The Rat Models of Gastric Cancer Using *Helicobacter pylori* Infection, *N*-methyl-*N*-nitrosourea, and High Salt Induced Carcinogenesis

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ABSTRACT

To clarify pathological and molecular mechanisms of gastric cancer, various experimental models have been purposed. *H. pylori* is one of the most important factors that classified as a class I carcinogen. *N*-nitroso compounds and salt have been proposed by several studies to be probable promoters of gastric carcinogenesis. The aim of our studies was to develop simplified gastric cancer models in rats using *H. pylori*, MNU and sodium chloride.

A total of 48 rats were divided into 4 experimental groups including: group 1: *H. pylori* inoculation + 30 ppm MNU in drinking water for 20 weeks, group 2: 30 ppm MNU in drinking water for 20 weeks, group 3: MNU 100 mg/kg by gavage feeding on day 1st and day 14th, and group 4: saturated NaCl solution fed twice a week for the 3 weeks + MNU 100 mg/kg by gavage feeding on day 1st and day 14th.

Results: In group 1, two rats developed gastric adenocarcinomas at week 52nd (50% incidence, n = 4), while in the group 2, no gastric adenocarcinoma was found. In group 3, could induce the development of squamous cell carcinoma (SCC) in forestomachs of 5 rats at week 9th, 13th, 16th, and 20th. Overall cancer incidence in group 3 was 50% (n = 10). Eight rats in group 4 developed forestomach SCC at week 6th, 9th, 13th, 16th, and 20th. Overall cancer incidence in group 4 was 47% (n = 17).

Conclusion: In this study, we could induce gastric adenocarcinoma by *H. pylori* infection and MNU administration in drinking water. In addition, short-term intragastric administration of MNU with or without saturated NaCl led to the development of forestomach SCC in rats. These rat models may be suitable in further studies to demonstrate molecular mechanisms and to evaluate efficacy of new modality of treatment options in stomach cancer.

Key words : rat model, gastric cancer, *H. pylori*, N-methyl-N-nitrosourea, high salt

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INTRODUCTION

Despite the falling of gastric cancer incidence and mortality in developed countries, stomach cancer is still the fourth most common cancer, and the second leading cause of cancer- related death worldwide.[1,2] There are distinct geographic variations in terms of mortality and incidence, with the highest incidence rates in Japan, China, Korea, and some countries in South America.[3,4] Additionally, gastric cancer incidence rates also vary according to sex, race, and socio-economic status.1 In Thailand, stomach cancer, one of the most common cancers, more than 90 percent of gastric tumors are adenocarcinomas.[5] Recent report showed the annual incidence rate of 7.1 cases per 100,000 people in this country.[6]

The histology of gastric cancer can be classified into two main subtypes: poorly-differentiated or diffuse type and well-differentiated or intestinal type.[7] Diffuse type, which contains neoplastic cell infiltration without glandular structure formation, usually originates from pangastritis without atrophy. In the contrary, Intestinal type characterized by intestinal gland-like structures replacing normal gastric tissues is believed to have sequential steps of carcinogenesis. These steps involve the transformation from corpus gastritis to gastric atrophy, intestinal metaplasia, dysplasia, and finally to carcinoma.[8]

Gastric cancer is a multifactorial disease. Many environmental and lifestyle factors have been proposed to be involved in cancer initiation or progression6. These include *Helicobacter pylori* infection, N-nitroso compounds, and high salt diet.

H. pylori, a gram-negative bacillus, is one of the most common chronic bacterial infections. Most people acquire *H. pylori* through oral ingestion during childhood period, and remain infected throughout life.[9] Interestingly, gastric cancer incidence tends to change in the same direction as *H. pylori* prevalence,[1,10,11] and many epidemiological studies have demonstrated compelling evidence supporting a relationship between *H. pylori* and gastric cancer development.[12-16] The International Agency for Research on Cancer has classified *H. pylori* as a definite carcinogen since 1994.[17]

Certain bacterial factors and host inflammatory responses put some *H. pylori*-infected patients at higher risk for gastric cancer. Virulent strains of *H. pylori*, such as those containing CagA, can activate or induce the production of proinflammatory mediators. These mediators, namely nuclear factor-κβ and interleukin-8, lead to inflammation, epithelial damage, apoptosis, cell proliferation and tumor progression.[18] People, who possess cytokine gene polymorphisms with elevated levels of IL-1β and TNF-α and decreased levels of IL-10, carry a 50-time increased risk of gastric cancer.[18,19]

