administration of 1 ml of 2.6 M NaCl. Experiments in Figures 5 and 6 were examined in different groups of rats. As with replicative DNA synthesis (liquid scintillation counting), the proportion of S-phase cells increased to a maximum at 17 h, but was still elevated at 48 h. Nonetheless, the results of liquid scintillation counting were reasonably well correlated with those of BrdU staining. For the studies of S-phase cells in the pyloric mucosa, it was noted that there was considerable variation in the proportion of S-phase cells over the area scored. The data used in the present study were from the areas of each specimen with the highest proportion of S-phase cells. This may explain why the control value in the pyloric mucosa in Figure 6 seems disproportionately higher than the [^{3}H]dThd-incorporation control of Figure 5.

Discussion

The present study clearly demonstrated that a high concentration of NaCl and not just total dose was responsible for induction of mucosal damage and temporary cell proliferation in rat stomach mucosa. This damage was apparent as early as 1 min after NaCl administration and resulted in the temporary rise in cell proliferation that peaked 17 h later. High dose (0.8 g/kg body weight) but low concentration (4 ml of 0.65 M NaCl) neither induced mucosal damage (Figure 1) nor replicative DNA synthesis (Figure 2). High concentration (1 ml of 1.3 M NaCl and 0.4 ml of 3.7 M NaCl) but low dose (0.4 g and 0.46 g/kg body weight, respectively) increased replicative DNA synthesis (Figures 3 and 4, respectively). This was similarly demonstrated by the histology of Figure 1c, 1d, and 1e and the replicative DNA synthesis in Figure 2 (2 ml of 1.3 M NaCl, 1 ml of 2.6 M NaCl and 0.7 ml of 3.7 M NaCl), namely a concentration-dependent induction of mucosal damage after 1 min and resulting replicative DNA synthesis after 17 h. Twenty years ago Tatematsu *et al.* suggested that affects of the mucous barrier by high concentration of NaCl enhanced stomach carcinogenesis (5).

