Effect of N-Methyl-N-Nitrosourea on Helicobacter-induced Gastric Carcinogenesis in C57BL/6 Mice

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Background: The aim of this study was to investigate the effect of N-methyl-N-nitrosourea (MNU) treatment followed by chronic Helicobacter pylori SS1 and H. felis colonization on the stomachs of C57BL/6 mice. The role of MNU and Helicobacter species in gastric carcinogenesis was also elucidated.

Methods: A total of 69 C57BL/6 mice at 4 weeks of age were divided into 6 groups according to MNU treatment and Helicobacter infection. The mice were sacrificed at 21 and 50 weeks. The degree of inflammation was determined by histopathology. The levels of gastric mucosal myeloperoxidase, TNF-α, and interleukin-1β (IL-1β) were measured by ELISA.

Results: In the H. felis groups with or without MNU, the incidence of gastric tumors was 21.1% and 35.0% at 21 and 50 weeks, respectively. No gastric tumors were observed in all control mice. At 50 weeks, 37.5% of gastric adenoma cases were observed in the H. felis alone and MNU + H. felis groups. Furthermore, 12.5% of gastric adenocarcinoma cases were observed in the MNU alone and MNU + H. felis group at 50 weeks, respectively, than that for control mice (P < 0.05). However, the effect of MNU on H. pylori SS1-induced gastric carcinogenesis was low compared to that on H. felis.

Conclusions: Administration of MNU before H. felis infection provokes severe inflammation through IL-1β, and eventually induces gastric cancer. However, the role of MNU in H. pylori SS1-induced gastric carcinogenesis model is minor.

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Key Words: Helicobacter felis, Helicobacter pylori, MethylNitrosourea, Gastric neoplasm
cinogenesis. In contrast, *H. pylori* SS1 failed to induce gastric adenocarcinoma in the majority of mouse species. We previously demonstrated that inflammation induced by *H. felis* infection was more severe than that from infection with the *H. pylori* SS1 strain, and it finally led to adenocarcinoma in C57BL/6 mouse models. Several animal studies demonstrated that the combination of MNU and *Helicobacter* spp. infection led to rapid induction of gastric cancer and to a certain extent mimics the proposed pathogenesis of human carcinogenesis.

The aim of this study was to investigate the effect of MNU treatment followed by chronic *H. pylori* SS1 and *H. felis* colonization on the stomachs of C57BL/6 mice. The role of MNU and *Helicobacter* spp. in inflammatory reactions of gastric carcinogenesis, including the production of pro-inflammatory cytokines, was also elucidated.

**MATERIALS AND METHODS**

1. **Animals**

Four-week-old male C57BL/6 mice (Orient Co., Ltd., Seoul, Korea) were used for the experiments. All mice were housed in cages maintained at 23°C with a 12/12-hour light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital (BA1401-144/001-02).

2. **Experimental design**

Mice were divided into the following six groups: Group 1, control; Group 2, MNU alone; Group 3, *H. pylori* SS1 alone; Group 4, *H. felis* alone; Group 5, MNU + *H. pylori* SS1; Group 6, MNU + *H. felis*. MNU (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in distilled water at a concentration of 200 ppm and administered in drinking water in light-shielded bottles ad libitum. Mice in the MNU groups (Groups 2, 5, and 6) were given drinking water containing 200 ppm MNU, biweekly, for a total of 10 weeks. After completion of MNU administration, mice in the *Helicobacter* species alone groups (Groups 3 and 4) and MNU + *Helicobacter* species group (Groups 5 and 6) were inoculated orogastrically with 1 × 10^10 colony-forming units/mL of *H. pylori* SS1 and *H. felis* (ATCC 49179), five times every alternate day (Fig. 1). The mice were sacrificed by CO₂ asphyxiation at 21 and 50 weeks after inoculation.

3. **Histopathology**

At necropsy, stomach tissue was taken from the greater curvature beginning at the squamocolumnar junction and ending at the gastroduodenal junction. Linear gastric strips were fixed in 10% formalin solution, processed by standard methods, embedded in paraffin, sectioned at 5 μm, and stained with H&E. The stomach mucosa was histologically examined for inflammatory and epithelial changes, and for the presence of *H. pylori* or *H. felis*. The degree of neutrophil infiltration, mononuclear cell infiltration, atrophy, and metaplasia was assessed according to...