H. pylori additionally promotes gastric carcinogenesis by reducing the gastric concentration of ascorbic acid, a potent antioxidant and an inhibitor of *N*-nitrosation. Moreover, *H. pylori* produces toxic substances, which include ammonia and cytotoxin. These chemicals impair host defenses and make gastric epithelial cells more vulnerable to be destroyed by other carcinogens, such as reactive oxygen species, and *N*-nitroso compound.[12]

H. pylori infection alone may not be sufficient to initiate gastric carcinogenesis.[20] Other nutritional factors are of important consideration. Data from case-control, cohort, and ecological studies supported the hypothesis that high intake of salt-preserved food and salt itself increases the risk of stomach cancer, while high intake of fruits and vegetables decreases it.[21,22] Two independent studies in Japan demonstrated an almost linear correlation between stomach cancer mortality and urinary salt excretion or salt intake.[23,24] The association between salty food and gastric cancer can be explained by the production of other carcinogens, such as *N*-nitroso compound, during the food preservation process. Furthermore, excessive salt intake can cause mucosal damage, which enhances the effect of chemical carcinogens, and promote persistent infection with *H. pylori*.[21,22]

N-nitroso compounds are formed in human stomach through the chemical reaction called nitrosation. This reaction requires nitrite as a substrate and is catalyzed either by gastric acid or intestinal bacteria. Nitrite can be obtained endogenously through the reduction of nitrate by oral bacteria. Nitrite and nitrate are also found in cured meat, preserved food, pickled vegetables, and drinking water.[22,25] Other than inducing gastric cancer in animal models, *N*-nitroso compounds are positively associated with stomach cancer risk in human studies.[22] In the north and north-eastern part of Thailand, gastric cancer is significantly related to the ingested amount of nitrite, nitrate, and *N*-nitrosodimethylamine.[26]

In the present study, we developed four distinct rat models of stomach cancer using *H. pylori* infec-

tion, *N*-methyl-*N*-nitrosourea (MNU), hypertonic salt, and the combination of these factors.

Materials and Methods

Forty-eight male Spraque-Dawley rats at the age of 6 weeks were purchased from National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. All animals were housed in stainless cages located in a temperature-controlled room with 12-hour light /12-hour dark cycle and they had freely access to regular rat chow and water. The animals were allowed to acclimate to the housing environment at least 7 days before experiments.

A total of 48 rats were divided into 4 experimental groups as described below.

Experimental group 1: *H. pylori* infection and MNU administration in drinking water (n = 12)

On the day of experiment, *H. pylori* suspension was administered to all rats using the protocol described later in this paper. Two weeks after inoculation, the animals received MNU in their drinking water with a concentration of 30 ppm for 20 weeks. Three rats were sacrificed at the 22nd week, three at the 32nd week, two at the 42nd week, and four at the 52nd week of the experiment.

Experimental group 2: MNU administration in drinking water (n = 9)

Each rat was given one milliliter of normal saline by oral gavage twice a day at an interval of four hours for three sequential days. After two weeks, the animals received MNU in their drinking water with a concentration of 30 ppm for 20 weeks. One rat was sacrificed at the 22nd week, two at the 32nd week, three at the 42nd week, and the last three at the 52nd week of the experiment.

Experimental group 3: intragastric MNU administration (n = 10)

Each rat was given 100 mg/kg of MNU by oral gavage on the first and the fourteenth day of experiment. One rat was sacrificed at the 3rd week, one at the 6th week, two at the 9th week, two at the 13th week, two at the 16th week, and two at the 20th week of the experiment.

Experimental group 4: Hypertonic salt and intragastric MNU administration (n = 17)

Each rat was given 100 mg/kg of MNU by oral gavage on the first and the fourteenth day of experiment. In addition, one milliliter of saturated NaCl was administered directly into the stomach of each animal twice a week for the first 3 weeks of the experiment. Three rats were sacrificed at the 3rd week, three at the 6th week, two at the 9th week, three at the 13th week, two at the 16th week, and four at the 20th week of the experiment.

