[ORIGINAL ARTICLE] Long-term administration of sterigmatocystin with drinking water in *Helicobacter pylori*- infected aged Mongolian gerbils enhances carcinogenesis in the gastric mucosa

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Keywords

1. *Helicobacter pylori*
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To elucidate the mechanisms of the development of gastric carcinogenesis, the effects of an *Aspergillus*-produced substance, sterigmatocystin (ST), were examined in stomach tissues in the *H. pylori*-infected Mongolian gerbil model of aging.

Five week old, *H. pylori*-infected male Mongolian gerbils were used in this study, and maintained under the ST-non-treated conditions until they were Seventy-five week old. Mongolian gerbils received ST ad libitum at a concentration of 100 ppb or 1000 ppb dissolved in drinking water for a period of 24 weeks.

The histopathological changes were scored on a scale of 0-3, and the expression of the proliferating cell nuclear antigen (PCNA) and p53 genes was also analyzed by immunostaining.

The mean indices of histopathological changes in the *H. pylori*-infected animals with various gastric diseases after ST-administration at a dose of 0 ppb (*H. pylori* control), 100 ppb, or 1000 ppb were as follows: For gastritis, the mean values were 1.9 for the *H. pylori* control, 2.1 for ST 100 ppb, and 2.5 for ST 1000 ppb. The mean indices in the ST-administered groups were increased compared with those of *H. pylori* control group; however, the differences were not significant.

Furthermore, the proliferating cell nuclear antigen (PCNA) expression rate in the *H. pylori*-infected animals (as indicated by the labeling index) were significantly greater in the ST-administered groups (100 ppb or 1000 ppb) than in the *H. pylori* control group.

In addition, in the *H. pylori*-infected animals, the p53 expression rates in the 1000 ppb ST-administered group was also significantly greater than in the *H. pylori* control group.

The results indicate that *H.pylori* induce changes in the gastric mucosa in the *H. pylori*-infected aged Mongolian gerbils, and suggest that ST enhances gastric carcinogenesis in these animals.

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Introduction

Previous studies in humans have clarified that *H. pylori*-infection induces the infiltration of inflammatory cells into the gastric mucosa,[1, 2] which leads to the development of chronic atrophic gastritis, erosions, gastric ulcer,[3] and gastric cancer.[4] Uemura et al.[5] reported that, in a follow-up of 1,526 subjects for about 8 years, gastric cancer developed in approximately 4.7% of *H. pylori*-infected gastritis patients, but in none of the uninfected subjects, indicating a close relationship between *H. pylori* infection and gastric cancer.

On the other hand, it is likely that, in addition to the direct action of Vacuolating toxin A (VacA toxin) on the gastric mucosa, the action of toxic environmental substances on the gastric mucosa in the presence of persistent inflammatory lesions induces gastric carcinogenesis.[6] We speculate that there must be other causal agents except *H. pylori* that produce gastric cancer. The causal agents of gastric cancer may include fungus toxins, which are universally distributed in Asian countries. The genus *aspergillus*, which is indigenous to human habitats, is known to grow in corn, barley, wheat, peanuts, and walnuts and to produce various mycotoxins.[7] In particular, the mycotoxin sterigmatocystin(ST), a substance of *Aspergillus versicolor*, the chemical structure of which has been elucidated, is a precursor of aflatoxin B1. It is also widely distributed in soil, agricultural crops, and grain crops.[8] Xie et al.[9] have shown that *Aspergillus*-inoculated corn flour induces typical hyperplasia of the glandular stomach in mice. In addition, an analysis of diet and gastric juice samples from patients with chronic gastritis in rural regions with high gastric cancer mortality showed that they were frequently contaminated with *Aspergillus versicolor*.

Pham et al.[10] reported that the gastric cancer mortality rates for men and women increase with age. Kimura et al.[11] showed that the extension of atrophic gastritis is closely correlated with aging. Asaka et al.[12] noted that the incidence of intestinal metaplasia increases with age in *H. pylori*-infected subjects. However, various factors involved in the development of atrophic gastritis, erosions, and progressive malignant transformation have not been elucidated.