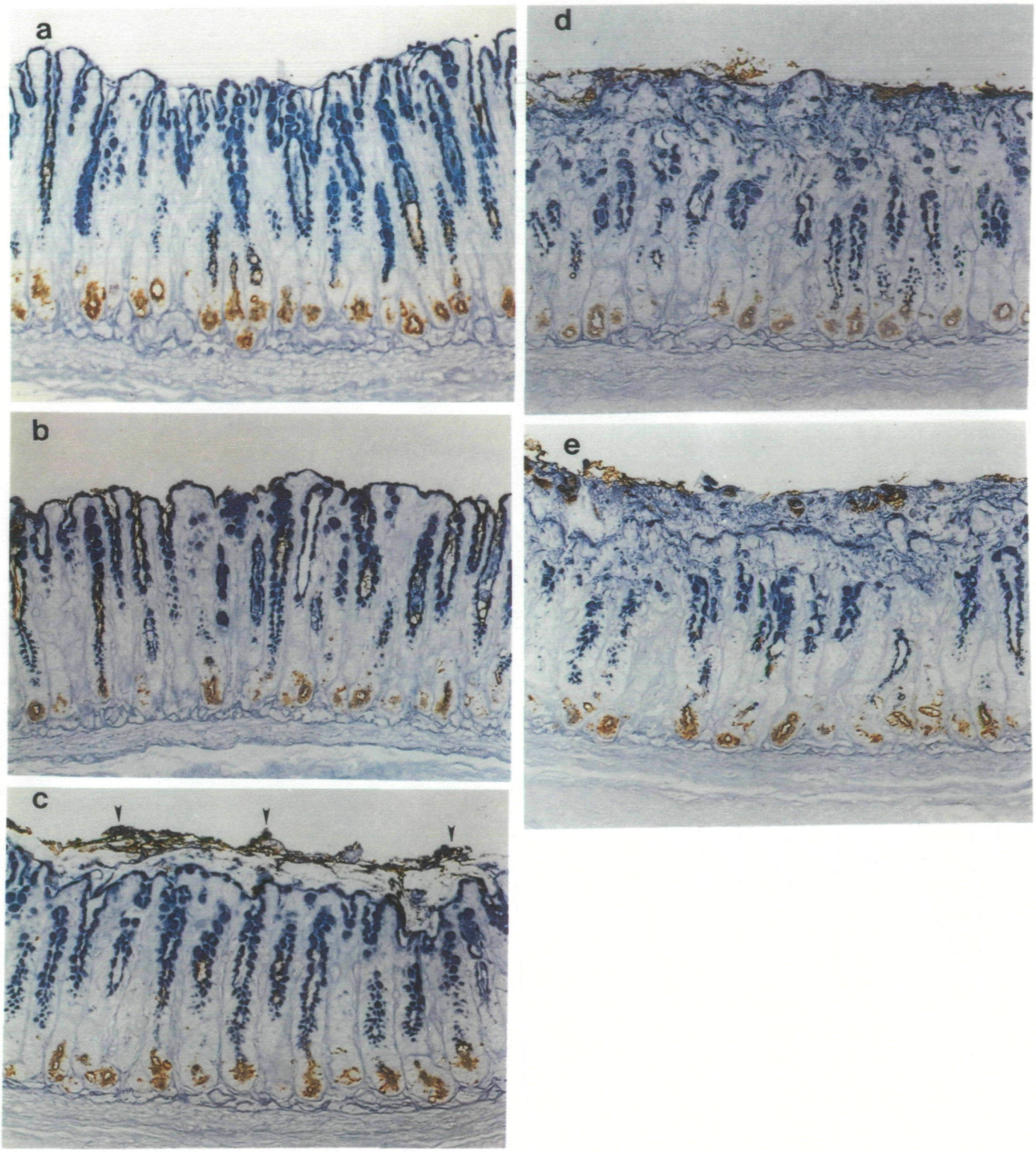

Fig. 1. Concentration-dependent tissue damage at the same NaCl dose with different volumes. F344 rats were given NaCl solution and killed after 1 min, the pyloric mucosa was then examined. Surface mucous cells stain blue with PATS, whereas pyloric gland cells stain brown with HRP-GSA-II. HRP-GSA-II and PATS. x184. (**a**) Control, 1 ml H_2O. (**b**) 0.65 M NaCl, 4 ml. (**c**) 1.3 M NaCl, 2 ml. Some surface mucous cells were desquamated (arrow). (**d**) 2.6 M NaCl, 1 ml. Foveolar epithelium is disrupted and some foveolar epithelial cells are desquamated. (**e**) 3.7 M NaCl, 0.7 ml. Disruption of foveolar epithelium is more evident than seen in (d).

In the present study we first used dual staining combining HRP-GSA-II and PATS to stain gland mucous cell type mucins and surface mucous cell type mucins, respectively. To stain surface mucous cell type mucins, galactose oxidase . cold Schiff reaction (GOS) or galactose oxidase cold thionin-Schiff reaction (GOTS) (19) has been used; however, in rat pyloric mucosa, surface mucous cells show weak reactivity to GOS or GOTS. In the rat gastric mucosa, the dual staining combining

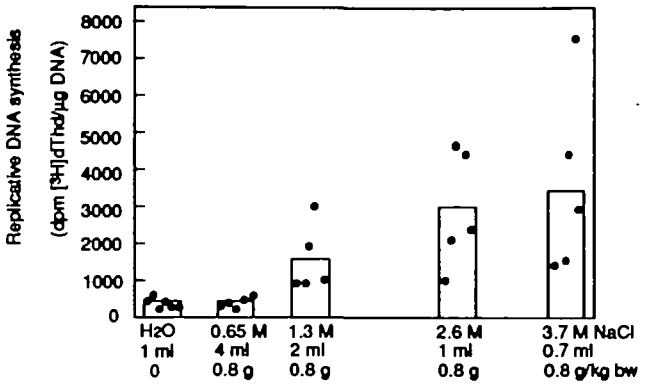

Fig. 2. Concentration-dependent increase in replicative DNA synthesis at the same NaCl dose with different volumes. Rats were given NaCl solution and after 17 h were killed and the pyloric mucosa examined. Results are for five individual rats in each group (six in control). Bars are means for each group. Values at 1.3 M, 2.6 M ($P < 0.05$) and 3.7 M ($P < 0.001$) were significantly different from the H_2O control.

