

Optimization of Infectious Conditions with *Helicobacter Pylori* in the Infection-highly Resistant Mongolian Gerbils Supplied in Japan

Katsuhito Kawato¹, B.Eng

Tetsurou Seita, PhD²

Takashi Kuribayashi, DVM, PhD²

Yoshiichi Takagi, DVM³

Motoo Matsuda, PhD⁴

Shizuo Yamamoto, DVM, PhD²

¹Laboratory of Immunology, School of Life and Environmental Science, Azabu University, 1-17-71 Fuchinobe, Chou-ku Sagamihara, Kanagawa 252-5201, Japan

²Graduate School of Environmental Health Science, Azabu University, 1-17-71 Fuchinobe, Chou-ku Sagamihara, Kanagawa 252-5201, Japan

³Graduate School of Veterinary Science, Azabu University, 1-17-71 Fuchinobe, Chou-ku Sagamihara, Kanagawa 252-5201, Japan

⁴Emeritus Professor, Azabu University, 1-17-71 Fuchinobe, Chou-ku Sagamihara, Kanagawa 252-5201, Japan

*Optimization of infectious with *H. pylori**

Address correspondence to: Shizuo Yamamoto, Graduate School of Environmental Health Sciences, Azabu University, 1-17-71 Fuchinobe, Chou-ku Sagamihara, Kanagawa 252-5201, Japan (Fax: +81-42-752-2461, E-mail: yamamoto@azau-u.ac.jp)

KEY WORDS: *H. pylori*, Mongolian gerbils, gastric pH, sodium bicarbonate, Japan

ABSTRACT

Optimization of infectious conditions with *Helicobacter pylori* (*H. pylori*) in the infection-highly resistant Mongolian gerbils was performed. Mongolian gerbils were inoculated with *H. pylori* without pre-treatment or administered with sodium bicarbonate prior to inoculation. Serum titers of IgG and IgM

against *H. pylori* measured by ELISA. *H. pylori* were isolated from the stomachs of Mongolian gerbils with increased IgM and IgG titers. On the other hand, *H. pylori* were not isolated from the stomachs of Mongolian gerbils with unchanged titers. All of the Mongolian gerbils obtained from supplier A were infected with *H. pylori* suspended in sterilized saline without pre-treatment, while none of the Mongolian gerbils obtained from supplier B were infected with *H. pylori* suspended in sterilized saline, while 42%

of the Mongolian gerbils inoculated with *H. pylori* suspended in brain heart infusion medium were infected. Furthermore, infection with *H. pylori* in Mongolian gerbils previously administered with famotidine, an H₂-blocker, was unsuccessful. Low infection rates were also observed in Mongolian gerbils administered 10% sodium bicarbonate solution prior to inoculation. However, infection with *H. pylori* in these Mongolian gerbils was successful after administration of 0.1% sodium bicarbonate solution prior to inoculation. The infection rate in Mongolian gerbils administered 0.1% sodium bicarbonate prior to inoculation with *H. pylori* suspended in brain heart infusion medium was 90%, and this rate was higher than with other pre-treatment methods. *H. pylori* infection of highly resistant Mongolian gerbils supplied in Japan is possible by adjusting gastric pH.

INTRODUCTION

Marshall was the first to report a link between *Helicobacter pylori* (*H. pylori*) infection and gastric cancer (Marshall 1983). *H. pylori* reportedly induces acute and chronic gastritis, gastric ulcer, duodenal ulcer and gastric cancer (Warren et al., 1983, McNulty et al., 1999, Allen 2001, Eslick 2006), and Karita et al. (Karita et al., 1991, Eslick 2006) established an animal model for human *H. pylori* infection using BALB/c nude mice, BALB/c euthymic mice or germ-free mice (Karita et al., 1991, 1994). A severe *H. pylori* infection and inflammation model was subsequently established in Mongolian gerbils (Karita et al., 1991, Yan et al., 2004, Takamoto et al., 2013). Thus, Mongolian gerbil models are essential experimental animals for infection experiments using *H. pylori*. Until recently, Mongolian gerbils supplied in Japan were readily infected *H. pylori* and infectious experiments were conducted without problems. However, this supplier has discontinued marketing Mongolian gerbils, leaving only one supplier of Mongolian gerbils in Japan. Unfortunately, Mongolian gerbils from this supplier are not readily infected with *H. pylori* using

standard inoculation methods (Hirayama et al., 1996), and infection experiments with *H. pylori* using Mongolian gerbils are therefore impossible in Japan at present. Thus, Mongolian gerbils purchased from this supplier require pre-treatment for infection with *H. pylori*. The aim of this study was to investigate suitable conditions for *H. pylori* infection in infection-highly resistant Mongolian gerbils supplied in Japan.