In particular, the interactions of aging with *H. pylori*-infection and the risk factors for gastric cancer have not been sufficiently studied, therefore, their relationship should be clarified.

In this study, to elucidate the mechanism of gastric carcinogenesis, we examined the relationship between gastric diseases and ST-administration or the combined effects of *H. pylori*-infection and ST-administration in the Mongolian gerbil model with aging, which allowed us to observe various gastric diseases, such as gastritis and erosions, that have similar characteristics to their counterparts in *H. pylori*-infected human subjects.

Materials and Methods

Animals

Five week old, male Mongolian gerbils (NOs/Sea, Kyudo, Kumamoto, Japan), weighing a mean of 69.4±5.57 g at the beginning of the experiment, were used in this study. They were housed 4-5/cage in an animal room maintained at 23 ±2°C with 55±5% humidity under a 12/12-h light-dark cycle, with free access to food (CE-2; CLEA Japan, Inc., Tokyo) and drinking water.

Five week old Mongolian gerbils were inoculated orally with *H. pylori* ATCC43504 strain (American Type Culture Collection, Manassa, VA, USA), after 8 weeks, the confirmation of bacterial colonization by ELISA method, and maintained under the above-described conditions until they became 75 weeks old. This study was based on the Guidelines for Animal Experimentation of the Faculty of Medicine, Oita University and was approved by its Ethics Committee.

Experimental protocol

Seventy five week old Mongolian gerbils were randomly assigned to the following groups: 1) non-treated control (*H. pylori*-uninfected and ST non-administration), 2) *H. pylori* control (*H. pylori*-infected and ST non-administration), 3) *H. pylori*+ ST(100 ppb), and 4) *H. pylori* + ST(1,000 ppb). These groups of animals in their respective cages showed no difference in body weight.

ST (Sigma Chemical Co., St. Louis, MO, USA) was administered at the specified concentrations in drinking water. After 24 weeks of ST administration, each groups of Mongolian gerbils were autopsied over 3 days. The stomach was opened along the greater curvature and fixed in 10% neutral formalin for more than 4 hours.

Histological examination

The stomach was divided longitudinally into two parts, embedded in paraffin, and cut into 7-μm thick sections, which were then stained with hematoxylin and eosin (HE) for morphological examination and with periodic acid-Schiff's reaction (PAS) and Alcian blue (AB, pH 2.5) for the detection of mucin-containing cells.

The histopathological findings of the stomach were classified as follows: 1) active gastritis, 2) invagination of glands, 3) intestinal metaplasia, and 4) lymphoid infiltration were scored on a scale of 0-3, where 0= normal, 1= mild, 2= moderate, and 3= marked.[13-15] And 5) erosion, 6) hyperplastic polyp were evaluated of positive or negative cases.[16, 17]

Table 1. Body weight and positive rates for anti-*H.pylori* immunostaining of Mongolian gerbils in the four groups.

Group	n	Body weight at 99 weeks (g) *	*H. pylori*-positive #
Non-treated control	14	99.8± 18.39	0/14
H. pylori control	14	96.9± 19.77	14/14†
H. pylori + ST (100 ppb)	14	101.1± 16.60	14/14†
H. pylori + ST (1,000 ppb)	13	95.2± 20.92	13/13†

* The results represent means ±SD. Data were analyzed by the multiple comparison test adjusted by Tukey.

\# The results were analyzed by the chi-square test.

† $P<0.05$, compared with the Non-treated control group.