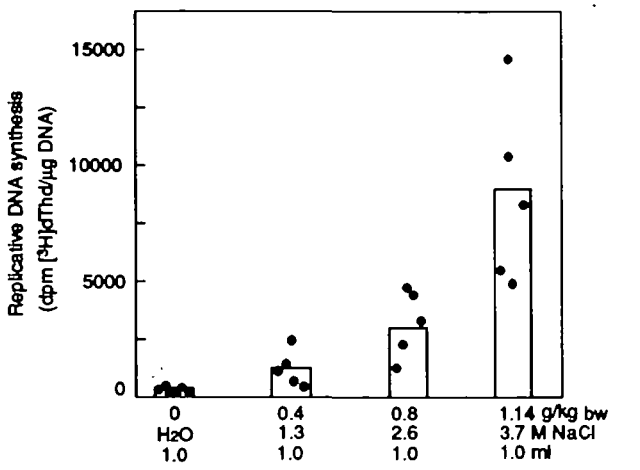

Fig. 3. Concentration-dependent increase in replicative DNA synthesis with the same volume of different NaCl concentrations. Rats were given NaCl solution and were killed after 17 h and the pyloric mucosa examined. Results are for five individual rats at each group (six in control). Bars are means for each group. Values at 1.3, 2.6 and 3.7 M ($P < 0.05$) were significantly different from control

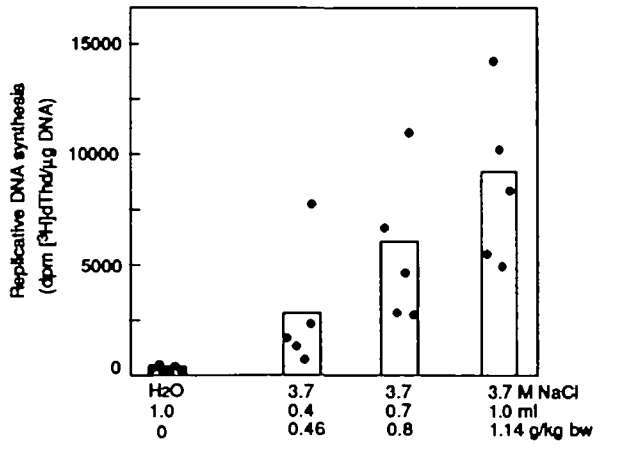

Fig. 4. Volume-dependent increase in replicative DNA synthesis by 3.7 M NaCl. Rats were given NaCl solution and were killed after 17 h and the pyloric mucosa examined. Results are for five individual rats at each group (six in control). Bars represent means in each group. Values at 0.4, 0.7 and 1.0 ml ($P < 0.05$) were significantly different from control.

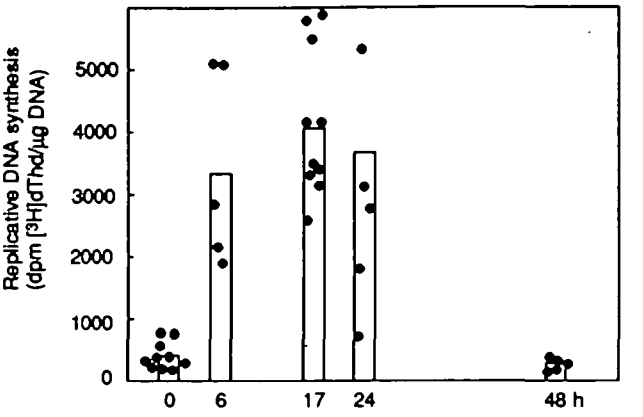

Fig. 5. Changes over time in replicative DNA synthesis in the pyloric mucosa after 2.6 M NaCl (1 ml) administration. Bars represent means of the individual rat values shown. Values at 6 h ($P < 0.02$), 17 h ($P < 0.001$) and 24 h ($P < 0.05$) were significantly different from those at time 0.

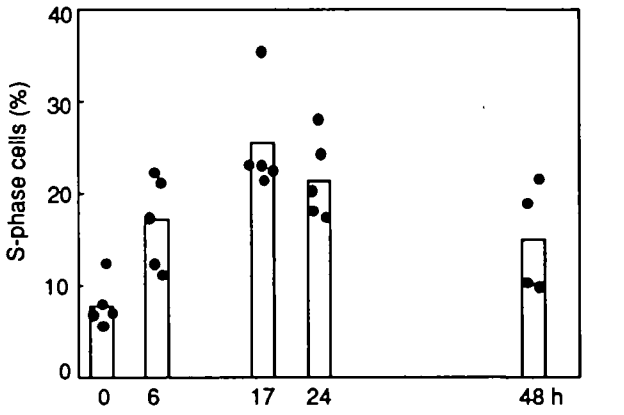

Fig. 6. Changes over time in the proportion of S-phase cells in the pyloric mucosa after 2.6 M NaCl (1 ml) administration. Results are for five individual rats at each time (four rats at 48 h). Bars are means at each time. Values at 6 h ($P < 0.01$), 17 and 24 h ($P < 0.001$) were significantly different from values at time 0.