![Figure 1](image-url)

Figure 1. Protocol of the study. Mice were divided into the six groups: Group 1, control; Group 2, N-methyl-N-nitrosourea (MNU) alone; Group 3, *Helicobacter pylori* SS1 alone; Group 4, *H. felis* alone; Group 5, MNU + *H. pylori* SS1; Group 6, MNU + *H. felis*. MNU was dissolved in distilled water at a concentration of 200 ppm and administered in drinking water in light-shielded bottles ad libitum biweekly, for a total of 10 weeks. After completion of MNU administration, mice in the *Helicobacter* species alone groups (Groups 3 and 4) and MNU + *Helicobacter* species group (Groups 5 and 6) were inoculated orogastrically with 1 × 10^10 colony-forming units/mL of *H. pylori* SS1 and *H. felis* (ATCC 49179), five times every alternate day. The mice were sacrificed by CO₂ asphyxiation at 21 and 50 weeks after inoculation.
the updated Sydney classification as follows: 0, absent; 1, minimal; 2, mild; 3, moderate; 4, marked.

4. Measurement of mucosal myeloperoxidase, TNF-α, and interleukin-1β

Scraped mucosa (10 mg) was homogenized for 30 seconds with a Polytron homogenizer in 200 μL of ice-cold lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris [pH 7.4], 10% glycerin, 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, and 28 μg/mL aprotinin). The cell suspensions were centrifuged at 13,000 rpm for 15 minutes and the resulting supernatant was assayed using a myeloperoxidase (MPO) ELISA kit (HyCult Biotechnology, Uden, The Netherlands). For TNF-α and interleukin-1β (IL-1β), the appropriate kits from R&D Systems (Minneapolis, MN, USA) were used following the manufacturer’s instructions. Protein concentration was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, MA, USA). The concentration of each cytokine was measured in picograms per milligram of protein. All assays were performed in triplicate.

5. Statistical analysis

Data are expressed as the mean ± SEM. Comparison between the two groups (experimental and control) was performed using the Mann-Whitney U-test. P < 0.05 was considered to indicate a statistically significant result. All statistical analyses were performed using IBM SPSS software ver. 20.0 (IBM Co., Armonk, NY, USA).

RESULTS

1. Gastric tumor incidence and histopathology

At 21 weeks, among 19 experimental mice, 4 mice (1 hyperplastic polyp and 1 adenoma in the H. felis alone group; 2 hyperplastic polyps in the MNU + H. felis group) had gastric tumors and the incidence of gastric tumors was 21.1%. At 50 weeks, among 40 experimental mice, 14 mice had gastric tumors and the incidence of gastric tumors was 35.0%. In the experimental group, 37.5% of gastric adenomas were observed in the H. felis and MNU + H. felis groups, respectively, and 25.0% of gastric adenomas were identified in the H. pylori group. Furthermore, 12.5% of gastric adenocarcinomas were observed in the MNU alone and the MNU + H. felis group, respectively. In contrast, no gastric tumors were observed in all control mice (Fig. 2A). The neutrophil and monocyte grades of Groups 2, 4, 5, and 6 were increased compared to those of the control group at 21 weeks and the neutrophil and monocyte grades of Groups 3, 4, and 6 were increased compared to those of the control group at 50 weeks (Fig. 2B).

2. Expression of pro-inflammatory cytokines

At 21 weeks, the gastric mucosal MPO level in H. felis-infected mice was significantly higher (P < 0.05) than that in control mice (Fig. 3A). At 50 weeks, the gastric mucosal MPO level in the MNU + H. pylori group was significantly higher (P < 0.05) than that in control mice (Fig. 3A). The gastric mucosal IL-1β level was significantly higher for the MNU + H. felis group at 21 weeks and the H. felis group at 50 weeks, respectively, than that for control mice.