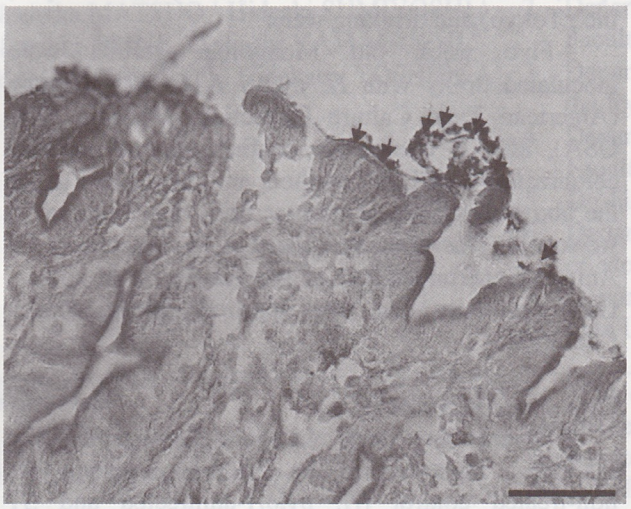

Fig. 1. *H. pylori* stained brown with immunostaining for anti-*H. pylori* antibody, and these present in the surface mucous gel layer. Arrows show *H. pylori* positive. (Anti-*H. pylori* antiboby immunostaining, *H.pylori* control group, Scale bar = 50 μm).

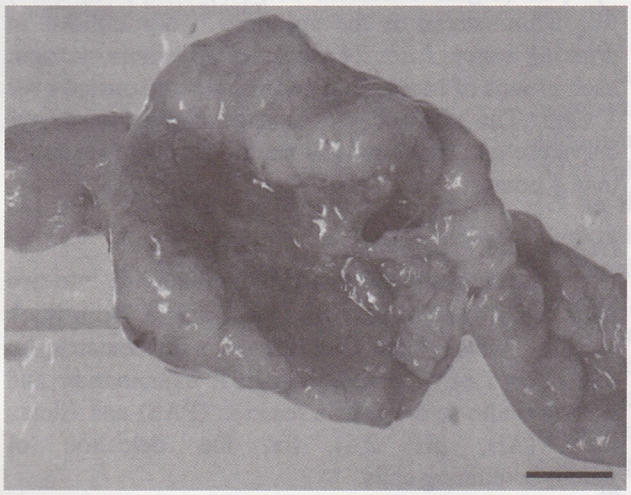

Fig. 2. Microscopic view of the gastric mucosa of *H.pylori*-uninfected Mongolian gerbils. (HE staining, Non-treated control group, Scale bar = 50 μm).

Immunohistochemistry

H. pylori expression was assessed by immunohistchemistry using rabbit anti- *H. pylori*

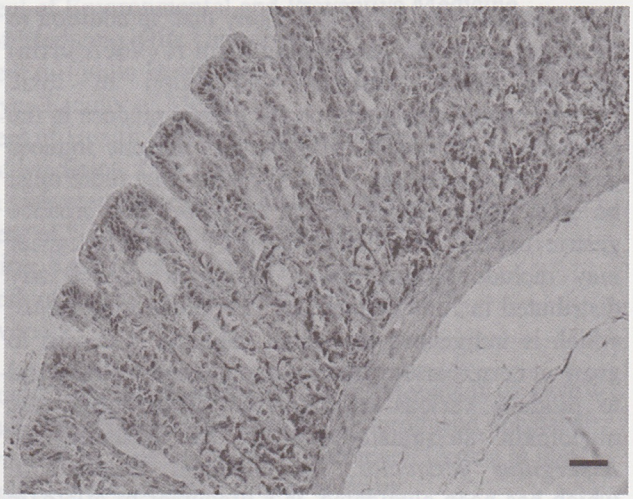

Fig. 3. Macroscopic image of *H. pylori*-infected Mongolian gerbils stomach. (ST 1,000 ppb-administrated group, Scale bar = 5 mm).

antibody (Nichirei, Japan). And gastric diseases were examined by histochemical staining with mouse anti-proliferating cell nuclear antigen (PCNA) antibody (Nichirei, Japan) and mouse anti-p53 antibody (Nichirei, Japan). The paraffin sections were autoclaved in TE buffer (pH 9.0) for 15 min to retrieve p53 antigenicity or microwaved in 10 mM citrate buffer (pH 6.0) for 15 min to retrieve PCNA antigen. However heating was not necessary for staining with anti- *H. pylori* antibody. The slide was subsequently incubated with the primary anti- *H. pylori* for 1 hr, and primary anti-PCNA for 1 hr at room temperature, and primary anti-p53 for over night at 4℃, together with their respective secondary antibodies (biotin-conjugated anti-immunoglobulin) at room temperature for 10 min, followed by reaction with the streptavidin-peroxidase method at room temperature for 5 min.

