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doi:10.3748/wjg.15.4907

World J Gastroenterol 2009 October 21; 15(39): 4907-4914
World Journal of Gastroenterology ISSN 1007-9327

ORIGINAL ARTICLE

Short-term Celecoxib intervention is a safe and effective chemopreventive for gastric carcinogenesis based on a Mongolian gerbil model

Chao-Hung Kuo, Huang-Ming Hu, Pei-Yun Tsai, I-Chen Wu, Sheau-Fang Yang, Lin-Li Chang, Jaw-Yuan Wang, Chang-Ming Jan, Wen-Ming Wang, Deng-Chyang Wu

Chao-Hung Kuo, Huang-Ming Hu, Division of Internal Medicine, Kaohsiung Municipal Hsiao-Kang Hospital, Kaohsiung 813, Taiwan, China
Chao-Hung Kuo, I-Chen Wu, Chang-Ming Jan, Wen-Ming Wang, Deng-Chyang Wu, Division of Gastroenterology, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan, China
Chao-Hung Kuo, Jaw-Yuan Wang, Chang-Ming Jan, Wen-Ming Wang, Deng-Chyang Wu, Department of Medicine, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan, China
Pei-Yun Tsai, Department of Nursing, Kaohsiung Municipal Hsiao-Kang Hospital, Kaohsiung 813, Taiwan, China
Sheau-Fang Yang, Department of Pathology, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan, China
Lin-Li Chang, Department of Microbiology, Kaohsiung Medical University, Kaohsiung 807, Taiwan, China
Jaw-Yuan Wang, Division of Gastrointestinal Surgery, Department of Surgery, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan, China
Author contributions: Kuo CH designed the study, collected and analyzed the data, supervised the collection, and wrote the manuscript; Hu HM, Tsai PY, Wu IC, Chang LL, Wang JY, Jan CM, Wang WM participated in the design and coordination of the work involved; Yang SF was the consultant overseeing the histopathological findings; Wu DC participated in the study design and coordination, and supervised the study; All authors have approved the final draft submitted.
Supported by A grant from Kaohsiung Medical University (Q096014), and Kaohsiung Municipal Hsiao-Kang Hospital (kmhk-96-005, kmhk-95-005). This study was approved by the Institutional Animal Care and Use Committee, Kaohsiung Medical University
Correspondence to: Deng-Chyang Wu, MD, PhD, Chief, Department of Gastroenterology, Kaohsiung Medical University Hospital, 100 Tz-You 1st Road, Kaohsiung City 807, Taiwan, China. dechwu@yahoo.com
Telephone: +886-7-3121101-7451 Fax: +886-7-3135612
Received: July 28, 2009 Revised: August 31, 2009
Accepted: September 6, 2009
Published online: October 21, 2009

Abstract

AIM: To evaluate the optimal intervention point of a selective cyclooxygenase-2 (COX-2) inhibitor, Celecoxib, for inhibiting *Helicobacter pylori* (*H pylori*)-associated gastric carcinogenesis in Mongolian gerbils (MGs).

METHODS: One hundred and twelve MGs were divided into six groups (A-F). One hundred gerbils were inoculated with *H pylori* (groups A-E). Twelve gerbils were inoculated with vehicle broth only (group F). After 4 wk, they were given N'-methyl-N'-nitro-N-nitroso-guanidine (MNNG) (50 μg/mL) in the drinking water for 20 wk. In groups B-E, the animals were given the stock Celecoxib (10 mg/kg per day) diet from the 21st, 31st, 21st and 41st week respectively. The periods of administering Celecoxib were 30, 20, 20, and 15 wk respectively. On the 51st week, the animals were sacrificed for histological examination. Local PCNA expression was examined by the immunohistochemistry method. The expression of COX-2 protein was assessed by Western Blot. Analysis used the χ^2 test. The difference was regarded as significant when P value was less than 0.05.

RESULTS: Seventeen percent (17/100) of *H pylori*-infected MGs developed gastric cancer. All of these lesions were well-differentiated adenocarcinoma. The incidence rates of adenocarcinoma in groups A-F were 40%, 0%, 0%, 20%, 25%, and 0% respectively. The inflammatory scores were higher in group B than in other groups. There was no inflammatory response noted in group F. Celecoxib treatment resulted in a significant reduction in the proliferation of *H pylori*-infected mucosal cells (groups B, C and D) ($P < 0.01$). The expression of COX-2 protein was significantly attenuated in the groups which were Celecoxib-treated for more than 20 wk (groups B, C, D). The groups treated with Celecoxib had a significantly lower rate of advanced gastric cancer (34% *vs* 75%, $P < 0.001$) There were no sudden deaths in any of the groups.