MATERIALS and METHODS

Bacterial strains

Six strains of *H. pylori* isolated from humans were used in this study. One strain was donated by Professor Yasuhiro Koga, Laboratory for Infectious Diseases, Tokai University School of Medicine and five strains were donated by Incorporated Foundation Tokyo Kenbikyo-in. *H. pylori* was cultured as described by Kabir et al. (Kabir et al., 1997). Colonies were confirmed using a commercial *H. pylori* urease kit (CLO Test; Sysmex Corporation, Hyogo, Japan). *H. pylori* was suspended in sterilized saline or brain heart infusion culture medium (Becton, Dickinson and Company, Franklin Lakes, NJ), at a pH of 7.4. Mongolian gerbils were inoculated with *H. pylori* within 1 hour of extraction from petri dishes.

Animals

Mongolian gerbils (body weight; 20 to 30 g) were purchased from two different suppliers in Japan. One supplier (supplier A) has since stopped supplying Mongolian gerbils. The other supplier (supplier B) is currently the only supplier of Mongolian gerbils in Japan. Mongolian gerbils were kept in cages at a temperature of $23 \pm 2^\circ\text{C}$, and a relative humidity of $55\% \pm 10\%$, on a 12/12 dark (18:00-6:00)/light (6:00-18:00) cycle with the air exchanged 12 times or more per hour. Mongolian gerbils were fed MF (Oriental Yeast Co., Ltd., Tokyo, Japan), and were allowed free access to water. For 18 hours before inoculation, Mongolian gerbils were deprived of food, but had free access to water.

All experiments were approved by the

Institutional Review Board of Azabu University and were conducted in accordance with the institute's Animal Experimentation guidelines (Japanese Association for Laboratory Animal Science, JALAS, 1987).

Experimental infection with *H. pylori*

In the first experiment, Mongolian gerbils from supplier A were inoculated with a 1-ml suspension of 5.0×10^7 CFU/ml *H. pylori* suspended in sterilized saline without pre-treatment. Mongolian gerbils from supplier B were inoculated with a 1-ml suspension of 5.0×10^7 CFU/ml *H. pylori* suspended in sterilized saline or brain heart infusion medium (Becton, Dickson and Company) without pre-treatment.

In the second experiment, all Mongolian gerbils from supplier B were inoculated with *H. pylori* after pre-treatment. First, *H. pylori* suspended in sterilized saline was given to Mongolian gerbils administered famotidine at 10 mg/kg body weight (Astellas Pharma Inc., Tokyo, Japan) 3 hours before inoculation. Furthermore, *H. pylori* suspended in brain heart infusion medium (Becton, Dickson and Company) including 0.2 mg/ml urea (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was given to Mongolian gerbils.

Next, Mongolian gerbils were inoculated with *H. pylori* after administration of sodium bicarbonate solution (Wako Pure Chemical Industries, Ltd.) as pre-treatment. *H. pylori* suspended in sterilized saline was inoculated into Mongolian gerbils administered 0.5 ml

of 10% sodium bicarbonate solution (Wako Pure Chemical Industries, Ltd.) 10 or 30 minutes before inoculation. Subsequently, *H. pylori* suspended in sterilized saline or brain heart infusion medium (Becton, Dickinson and Company) was inoculated into Mongolian gerbils administered 0.3 ml of 0.1% of sodium bicarbonate solution 10 minutes before inoculation. *H. pylori* was inoculated either once or once a day for two days.

Measurement of antibodies against *H. pylori*

Infection was confirmed by elevation of serum titers of IgG and IgM against *H. pylori*. Blood was collected by ventricular puncture before inoculation and at 1 week after inoculation, under anesthesia with pentobarbital (Kyoritsu Seiyaku Corporation, Tokyo, Japan).