The expressions of the PCNA-labeling index (LI) and p53-positive index (PI) were evaluated by light microscopy. [18, 19] Three microscopic fields of the gastric body and pyloric region that contained a large number of glands were photographed, and each index was calculated by counting about 1,000 positive and ormal nuclei in the selected fields.

Table 2. Histopathological changes in the glandular stomach of Mongolian gerbils in the four groups.

Group	n	Active gastritis*	Invagination of glands*	Intestinal metaplasia*	Lymph infiltration*
Non-treated control	14	0.3±0.61	0 0	0 0	0 0
H. pylori control	14	1.9±1.07†	1.8 ±0.70†	1.9 ±0.53†	2.1 ±1.03†
H. pylori + ST (100 ppb)	14	2.1±0.92†	1.6 ±1.02†	2.1 ±0.66†	1.2 ±0.58†
H. pylori + ST (1,000 ppb)	13	2.5±0.66†	2.2 ±0.93†	2.0 ±0.71†	2.1 ±0.95†

* The results represent means ±SD. Data were analyzed by the nonparametric Mann-Whitney U test.
†P<0.05, compared with the Non-treated control group.

Table 3. Erosions and hyperplastic polyps in the stomach of Mongolian gerbils in the four groups.

Group	n	Erosions *	Hyperplastic polyps *
Non-treated control	14	1/14	0/14
H. pylori control	14	13/14 †	11/14 †
H. pylori + ST (100 ppb)	14	14/14 †	12/14 †
H. pylori + ST (1,000 ppb)	13	13/13 †	10/13 †

* The results show positive cases. Data were analyzed by chi-square test.
† P<0.05, compared with the Non-treated control group.