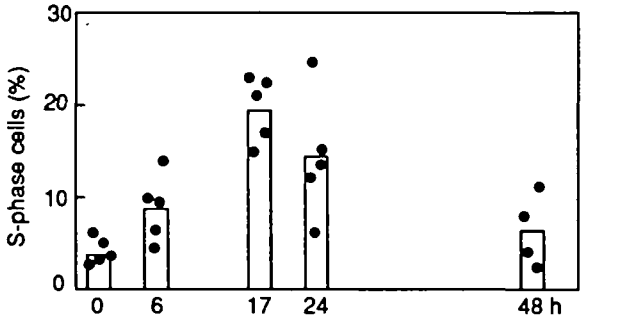

Fig. 7. Changes over time in the proportion of S-phase cells in the fundic mucosa after 2.6 M NaCl (1 ml) administration. Results are for five individual rats at each time (four rats at 48 h). Bars are means at each time. Values at 6 h ($P < 0.05$), 17 h ($P < 0.001$) and 24 h ($P < 0.01$) were significantly different from values at time 0.

HRP-GSA-II and PATS stained gland mucous cells brown and surface mucous cells blue, respectively. The dual staining was useful to differentiate rat surface mucous cells and gland mucous cells. After administration of high concentration of NaCl, disruption of foveolar epithelium was clearly demonstrated by the dual staining. The dual staining is also useful to show sites of lesion and degree of damage.

The BrdU staining method is widely used and proved excellent for showing cell proliferation in the tissues studied here. We also determined replicative DNA synthesis using [^{3}H]dThd incorporation and liquid scintillation counting. This latter method has still several benefits. For example, it is quicker, as it takes only 3 days to obtain a final result using 25 rats. Also, as more than half of the pyloric mucosa from each rat was used for measuring, a mean value was obtained for each rat. On the other hand, the number of S-phase cells in the stomach mucosa varied considerably from area to area when the BrdU method was used. Nonetheless, the present study revealed that data from the liquid scintillation counting method were well correlated with those from the BrdU method.

Previously we showed that all four glandular stomach tumor promoters examined and all eight glandular stomach carcinogens examined increased replicative DNA synthesis dose-dependently in the pyloric mucosa of male F344 rats with maximum effect 16–24 h after administration. These glandular stomach tumor promoters were NaCl (12), taurocholate (20), $K_2S_2O_5$ (21) and formaldehyde (22). The glandular stomach carcinogens studied were *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (23), *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (23), *N*-propyl-*N'*-nitro-*N*-nitrosoguanidine (23), *N*-methyl-*N*-nitrosourea (24), *N*-nitroso-*N*-methylurethane (23), 4-nitroquinoline 1-oxide (23), catechol (25) and *p*-methylcatechol (26). In addition, an increase in replicative DNA synthesis in rat pyloric mucosa was observed with glyoxal (27) and KCl (21), and these two chemicals were subsequently proven to enhance rat glandular stomach carcinogenesis (28,29). Conversely, the liver carcinogens 2-acetylaminofluorene (23), dimethylnitrosamine (23) and Trp-P-2 (23) did not induce replicative DNA synthesis in rat pyloric mucosa. In addition, ethanol, which induced acute ulcers in rat stomach mucosa but did not enhance stomach carcinogenesis, did not induce replicative DNA synthesis (23). These results strongly implicate the requirement for replicative DNA synthesis and cell proliferation in rat stomach carcinogenesis enhanced by NaCl and other stomach tumor promoters and carcinogens. Even when administered alone in long-term feeding studies, NaCl induces hyperplasia in rat stomach mucosa (6).

NaCl intake in the Japanese diet is still high. In 1994, the average daily intake was 12.8 g per person; this is more than twice the intake in the Untied States. The Ministry of Health and Welfare of Japan recommends a decrease of NaCl intake to <10 g/day. The present study suggests that not only should the total amount of NaCl be decreased, but also that NaCl concentration in each food should be decreased.

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