H. pylori preparation and inoculation

H. pylori were taken from peptic ulcer patients during they were performed the endoscopy at King Chulalongkorn Memorial hospital. The bacteria were grown in Columbia agar plate supplemented with sheep blood for 72 hours at 37°C in an automatic CO_2-O_2 incubator under microaerophilic conditions (85%N_2, 10%CO_2, and 5%O_2). On the day of experiment, *H. pylori* colonies were swabbed into normal saline to form the suspension with the concentration of 5×10^8 to 5×10^{10} colony forming unit/ml (CFU/ml).

H. pylori inoculation was performed according to our protocol in previous study.[27] Streptomycin suspended in tap water (5 mg/ml) had been administered to each rat for three days before inoculation. After 18-hour fast, one milliliter of *H. pylori* suspension had been inoculated into each animal by oral gavage twice daily at the interval of four hours for three consecutive days. Before the next day of inoculation, the rats were fed with the regular food for 2 hours. After the last inoculation, these animals were given free access to water and standard food.

Chemical preparations

In the experimental group 1 and 2, MNU was dissolved in distilled water at a concentration of 30 ppm. Solution was freshly prepared three times a week, and was administered *ad libitum* in a light-shielded bottle as the drinking water.

In the experimental group 3 and 4, MNU was dissolved in citrate buffer (pH 4.5) at the amount of 100 mg per kg rat body weight. For using in experimental group 4, saturated NaCl solution (5.2 M NaCl) was prepared by dissolving NaCl salt in distilled water.

Gastric excision was done under anesthesia using intraperitoneal injection of thiopental (60 mg/kg). All tissues were washed twice with ice-cold phosphate-buffered saline at the concentration of 0.1 mol/L and pH 7.4, fixed in 4% phosphate-buffer paraformaldehyde, and then embedded in paraffin for histological studies. Each stomach was cut along the greater curvature into multiple 5 μm-thick sections, which were

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later stained with hemotoxylin and eosin (H&E). Histopathological analyses were performed by a pathologist to evaluate the degree of inflammation, gastric epithelial cell changes, and cancer development.

This study was conducted in accordance with the guidelines of animal experimentation established by the National Research Council of Thailand (NRCT), 1999.

Statistical analysis

The incidence of cancer in each experimental group was shown in percentage. The differences in cancer development among groups were determined using Fisher's exact test. *P*-value at less than 0.05 was considered statistically significant.

RESULT

One of the rats in experimental group 1 died from aspiration at the 42nd week of the experiment without gastric cancer development. Others were sacrificed at aforementioned time. All data concerning histological findings in each group were demonstrated in Table 1 and 2. In group 1 (*H. pylori* infection with MNU *ad libitum*), all stomach tissues obtained at the 22nd week showed normal gastric mucosa, while the sample from one rat sacrificed at the 32nd week showed changing of gastric gland distortion (Figure 1). Two rats in this group developed adenocarcinoma of glandular stomach (Figure 2) at the 52nd week. Cancer incidence rates at the 22nd, 32nd, 42nd, and 52nd week were 0% (0/3), 0% (0/3), 0% (0/2), and 50% (2/4) respectively.

In group 2 (MNU *ad libitum*), non of the rats developed gastric cancer; however, one of those sacrificed at the 52nd week had glandular distortion of the stomach. In group 3 (intragastric MNU administration), stomach tissues from one rat at the 3rd and 16th week showed hyperkeratosis of forestomach (Figure 3), which was an early abnormal change of squamous cell carcinoma (Figure 4). Moreover, one rat from the 9th week, one from the 13rd week, one from the 16th week, and two from the 20th week developed squamous cell carcinomas of forestomachs. Cancer incidence rates at the 3rd, 6th, 9th, 13rd, 16th, and 20th week were 0% (0/1), 0% (0/1), 50% (1/2), 50% (1/2), 50% (1/2), and 100% (2/2) respectively.

In group 4 (intragastric MNU administration and saturated NaCl), gastric tissues obtained from five rats at the 3rd, 6th, 9th, and 13rd week showed hyperkeratosis of forestomach, and one specimen collected at the 16th week exhibited low-grade squamous dysplasia. In addition, one rat from the 6th week, one from the 9th week, one from the 13rd week, one from the 16th week and four from the 20th week developed squamous cell carcinomas of forestomachs. Cancer incidence rates at the 3rd, 6th, 9th, 13rd, 16th, and 20th week were 0% (0/3), 33% (1/3), 50% (1/2), 33% (1/3), 50% (1/2), and 100% (4/4) respectively.