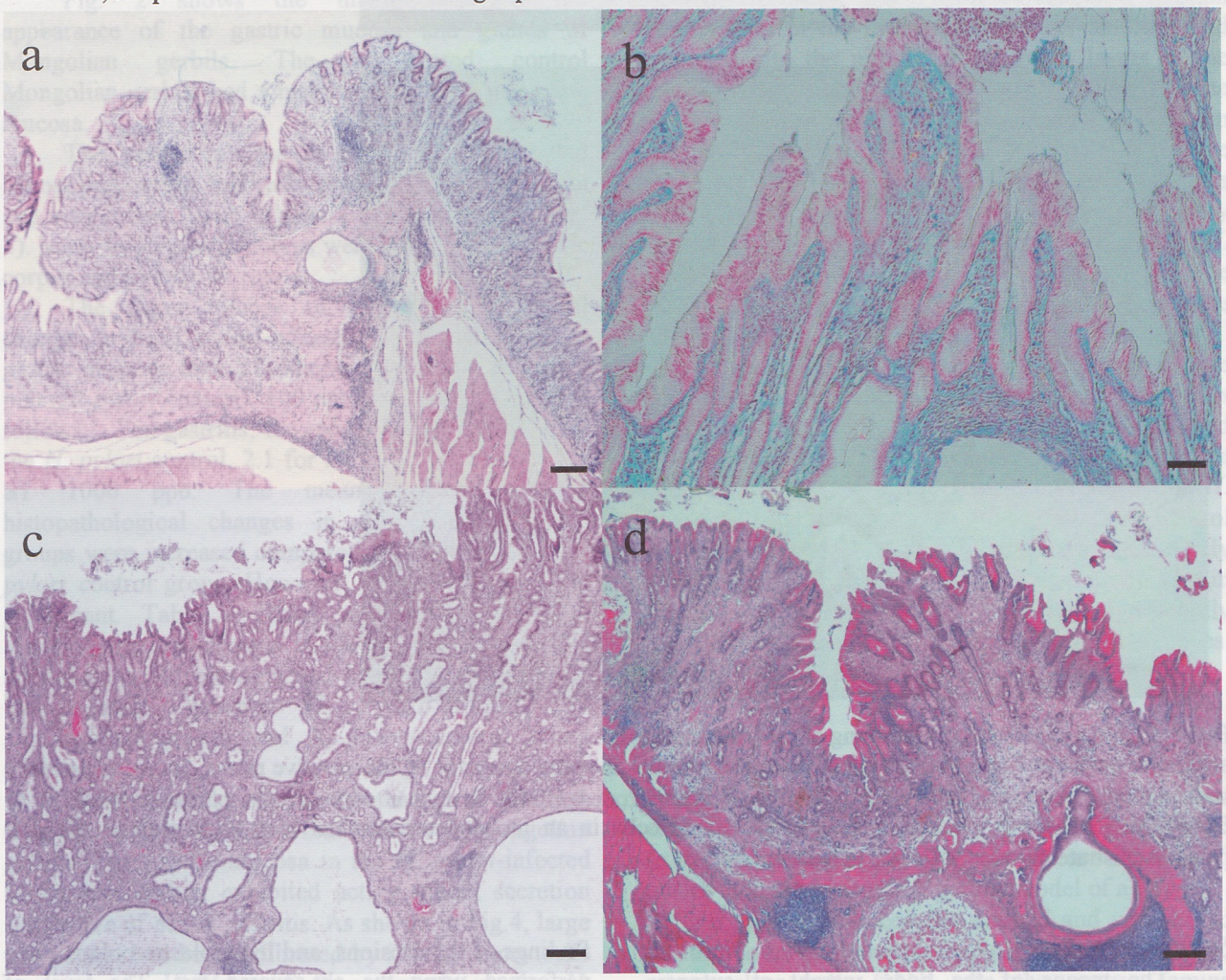

Fig.4. Histopathological images in the gastric mucosa of *H. pylori*-infected Mongolian gerbils.
a: Severe infiltration of inflammatory cells and polyps. (HE staining, ST 1,000 ppb-administrated group, Scale bar = 250 μm). b: Intestinal metaplasia lesions lined with Alcian blue-stained goblet cells. (Alcian blue at pH 2.5 staining, ST 1,000 ppb-administrated group, Scale bar = 100 μm). c: Erosions formation in the antrum mucosa, with some dilated glands in the submucosa. (HE staining, ST 100 ppb-administrated group, Scale bar = 250 μm). d: The mucosa is thick with dilated glands. Many dilated glands can be seen in the submucosa. (HE staining, ST 1,000 ppb-administrated group, Scale bar = 250 μm).