Serum titers of IgG and IgM against *H. pylori* were measured by enzyme-linked immunosorbent assay (ELISA). *H. pylori* were disrupted with ultrasonic treatment, and were dissolved in phosphate buffered saline for use as somatic antigen. Somatic antigen was diluted in 0.05 M sodium bicarbonate buffer (pH 9.6) and was incubated at 100 µl/ml at room temperature for 1 hour. After blocking with 1% bovine serum albumin in sodium bicarbonate buffer (pH 9.6), sera from Mongolian gerbils inoculated with *H. pylori* were added at 100 µl/well. Plates were incubated at room temperature for 1 hour. HRPO conjugated goat anti mouse IgG (Bethyl Laboratories Inc., Montgomery,

Table 1 Infection rates of *Helicobacter pylori* in Mongolian gerbils

Breeding supplier	Suspension with <i>H. pylori</i>	Inoculation frequency	n	Case of infection	Infection rate (%)
A	Sterilized saline		27	27	100
B	Sterilized saline	once a day	40	0	0
B	Brain heart infusion medium		12	5	42

H. pylori was suspended suspension of 5.0×10^7 CFU/ml.

Figure 1. Typical cultivation observations of stomachs from Mongolian gerbils inoculated with *Helicobacter pylori*. (A) Stomach from Mongolian gerbil showing increased titers of IgG and IgM against *H. pylori*. (B) Stomach from Mongolian gerbil showing no increase in titers of IgG and IgM against *H. pylori*.



TX) or HRPO-conjugated goat anti mouse IgM antibodies (American Qualex, San Clemente, CA) were added at 100 µl/well. After incubation for 1 hour, peroxidase conjugated rabbit anti-mouse IgG antibodies were added at 100 µl/well. Substrate was 2, 2-azino-di-(3-ethyl-benzthiazoline sulphonic acid-6) (ABTS), and after incubation for 1 hour (Zymed Laboratories, South San Francisco, CA), absorbance at 415 nm was measured.

Cultivation of *H. pylori* from Mongolian gerbils

Two groups of Mongolian gerbils were sacrificed after measurement of antibody titers against *H. pylori* in order to assess the differences in antibodies against *H. pylori* and isolated *H. pylori* from the stomach. One group was administered 0.1% sodium bicarbonate prior to inoculation once a day for two days and the other group was administered famotidine prior to inoculation. Stomachs of these groups were removed and homogenized with sterilized phosphate buffered saline (PBS, pH 7.4). *H. pylori* was then cultured according to the method de-

scribed by Kabir et al. (Kabir et al., 1991).

Measurement of gastric pH

When Mongolian gerbils were sacrificed, gastric pH was measured using a pH Spear for food testing (Eutech Instruments Pte, Ltd., Singapore). Furthermore, the pH of various suspended solutions containing hydrochloric acid (Wako Pure Chemical Industries, Ltd.) to simulate gastric pH (1.52) was measured.

Statistical analysis

Serum titers of IgG and IgM against *H. pylori* between pre-inoculation and post-inoculation were analyzed using the paired Student's t-test. Differences in p values <0.01 were considered significant.

RESULTS

Infection rates in Mongolian gerbils purchased from supplier A or B without pre-treatment after inoculation with *H. pylori* are shown Table 1. All Mongolian gerbils purchased from supplier A were infected with *H. pylori*. On the other hand, Mongolian gerbils purchased from supplier B were not infected with *H. pylori* suspended in sterilized saline, while 42% of Mongolian gerbils inoculated with *H. pylori* suspended in brain heart infusion medium (Becton, Dickson and Company) were infected without pre-treatment.

The infection rate in Mongolian gerbils administered famotidine was only 10%, while it was 70% in Mongolian gerbils inoculated with *H. pylori* suspended in brain

Table 2 Infection rate of *Helicobacter pylori* in the infection highly resistant mongolian gerbils with pre-treatment

Pre-treatment method	Suspension with <i>H. pylori</i>	Inoculation frequency	n	Case of infection	Infection rate (%)
H ₂ -blocker (famotidine)	Sterilized saline	once a day	20	2	10
Urea			10	7	70

All mongolian gerbils were purchased from supplier B. *H. pylori* was suspended suspension of 5.0×10^7 CFU/ml.