Table 1. Histological findings in experimental group 1 and 2.

| Histological findings | Week of experiment | | | | | | | |
| | Week 22 | | Week 32 | | Week 42 | | Week 52 | |
	No. of case	Cancer incidence	No. of case	Cancer incidence	No. of case	Cancer incidence	No. of case	Cancer incidence
Group 1								
Normal gastric tissue	3		2		2		2	
Gastric gland distortion	0		1		0		0	
Adenocarcinoma	0		0		0		2	
Total	3	0%	3	0%	2	0%	4	50%
Group 2								
Normal gastric tissue	1		2		3		2	
Gastric gland distortion	0		0		0		1	
Adenocarcinoma	0		0		0		0	
Total	1	0%	2	0%	3	0%	3	0%

group 1 = *H. pylori* infection with MNU ad *libitum*
group 2 = MNU ad *libitum*

Table 2. Histological findings in experimental group 3 and 4.

	Week of experiment											
	Week 3		Week 6		Week 9		Week 13		Week 16		Week 20	
	No.	Cancer incidence	No.	Cancer incidence	No.	Cancer incidence	No.	Cancer incidence	No.	Cancer incidence	No.	Cancer incidence
Group 3												
Normal gastric tissue	1		0		1		1		0		0	
Hyperkeratosis	0		1		0		0		1		0	
SCC	0		0		1		1		1		2	
Total	1	0%	1	0%	2	50%	2	50%	2	50%	2	100%
Group 4												
Normal gastric tissue	2		1		0		0		0		0	
Hyperkeratosis	1		1		1		2		0		0	
Squamous dysplasia	0		0		0		0		1		0	
SCC	0		1		1		1		1		4	
Total	3	0%	3	33%	2	50%	3	33%	2	50%	4	100%

group 3 = intragastric MNU administration
group 4 = intragastric MNU administration and saturated NaCl
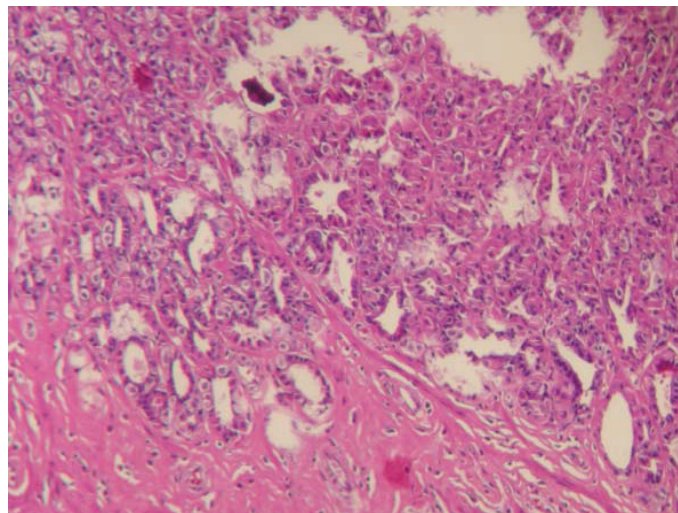

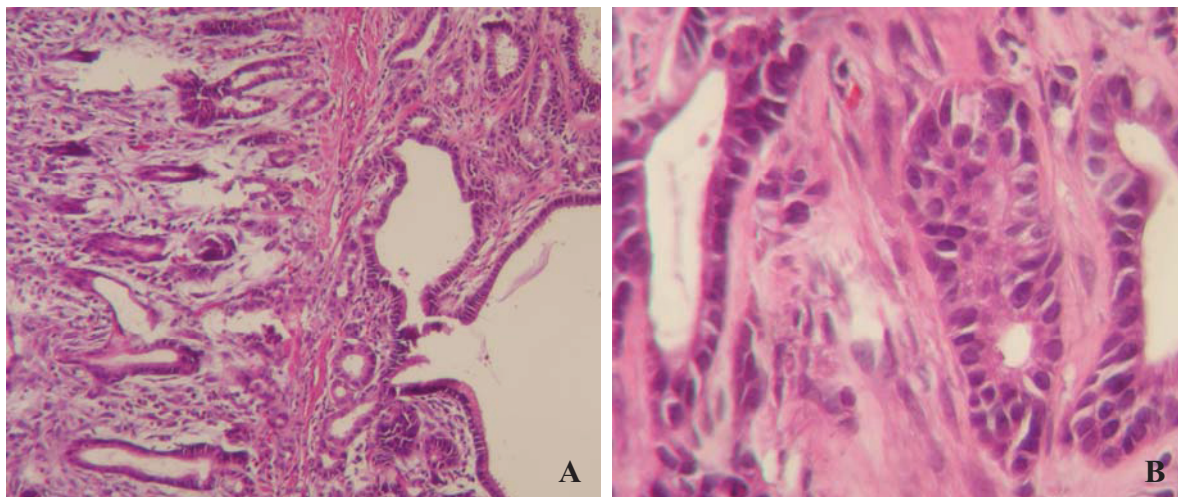**Figure 1.** Gastric gland distortion in experimental group 1.