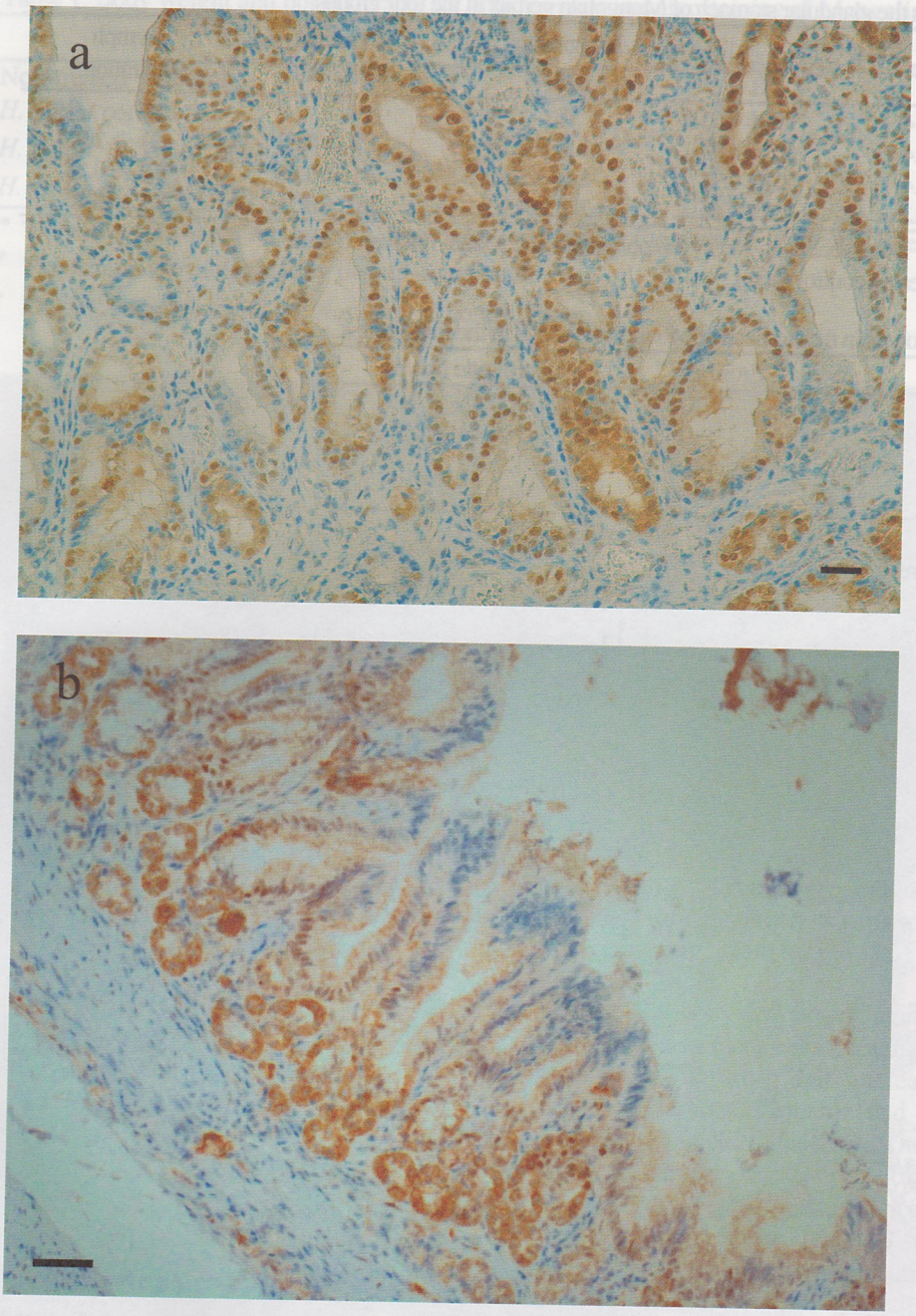

Fig. 5. PCNA and p53 immunostaining of the gastric mucosa.
a: Gastric mucosa of *H. pylori*-infected Mongolian gerbils showing abundant PCNA-positive nuclei. Many nuclei were stained in the extended glands. (ST 1,000 ppb-administrated group, Scale bar =50 μm). b: p53 expression in *H. pylori*-infected gastric mucosa. A cluster of stained cells in an incomplete intestinal metaplasia region. (ST 100 ppb-administrated group, Scale bar =50 μm).

Statistical analysis

The means of the body weight at autopsy, histopathological index (0-3 points) and p53 PI were compared between the groups using the Mann-Whitney U Test. PCNA LI were compared by one-way ANOVA followed by Tukey's multiple comparison test. Qualitative evaluations, such as to assess *H. pylori*-positive rates, and histopathological findings of the erosions, and hyperplastic polyps were performed using the chi-squared test. The level of significance was set at $p<0.05$.

Results

H. pylori expressions

Anti-*H. pylori* antibody was positive in all *H. pylori*-infected Mongolian gerbils (Table 1). Thus, none

Table 4. Proliferating cell nuclear antigen (PCNA) LI and p53 PI in the four groups.

Group	n	PCNA LI (%) *	P53 PI (%) #
Non-treated control	14	13.9 ± 9.68	0 ± 0.07
H.pylori control	14	48.3 ± 10.89 [a]	33.4 ± 16.02 [a]
H.pylori + ST (100 ppb)	14	60.8 ± 8.45 [ab]	37.4 ± 11.87 [a]
H.pylori + ST (1,000 ppb)	13	62.0 ± 7.67 [ab]	41.7 ± 11.36 [ab]

* The results represent means ± SD. Data were analyzed by multiple comparison test adjusted by Tukey.
\# The results represent means ±SD. Data were analyzed by Mann-Whitney U test.
[a] $p < 0.05$, compared with the Non-treated control group
[b] $p < 0.05$, compared with the *H. pylori* control group.

of the Mongolian gerbils in the non-treated control group were positive for anti- *H. pylori* antibody. Fig 1 shows *H. pylori* stained brown with immunostaining for *H. pylori*.