Table 3 Infection rates of *Helicobacter pylori* in the infection-highly resistant mongolian gerbils administered before sodium bicarbonate solution

Pre-treatment method			Inoculation method				
Concentration of sodium bicarbonate (%)	Administration volume of sodium bicarbonate (ml)	Suspension with <i>H. pylori</i>	Time after pre-treatment (minutes)	Inoculation frequency	n	Case of infection	Infection rate (%)
10	0.5	Sterilized saline	30	once a day	20	5	25
10	0.5	Sterilized saline	10	twice a day	10	4	40
10	0.5	Sterilized saline	10	once a day for 2 days	10	5	50
0.1	0.3	Sterilized saline	10	once a day	10	7	70
0.1	0.3	Sterilized saline	10	once a day for 2 days	10	8	80
0.1	0.3	Brain heart infusion medium	10	once a day	10	8	80
0.1	0.3	Brain heart infusion medium	10	once a day for 2 days	10	9	90

All mongolian gerbils were purchased from supplier B. *H. pylori* was suspended suspension of 5.0×10^7 CFU/ml.

heart infusion medium (Becton, Dickson and Company) including urea (Table 2).

Infection rates in Mongolian gerbils purchased from supplier B administered sodium bicarbonate before inoculation of *H. pylori* are shown in Table 3. The highest infection rate was 90% in Mongolian gerbils administered 0.1% sodium bicarbonate before inoculation with *H. pylori* suspended in brain heart infusion medium.

Titers of IgG and IgM against *H. pylori* did not increase in 18 Mongolian gerbils prior to inoculation with famotidine. *H. pylori* were not isolated from the stomach of ten randomly selected Mongolian gerbils from the 18 showing no changes in titer (Fig. 1). However, titers of IgG and IgM against *H. pylori* in nine Mongolian gerbils increased after administration of 0.1% sodium bicarbonate solution once a day for two days prior to inoculation of *H. pylori* suspended in brain heart infusion medium. Furthermore, these titers showed significant differences between pre-inoculation and post-inoculation. Moreover, *H. pylori* was isolated from the stomachs of these Mongolian gerbils (Fig. 1). On the other hand, *H. pylori* was not isolated from the stomach of one Mongolian gerbil that did not show increased titers against *H. pylori* (Fig. 1). The infection rates for other *H. pylori* strains isolated from humans, donated by Incorporated Foundation Tokyo Kenbikyo-in, are shown in Table 4. One of the three Mongolian gerbils inoculated with two strains showed increased titers of IgM and IgG. The other strains did not show increased titers.

The pH of hydrochloric acid-containing simulated gastric solutions with various suspended solutions is

Table 4 Infection rates of *Helicobacter pylori* isolated from human in the infection-highly resistant mongolian gerbils without pre-treatment

Strain number of <i>H. pylori</i>	n	Suspension with <i>H. pylori</i>	Inoculation frequency	Case of infection	Infection rate (%)
1	3			1	33
2	3			0	0
3	3	Sterilized saline	once a day	1	33
4	3			0	0
5	3			0	0

All mongolian gerbils were purchased from supplier B. These five strains were donated by Incorporated Foundation Tokyo Kenbikyo-in. *H. pylori* was suspended suspension of 5.0×10^7 CFU/ml.

shown in Table 5. The pH of the solution with saline was 2.13. However, the pH of the *H. pylori* suspension with bicarbonate sodium or brain heart infusion was almost neutral.

DISCUSSION

H. pylori are considered to induce various gastric disorders in humans (Mrshall 1994, Konturek et al., 2009). Mongolian gerbils are used as pathologic models for *H. pylori* infection (Hirayama et al., 1996). However, it is now difficult to obtain *H. pylori*-sensitive Mongolian gerbils in Japan. Thus, suit-

able conditions for infection with *H. pylori* in the infection-highly resistant Mongolian gerbils need to be elucidated.

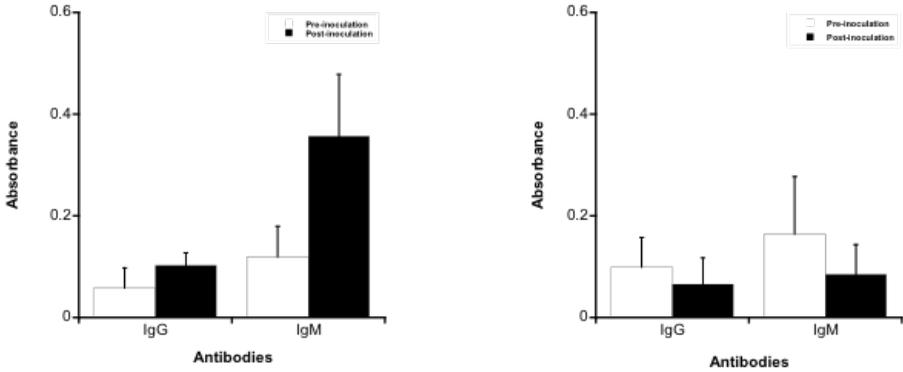
Titers of IgG and IgM against in Mongolian gerbils isolated *H. pylori* from stomach increased significantly. A correlation between increases in IgG and IgM titers against *H. pylori* and isolation of *H. pylori* from stomach was confirmed. These results suggest that establishment of infection with *H. pylori* could be confirmed by detecting increases in these titers.