Figure 2. Adenocarcinoma of glandular stomach in experimental group1 (A×40, B×100).

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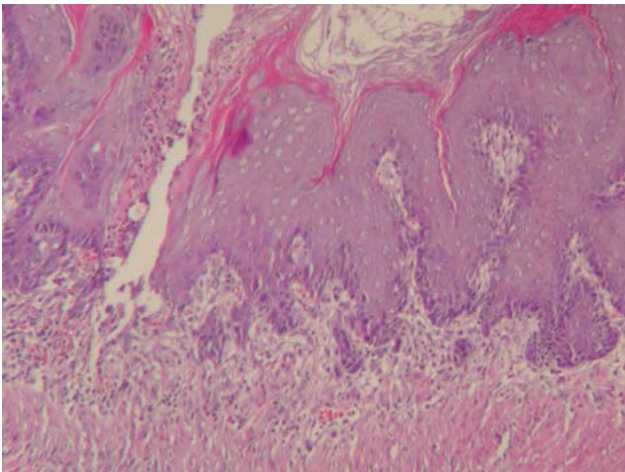*N-methyl-N-nitrosourea, and High Salt Induced Carcinogenesis*

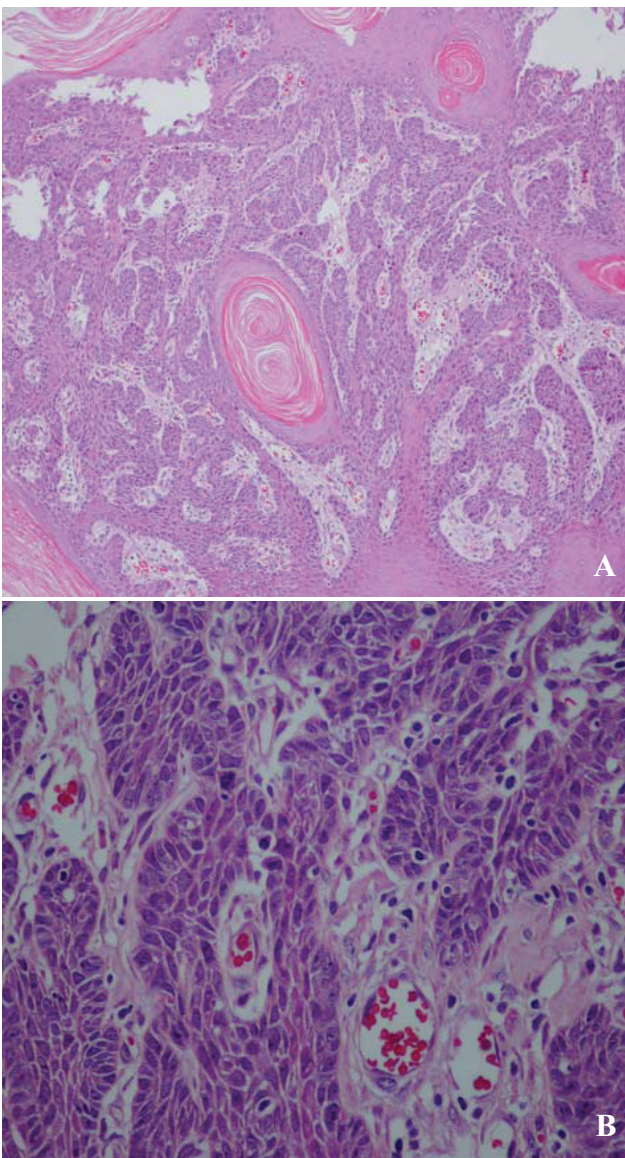**Figure 3.** Hyperkeratosis in experimental group 3.