Histopathological findings

Fig. 2 shows the histologically normal appearance of the gastric mucosa and glands of Mongolian gerbils. The non-treated control Mongolian gerbils had a histologically normal gastric mucosa.

The gastric mucosa of the *H. pylori*-infected Mongolian gerbils markedly protruded into the lumen and was covered with a large amount of mucus (Fig. 3). Many hyperplastic polyps were found in the gastric corpus and pyloric antrum.

The mean indices of the histopathological changes in the *H. pylori*-infected animals with various gastric diseases after ST-administration at a dose of 0 ppb (*H. pylori* control), 100 ppb, or 1000 ppb were as follows: For gastritis, the mean values were 1.9 for the *H. pylori* control, 2.1 for ST 100 ppb, and 2.5 for ST 1000 ppb. The mean indices of the histopathological changes in the ST-administered groups were increased compared with those of the *H. pylori* control group. However, the increase was not significant. Table 2 shows the mean indices of histopathological changes in the stomach. There was no difference of erosions and hyperplastic polyps in the stomach between *H. pylori*-infected ST-administered groups and *H. pylori* control group (Table 3).

Fig.4 demonstrates histopathological findings from *H. pylori*-infected, ST-administered Mongolian gerbils. The gastric mucosa in the *H. pylori*-infected Mongolian gerbils exhibited active mucin secretion suggestive of active gastritis. As shown in Fig.4, large polyps that had been infiltrated by inflammatory cells were observed. Most of the stomach in the *H. pylori*-infected Mongolian gerbils exhibited severe progressive gastritis, polyps of varying size, erosions, and intestinal metaplasia (Figs.4 a, b, c, and d).

In all *H.pylori*-infected Mongolian gerbils, marked intestinal metaplasia occurred not only in the pyloric antrum but also in the body of the stomach. Severe inflammatory infiltration was observed in all *H. pylori*-infected Mongolian gerbils, but not in the non-treated animals (Table 2).

PCNA LI and p53 PI expression

PCNA-positive cells were more abundant in the gastric mucosa of *H. pylori*-infected Mongolian gerbils than in the non-treated control animals, and extended into the middle of the basal layers of the mucosa. Fig. 5a shows PCNA immunostaining of the gastric mucosa of *H. pylori*-infected Mongolian gerbils. It should be noted that many PCNA-positive nuclei are seen around the markedly dilated glands. As shown in Table 4, the PCNA LI was significantly higher in *H. pylori*-infected than in *H. pylori*-uninfected Mongolian gerbils. In addition, the PCNA LI was significantly higher in the ST-administered Mongolian gerbils.

Expression of p53 was not detected in the normal part of the gastric mucosa. p53 positive cells were observed due to the presence of faintly-stained nuclei (Fig. 5b). The mean indices of p53 were significantly greater in the gastric mucosa of the *H. pylori*-infected groups than in the non-treated control group, and the mean indices of p53 were significantly greater in the ST 1000 ppb administered group than in the *H. pylori* control group, showing significantly higher expression rates of tumor suppressor genes. Table 4 shows p53 positive indices.

Discussion

The present study demonstrated that long-term administration of the mycotoxin ST had a metaplastic effect including increases in the PCNA and p53 expression rates in the gastric mucosa in the *H. pylori*-infected Mongolian gerbils model of aging.

H. pylori produced the marked and widespread infiltration of polymorphonuclear cells (PMN) and mononuclear cells (MN) into the gastric lamina propria excluding areas that are covered by the stratified squamous epithelium. PMN and MN infiltrated into the pyloric gland area locally as well as into most of the submucosa and muscular layer, inducing lymph follicle formations, and leading to erosive gastritis, gastric mucosal atrophy, and

intestinal metaplasia.