Figure 2. The incidence of gastric hyperplastic polyps, adenoma, and adenocarcinoma at 50 weeks and histologic findings at 21 and 50 weeks. Stomach tissue was taken from the greater curvature beginning at the squamocolumnar junction and ending at the gastro-duodenal junction. (A) Hyperplastic polyp, adenoma, and adenocarcinoma were detected using a stereomicroscope and histopathological examination. The incidence was calculated as percentage of tumor-bearing mice/total mice in each experimental group. (B) The degree of histological neutrophil and mononuclear cell infiltration (0, absent; 1, minimal; 2, mild; 3, moderate; 4, marked) in the stomach mucosa of mice treated N-methyl-N-nitrosourea (MNU), Helicobacter felis and/or H. pylori. Data are presented as means ± SEMs. *P < 0.05 compared with controls at the same time-point.
Figure 3. The levels of gastric mucosal myeloperoxidase, interleukin-1β, and TNF-α at 21 and 50 weeks. Scraped mucosa (10 mg) was homogenized and the cell suspensions were centrifuged. (A) Myeloperoxidase (MPO), (B) TNF-α, and (C) interleukin (IL)-1β levels in the supernatants were measured with ELISA kit following the manufacturer’s instructions. Data are presented as means ± SEMs. MNU, N-methyl-N-nitrosourea. *P < 0.05 compared with control mice at the same time-point.

In this study, we observed that the incidence of gastric tumors, including adenomas and adenocarcinomas, was higher in the groups of mice infected with *H. felis* with or without MNU. *H. pylori* SS1 alone could cause chronic gastritis with hyper-proliferation of the gastric mucosa; however, it did not cause gastric cancer at 50 weeks. In contrast, *H. felis* provoked more severe inflammation, glandular atrophy, metaplasia, dysplastic change, and with MNU, *H. felis* caused gastric cancer at 50 weeks. In addition, we analyzed the levels of gastric mucosal pro-inflammatory cytokines such as IL-1β and TNF-α.

MNU-induced tumors in mice are located mainly in the gastric antrum, and pathologically are uniformly well differentiated or moderately differentiated adenocarcinomas. MNU treatment (0.5 mg, weekly, intragastric intubation) in BALB/c mice after removal of the forestomach induced well-differentiated adenocarcinoma in the glandular stomach of mice with 100% incidence within 40 weeks. The efficiency of tumor induction by MNU was found to depend on its concentration rather than total intake, and MNU administered in drinking water at 240 ppm in alternate weeks (total exposure: 5 weeks) was effective in inducing gastric cancer in six strains of mice that were studied.

However, the MNU model of gastric carcinogenesis does not proceed through a classical atrophy-metaplasia-dysplasia sequence and this is a drawback of the MNU mouse model. Several studies showed that pretreatment with MNU prior to Helicobacter infection caused more severe pre-neoplastic changes and increased the occurrence of gastric cancer. Han et al. found that > 80% of experimental C57BL/6 mice exhibited adenoma or adenocarcinoma in their glandular stomach in 12 months after treatment of MNU + *H. pylori* SS1, suggesting that *H. pylori* infection clearly enhanced the development of gastric carcinogenesis. Tomita et al. demonstrated that nearly 100% of experimental mice presented with pre-neoplastic lesions or adenocarcinoma following 36 weeks of treatment in the MNU + *H. felis* group. It is also reported that MNU + Helicobacter spp. infection induced a high proportion of gastric cancer than those of MNU only in Mongolian gerbils. Therefore Helicobacter spp. infection followed by MNU treatment thought similar to the pathogenesis of human gastric cancer.

In this study, the incidence of gastric adenoma or adenocarcinoma was higher in the *H. felis-infected* mouse group with or without MNU compared with those of the control group or *H. pylori-infected* group. While a chronic colonization of the mouse stomach by *H. felis* and *H. pylori*SS1 leads to chronic gastritis and
SS1-induced gastric carcinogenesis is lower than that of H. felis. We observed H. pylori SS1-infected C57BL/6 mice for 80 weeks and funded by the Korea government (MSIP) (No. 2011-0030001). (NRF) of Korea grant for the Global Core Research Center (GCRC) 2012-142-531-42.

Secondly, we inoculated Helicobacter species five times every alternate day rather than three times every alternate day, which was used in other studies. According to our previous experimental results, inoculation five times every alternate day increased Helicobacter density and therefore we used this protocol. However, it is not clear whether five times inoculation increases gastric inflammation.

In conclusion, administration of MNU before H. felis infection provokes severe inflammation, and eventually induces gastric cancer. However, it seems that the effect of MNU in H. pylori SS1-induced gastric carcinogenesis is lower than that of H. felis. IL-1β plays an important role in H. felis gastric inflammation; however, TNF-α is not involved in the inflammatory process.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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