Figure 4. Squamous cell carcinoma of forestomach in experimental group 3, and 4 (A×40, B×100).

DISCUSSION

As an important medical problem, gastric cancer has been a topic of interest in many researches. Animal studies on pathological and biological aspects of stomach cancer have been done to determine the potential risk factors, prevention measures, and treatment options. Those risk factors include *H. pylori* infection, *N*-nitroso compound, and salt. In the present study, we demonstrated the effect of carcinogen exposure on gastric carcinogenesis.

In several models of gastric cancer, *H. pylori* could enhance the carcinogenic effect of *N*-nitroso compound. Shimizu N *et al*.[28] found that the incidence of gastric adenocarcinoma in Mongolian gerbils treated with *H. pylori* and MNU was significantly higher than those treated with MNU alone. This was in accordance with the result from Sugiyama A *et al*.[29], which illustrated the enhancing effect of *H. pylori* infection on gastric cancer development in Mongolian gerbils received MNU. In spite of insignificant difference, our result indicated that *H. pylori* infection along with MNU administration increased the incidence of adenocarcinoma in rats, compared to another group treated with MNU alone. *H. pylori* and MNU worked synergistically to promote gastric carcinogenesis. *H. pylori* infection led to inflammation and epithelial cell destruction, which made gastric tissues more prone to chemical carcinogen exposure. In the same time, mucosal damage caused by MNU could increase the rate of *H. pylori* infection in rats.[28] Our insignificant result might be due to small sample size, and the fact that Mongolian gerbils used in aforementioned studies were more susceptible for persistent *H. pylori* infection than rats and mice.

We also found that rats treated with intragastric MNU administration developed more cases of gastric cancer than those received MNU through their drinking water. Interestingly, the type of cancer in this group was a squamous cell carcinoma of the forestomach, not an adenocarcinoma of glandular stomach. From the study by Maekawa A *et al*.[30], both squamous cell carcinoma of forestomach and adenocarcinoma of glandular stomach were induced in F344 rats treated with MNU 100 ppm in drinking water. The same findings were also demonstrated in other studies using MNU in drinking water to induce glandular stomach cancer in rats and mice.[31,32] In our study, MNU in drinking water could not produce gastric cancer neither in forestomach nor in glandular stomach. This result could be ex-

plained by the lower dose of MNU in relation to other studies, which mostly used 100 ppm of MNU.[30-32] Intragastric MNU administration might increase the likelihood of direct contact with forestomach mucosa. This could explain the greater incidence of squamous cell carcinoma compared to that of adenocarcinoma in this model. Carcinogenic effect of N-nitroso compounds could be associated with DNA alkylation and reaction with various nucleophiles, especially amines.[33] These substances could induce GC → AT transition, which led to cell-cycle arrest in G2.[34] Rodent gastric cancer induced by MNNG or MNU in drinking water or by oral gavage possessed similar genetic alterations to those in human stomach cancers. These genetic changes included mutations of oncogene, β-*catenin*, ras, and tumor suppressor gene, *p*53.[35,36]

According to various studies in human, salt and salted food pose a possible risk to gastric carcinogenesis.[22] Some animal studies also demonstrated the same results. Tatematsu M *et al*. found that NaCl increased the occurrence of glandular stomach cancer in rats treated with MNNG and 4-NQO.[37] Takahashi M *et al*. illustrated that NaCl could promote gastric carcinogenesis in a dose-dependent manner.[38] In the study by Sorbye H *et al*.[39], Incidence of forestomach squamous cell carcinoma increased significantly in rats pretreated with hypertonic NaCl compared to animals given MNU alone. In contrast with previous researches, our study failed to demonstrate the promoting effect of saturated NaCl on stomach cancer development. Although there was no difference in cancer incidence between a group given NaCl and MNU, and the one received MNU alone, the incidence of abnormal lesion, namely hyperkeratosis, was higher in NaCl-treated group. Moreover, squamous cell carcinoma tended to develop earlier in rats receiving both MNU and NaCl. Cancer-inducing effect of salt could be explained by its ability to increase cancinogen uptake by gastric mucosa[40], and its stimulating effect on DNA synthesis, which increased cell susceptibility to mutagens[41]. Hypertonic NaCl also decreased gastric mucin viscosity, thereby decreasing protective ability of mucous barrier.[20] Possible explanations for negative results in our study might be a small sample size, and short duration of exposure or observation. In the study by Takahashi M *et al*.[38], they had administered NaCl solution for 82 weeks before sacrificing the animals. Longer exposure time might be needed before sodium chloride exerted its carcinogenic effect.