Atrophic gastritis involved the body of the stomach in the *H. pylori*-infected, ST-administered Mongolian gerbils. These results were similar to those reported by Correa et al.[20] They found that *H. pylori* induced atrophic gastritis and intestinal metaplasia, and that mucosal atrophy developed in the lesser curvature of the stomach and extended to the greater curvature.

Histopathological examinations confirmed that the gastric mucosa of *H. pylori*-infected Mongolian gerbils underwent metaplastic changes. It is suggested that *H. pylori* infection is a major etiologic factor for gastric mucosal atrophy, it grows to be high risk of intestinal metaplasia and gastric cancer. [16, 21] In our study, the mean indices of the histopathological changes in the ST-administered groups were increased compared with those of the *H. pylori* control group. However, the increase was not significant. Scott et al.[22] suggested that in human, excessive proliferation of the gastric mucosa promoted the progression of normal to dysplastic gastric epithelia. On the other hand, Ma et al. [17] reported that ST enhanced the development of *H. pylori*-induced gastric mucosal injury in Mongolian gerbils.

PCNA is an auxiliary protein of DNA polymerase delta and is synthesized in the cell nucleus in the late G1 and S phases of the cell cycle.[23] It is a very reliable indicator of cell proliferative activity in tumorous and non-tumorous tissues. [24,25] We evaluated PCNA expression in several experimental groups. The PCNA LI rate was high in the *H. pylori*-infected groups and was significantly greater in the *H. pylori* +ST(100 ppb) and *H. pylori* +ST(1,000 ppb) groups than in the *H. pylori* control group. These results indicate that ST changes the patterns of active cell proliferation in gastric glands.

The p53 gene is a tumor suppressor gene which is most frequently expressed at the time of malignant transformation of cells, including to those in gastric cancer. [26] In this study, the p53 gene expression rate was significantly higher in the gastric mucosa in the *H. pylori*-infected groups than in the non-treated control group, and that in the *H. pylori* +ST(1,000 ppb) group was significantly greater than in the *H. pylori* control group. The p53 expression was identified in occasional epithelial cells that were concentrated in the neck region, as reported by previous study. [27] This suggests that the combination with *H. pylori* and ST increase DNA damage in epithelial cells. p53 gene expression has been reported in precancerous lesions such as intestinal metaplasia. [18, 28] *H. pylori*-induced gastritis is known to destabilize genes, as indicated by p53 gene expression. [29, 30] Furthermore, Xie et al. [31] reported that, in an *in vitro* study to elucidate the mechanism of ST-induced carcinogenesis in mouse embryonic fibroblasts, the ST-induced activation and overexpression of MDM2 led to the suppression or inhibition of p53 gene function, and impairment of the DNA-repair function resulted in the failure of G1 arrest; thus, ST induced the loss of genomic integrity, thereby increasing carcinogenicity. Therefore, we speculated that the high-level of PCNA and p53 gene expression confirmed in this study, indicates that ST promotes the malignant transformation of cells.

We administered ST to aged Mongolian gerbils to examine its carcinogenicity. The mean lifespan of Mongolian gerbils is reportedly about 3 years. [32] If a crude extrapolation could be made, seven weeks corresponds to a human age of 3-4 years, 26 weeks to 14 years old, and 1 year to 25 years old. [33] Therefore, the Mongolian gerbils at the start of this study were estimated to be in their fifties in terms of human age. Pham et al. [10] have reported that the gastric cancer mortality rates for men and women increase markedly with age. Furthermore, aging factors are closely involved in the extension of atrophic gastritis. [11] The results of this study indicate that *H. pylori* induce changes in the gastric mucosa in *H. pylori* - infected aged Mongolian gerbils, and suggest that an environmental toxic substance such as ST enhances gastric carcinogenesis in *H. pylori*-infected Mongolian gerbils model of aging, however further, we need to study in the age of youth and middle. The mechanism of ST-induction of histopathological changes remains to be clarified, and detailed studies of the base arrangements of genes with carcinogenic effects are needed.

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