All Mongolian gerbils purchased from supplier A, which has since left the market, were infected with *H. pylori* without pre-treatment. On the other hand, Mongolian gerbils purchased from supplier B, the only supplier in Japan at present, were not infected with *H. pylori* without pre-treatment. The other five *H. pylori* strains isolated from humans, donated from Incorporated Foundation Tokyo Kenbikyo-in, were given to Mongolian gerbils from supplier B. The purpose of this experiment was to evaluate differences in strain sensitivity among the Mongolian gerbils purchased from supplier B. One-third of Mongolian gerbils inoculated with two strains were infected. Differences in infection rates between strains were assumed to depend on the ease of establishing infection; however, it was not possible to stably infect Mongolian gerbils purchased from supplier B. Differences in strain were

Table 5 The ph of hydrochloric solution adjusted gastric pH added with various suspended solution

Added solution	pH
Bicarbonate sodium	6.90
Urea	1.94
Brain heart infusion medium	6.83
Bicarbonate sodium and Brain heart infusion medium	6.92
Saline	2.13
Bicarbonate medium and saline	6.56
Urea and Brain heart infusion medium	6.86

Figure 2. Titers of IgG and IgM against *H. pylori* pre- and post-inoculation. (A) *H. pylori* was isolated from the stomach (B) *H. pylori* was not isolated from stomach : significant difference between pre- and post-inoculation ($p < 0.01$).



not considered to be a principal factor. *H. pylori* survives in the gastric environment by secreting urease to decompose ammonia and carbon dioxide from urea (Ha et al., 2001, Amin et al., 2010, Fahy et al., 2013). Mongolian gerbils administered antibodies against fragments of *H. pylori* urease could be infected with *H. pylori* (Mahdieh et al., 2012). Moreover, the survival of *H. pylori* incubated for 1 hour at pH 7.4 was higher than at pH 3.0 (Marcus et al., 2013). The pH of gastric juice was 1.52 in Mongolian gerbils in this study. Gastric pH was thus considered to be an important factor in *H. pylori* infection of highly resistance Mongolian gerbils.

Administration of 0.1% sodium bicarbonate solution to Mongolian gerbils inoculated with *H. pylori* suspended in brain heart infusion medium once daily for two days showed the highest infection rate. Infection rates increased with administration of sodium bicarbonate solution or urea prior to inoculation. In vitro, the pH of hydrochloric acid solution-containing simulated gastric solution increased by adding sodium bicarbonate, brain heart infusion medium or urea. Thus, it is possible to infect highly resistance Mongolian gerbils by elevating gastric pH prior to inoculation. The famotidine is an efficacious H₂-blocker that elevates

gastric pH by inhibiting gastric acid (Özer et al., 2012, Okabe et al., 2001). However, infection rates were lower with famotidine than with other pre-treatment methods. This may be because famotidine did not sufficiently adjust gastric pH for infection with *H. pylori*.

Mongolian gerbils are essential experimental animals for infection studies with *H. pylori*. However, it is now difficult to obtain Mongolian gerbils that can be stably infected with *H. pylori* in Japan. In the present study, infection-highly resistant Mongolian gerbils could be infected with *H. pylori* by elevating gastric pH with pre-treatment. Thus, gastric pH is an important factor in *H. pylori* infection of highly-resistant Mongolian gerbils.

ACKNOWLEDGMENTS

This research was partially supported by a research project grant awarded by Azabu University. We are also indebted to Professor Yasuhiro Koga, Laboratory for Infectious Diseases, Tokai University School of Medicine and Incorporated Foundation Tokyo Kenbikyoin for donating the clinical isolates of *H. pylori*.