In conclusion, in rats model, *H. pylori* along with MNU in drinking water could induce adenocarcinoma of glandular stomach, while intragastric MNU administration with or without hypertonic NaCl led to the development of squamous cell carcinoma of forestomach. A simplified models of gastric cancer presented in this study can be used to determine risk factors, mechanisms, and to evaluate efficacy and effectiveness of various prevention measures and treatment options for the further study.

REFERENCES

1. Crew KD, Neugut AI. Epidemiology of gastric cancer. World J Gastroenterol 2006;12(3):354-62.
2. Parkin DM, Bray FI, Devesa SS. Cancer burden in the year 2000. The global picture. Eur J Cancer 2001;37(Suppl 8):4-66.
3. Pisani P, Bray F, Parkin DM. Estimates of the world-wide prevalence of cancer for 25 sites in the adult population. Int J Cancer 2002;97:72-81.
4. Ferlay J, Bray F, Pisani P, *et al*. Globocan 2000: cancer incidence, mortality, and prevalence worldwide. Version 1.0. IARC Cancer Base No. 5. Lyon: IARC Press; 2001.
5. Thong-Ngam D, Tangkijvanich P, Mahachai V, *et al*. The current status of gastric cancer in Thai patients. J Med Assoc Thai 2001;84(4):475-82.
6. Singh K, Ghoshal UC. Causal role of *Helicobacter pylori* infection in gastric cancer: an Asian enigma. World J Gastroenterol 2006;12(9):1346-51.
7. Lauren P. The two histological main types of gastric carcinoma: Diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 1965;64:31-49.
8. Correa P. *Helicobacter pylori* and gastric cancer: state of the art. Cancer Epidemiol Biomarkers Prev 1996;5:477-81.
9. Suerbaum S, Michetti P. *Helicobacter pylori* infection. N Engl J Med 2002;347(15):1175-86.
10. Parsonnet J. The incidence of *Helicobacter pylori* infection. Aliment Pharmacol Ther 1995;9(2):45-51.
11. Howson CP, Hiyama T, Wynder EL. The decline in gastric cancer: epidemiology of an unplanned triumph. Epidemiol Rev 1986;8:1-27.
12. Cheung TK, Xia HHX, Wong BCY. *Helicobacter pylori* eradication for gastric cancer prevention. J Gastroenterol 2007;42(17):10-5.
13. Eslick GD, Lim LL, Byles JE, *et al*. Association of *Helicobacter pylori* infection with gastric carcinoma: a meta-analysis. Am J Gastroenterol 1999;94:2373-9.
14. Limburg P, Qiao Y, Mark S, *et al*. *Helicobacter pylori* seropositivity and subsite-specific gastric cancer risk in Linxian, China. J Natl Cancer Inst 2001;93:226-33.

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15. El-Omar EM, Oien K, Murray LS, *et al*. Increased prevalence of precancerous changes in relatives of gastric cancer patients: critical role of *H. pylori*. Gastroenterology 2000;118: 22-30.

16. Uemura N, Okamoto S, Yamamoto S, *et al*. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med 2001;345:784-9.

17. International Agency for Research on Cancer Working Group on the Evaluation of Carcinogenic Risks to Humans. Schistosomes, Liver Flukes, and *Helicobacter pylori*. Lyon: Internation Agency for Research on Cancer 1994:177-240.

18. Li H, Stoicov C, Cai X, *et al*. *Helicobacter* and gastric cancer disease mechanisms: host response and disease susceptibility. Current Gastroenterology Reports 2003;5:459-67.

19. El-Omar EM, Rabkin CS, Gammon MD, *et al*. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. Gastroenterology 2003;124: 1193-201.

20. Tsukamoto T, Mizoshita T, Tatematsu M. Animal models of stomach carcinogenesis. Toxicology Pathology 2007;35:636-48.

21. Tsugane S, Sasazuki S. Diet and the risk of gastric cancer: review of epidemiological evidence. Gastric Cancer 2007;10: 75-83.

22. Kono S, Hirohata T. Nutrition and stomach cancer. Cancer Causes Control 1996;7:41-55.

23. Tsugane S, Akabane M, Inami T, *et al*. Urinary salt excretion and stomach cancer mortality among four Japanese populations. Cancer Causes Control 1991;2:165-8.

24. Hirohata T. Analytic epidemiologic studies on the association between lifestyle and environmental factors and occurrence of cancer (in Japanese), Tokyo, Japan: Report of the studies in 1991 Supported by the Ministry of Health and Welfare of Japan (1-6):9-12.

25. Tricker AR, Preussmann R. Carcinogenic *N*-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. Mutat Res 1991;259:277-89.

26. Mitacek EJ, *et al*. Geographic distribution of liver and stomach cancers in Thailand in relation to estimated dietary intake of nitrate, nitrite, and nitrosodimethylamine. Nutr Cancer 2008;60(2):196-203.

27. Thong-Ngam D, *et al*. A simple rat model of chronic *Helicobacter pylori* infection for research study. Thai J Gastroenterol 2005;6(1):3-7.

28. Shimizu N, *et al*. *Helicobacter pylori* infection enhances glandular stomach carcinogenesis in Mongolian gerbils treated with chemical carcinogens. Carcinogenesis 1999;20:669-76.

29. Sugiyama A, Maruta F, Ikeno T, *et al*. *Helicobacter pylori*. Cancer Res 1998;58:2067-9.

30. Maekawa AC, Matsuoka H, Onodera. Organospecific carcinogenicity of *N*-methyl-*N*-nitrosourea in F344 and ACI/N rats. J Cancer Res Clin Oncol 1985;109:178-82.

31. Hirota N, *et al*. Selective induction of glandular stomach carcinoma in F344 rats by *N*-methyl-*N*-nitrosourea. Jpn J Cancer Res 1987; 78: 634-8.

32. Tatematsu M, *et al*. Induction of glandular stomach cancers in C3H mice treated with *N*-methyl-*N*-nitrosourea in the drinking water. Jpn J Cancer Res 1993; 84: 1258-64.

33. IARC. Some aromatic amines, hydrazine and related substances, *N*-Nitroso compounds and miscellaneous alkylating agents, in IARC monographs on the evaluation of carcinogenic risk of chemicals to humans. International Agency for Research on Cancer: Lyon, France 1974; 286.

34. Jiricny J. The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol 2006;7:335-46.

35. Matsumoto K, *et al*. Demonstration of ras and p53 gene mutations in carcinomas in the forestomach and intestineand soft tissue sarcomas induced by N-methyl-N-nitrosourea in the rat. Jpn J Cancer Res 1997;88:129-36.

36. Niwa T, *et al*. Whole-genome analyses of loss of heterozygosity and methylation analysis of four tumor-suppressor genes in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine induced rat stomach carcinomas. Cancer Sci 2005;96(7):409-13.

37. Tatematsu M, Takahashi M, Fukushima S, *et al*. Effects in rats of sodium chloride on experimental gastric cancers induced by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine or 4-nitroquinoline-1-oxide. J Natl Cancer Inst 1975;55:101-6.

38. Takahashi M, *et al*. Dose-dependent promoting effects of sodium chloride (NaCl) on rat glandular stomach carcinogenesis initiated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Carcinogenesis 1994;15:1429-32.

39. Sorbye H, Maartmann-Moe H, Svanes K. Gastric carcinogenesis in rats given hypertonic salt at different times before a single dose of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. J Cancer Res Clin Oncol 1994; 120:159-63.

40. Capoferro R, Torgersen O. The effect of hypertonic saline on the uptake of tritiated 7,12-dimethylbenz(*a*)-anthracene by the gastric mucosa. Scand J Gastroenterol 1974;9:343-9.

41. Charnley G, Tannenbaum SR. Flow cytometric analysis of the effect of sodium chloride on gastric cancer risk in the rat. Cancer Res 1985;45:5608-16.