REFERENCES

1. Allen P. What's the story *H. pylori*? *Lancet* 2001; 357: 694.
2. Amin M, Iqbal MS, Hughes RW, Khan SA,

- Reynolds PA, Enne VI, Sajjad-ur-Rahman, Mirza AS. Mechanochemical synthesis and in vitro anti-Helicobacter pylori and urease inhibitory activities of novel zinc(II)-famotidine complex. *J Enzyme Inhib Med Chem* 2010; 25: 383-389.
3. Eslick GD. Helicobacter pylori infection causes gastric cancer? A review of the epidemiological, meta-analytic, and experimental evidence. *World J Gastroenterol* 2006; 21: 2991-2999.
 4. Fahy JW, Stepheson KK, Wade KL, Talaly P. Urease from Helicobacter pylori is inactivated by sulforaphane and other isothiocyanates. *Biochem Biophys Res Commun* 2013; 435: 1-7.
 5. Ha N, Oh S, Sung JY, Cha KA, Lee M, Oh B. Supramolecular assembly and acid resistance of Helicobacter pylori urease. *Nat Struct Mol Biol* 2001; 8: 505-509.
 6. Hirayama F, Takagi S, Yokoyama Y, Iwao E, Ikeda Y. Establishment of gastric Helicobacter pylori infection in Mongolian gerbils. *J Gastroenterol* 1996; 31(Suppl 9): 24-28.
 7. Kabir AMA, Aiba Y, Takagi A, Kamiya S, Miwa T, Koga Y. Prevention of Helicobacter pylori by lactobacilli in a gnotobiotic murine model. *Gut* 1997; 41: 49-55.
 8. Karita M, Kouchiyama T, Okita K, Nakazawa T. New small animal model for human gastric Helicobacter pylori infection: success in both nude and euthymic mice. *Am J Gastroenterol* 1991; 86: 1596-1603.
 9. Karita M, Kouchiyama T, Okita K. New small animal model for human gastritis Helicobacter pylori infection: success in both nude and euthymic mice. *Am J Gastroenterol* 1991; 86: 1596-1603.
 10. Karita M, Morshed MG, Ouchi K, Okita K. Bismuth-free triple therapy for eradicating Helicobacter pylori and reducing the gastric ulcer recurrence rate. *Am J Gastroenterol* 1994; 89: 1032-1035.
 11. Konturek PC, Konturek SJ, Brzozowski T. Helicobacter pylori infection in gastric cancerogenesis. *J Physiol Pharmacol* 2009; 69: 3-21.
 12. Mahdieh ASR, Seyed LMG, Iraj R, Mohammadreza JN, Walead E. Inhibition of H. pylori colonization and prevention of gastritis in murine model. *World J Microbiol Biotechnol* 2012; 28: 2513-2519.
 13. Marcus EA, Sachs G, Scott DR. The role of ExbD in periplasmic pH homeostasis in Helicobacter pylori. *Helicobacter* 2013; 18: 363-372.
 14. Marshall BJ. Helicobacter pylori. *Am J Gastroenterol* 1994; 89: S116-128.
 15. Marshall BJ. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983; 1: 1273-1275.
 16. McNulty CA. The discovery of Campylobacter-like organisms. *Curr Top Microbiol Immunol* 1999; 241: 1-9.
 17. Okabe S, Shimosaka K, Amagase K. Pharmacological regulation of gastric acid secretion in the apical membrane of parietal cells; a new target for antisecretory drugs. *J Physiol Pharmacol* 2001; 52: 639-656.
 18. Tsukamoto T, Toyoda T, Mizoshita T, Tatematsu M. Helicobacter pylori infection and gastric carcinogenesis in rodent models. *Semin Immunol* 2013; 35: 177-190.
 19. Warren RJ, Marshall BJ. Unidentified curved bacilli in gastric epithelium in active chronic gastric. *Lancet* 1983; 1: 1273.
 20. Yan J, Luo YH, Mao YF. Establishment of Helicobacter pylori infection model in mongolian gerbils. *World J Gastroenterol* 2004; 15: 852-855.
 21. Özer M, Duman M, Tas S, Demirci Y, Aydin MF, Reyhan E, Atici AE, Bostanci EB, Akoğlu M, Genç E. In vitro effects of famotidine and ranitidine on lower esophageal sphincter tone in rats. *Turk J Gastroenterol* 2012; 23: 438-443.