CONCLUSION: Short-term treatment with Celecoxib has an anti-carcinogenic effect, and resulted in less severe inflammation and inhibited the invasive degree of gastric cancer.

Key words: Cyclooxygenase-2; Chemoprevention; *Helicobacter pylori*; Mongolian gerbil

Peer reviewer: Yuan Yuan, Professor, Cancer Institute of

China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province, China

Kuo CH, Hu HM, Tsai PY, Wu IC, Yang SF, Chang LL, Wang JY, Jan CM, Wang WM, Wu DC. Short-term Celecoxib intervention is a safe and effective chemopreventive for gastric carcinogenesis based on a Mongolian gerbil model. *World J Gastroenterol* 2009; 15(39): 4907-4914 Available from: URL: http://www.wjgnet.com/1007-9327/15/4907.asp DOI: http://dx.doi.org/10.3748/wjg.15.4907

INTRODUCTION

Since the isolation and culture of *Helicobacter pylori* (*H pylori*) in 1983, this bacterium has become accepted as an important human pathogen for the development of gastritis, peptic ulcer, and gastric cancer[1]. The worldwide prevalence rate of *H pylori* infection is approximately 50%, with the highest being in developing countries. In Taiwan, the overall prevalence rate is 54% and this rises with age[2]. Seven-day triple therapy [proton pump inhibitor (PPI), amoxicillin and clarithromycin] has been the main first-line therapy for *H pylori* infection in Taiwan, Europe and many other countries as guided by the Maastricht-22 000 consensus[3,4]. Despite this, although the eradication of *H pylori* is still the most cost-effective method for prevention of gastric carcinogenesis, the antibiotic treatment of *H pylori* infection is confronting a significant challenge associated with resistance to antibiotics. The first-line regimen continues to have a 10%-23% failure rate[5-10]. Unfortunately, several studies have shown that rescue regimens have failed in 5%-63% of patients whose *H pylori* cannot be eradicated by standard PPI-based triple therapies[11,12].

If refractory *H pylori* infection persists, *H pylori*-dependent induction of cyclooxygenase-2 (COX-2) is associated with enhanced production of multidrug resistance-1 (MDR-1) and Bcl-xL proteins that may contribute to gastric tumorigenesis and resistance to therapy[13]. Therefore, it is important to find an alternative chemoprevention for these patients.

COX-2 is a prostaglandin-synthesizing enzyme. Elevated expression of COX-2 is observed in a wide variety of human malignancies, including gastric cancer. Various *in vitro* and *in vivo* studies strongly suggest that COX-2 is involved in a major early oncogenic event in these human malignancies[14-18]. *H pylori*-induced chronic gastritis also shows elevated levels of COX-2 expression in the stomach mucosa[17-22]. Enhanced expression of COX-2 is also observed in intestinal metaplasia, dysplasia, and gastric adenoma, which are regarded as precancerous lesions[14,23]. Moreover, previous studies showed that regular intake of either nonselective or selective COX-2 inhibitors reduces the risk of several human cancers[24-26].

The selective COX-2 inhibitors have been reported to prevent chemical carcinogen-induced carcinogenesis in C57/BL6 mice[22] and Mongolian gerbils (MGs)[27-29]. However, the adverse effects of COX-2 inhibitors on the cardiovascular system inhibit the application of this chemoprevention[30-33]. There is no animal study focusing on the optimal therapeutic period of COX-2 inhibitors to prevent these possible severe adverse events.

In the present study, our aim is to evaluate the optimal intervention point of Celecoxib in order to inhibit *H pylori*-associated gastric carcinogenesis in MGs. In addition, we also investigated the effects on tumor invasion.

MATERIALS AND METHODS

The experimental design was approved by the Animal Research Committee of Kaohsiung Medical University. One hundred and twelve gerbils were divided into six groups (A-F), and were inoculated with *H pylori* [CagA(+)/VacA(+)] (groups A-E; n = 20 in every group) or vehicle (Brucella broth) alone (group F; n = 12). After 1 wk, groups A-E were given N'-methyl-N'-nitro-N-nitroso-guanidine (MNNG) at a concentration of 50 μg/mL in the drinking water for 20 wk (as shown in Figure 1). Then, all groups were switched to autoclaved distilled water as drinking water. In groups B-E, the animals started to be given the stock Celecoxib diet from the 21st, 31st, 21st and 36th week. The periods during which Celecoxib was given were 30, 20, 20, and 15 wk respectively. However, the animals in groups A and F received the control diet. The daily-administered dosage of Celecoxib was 10 mg/kg per day in groups B-E. On the 51st experimental week, the animals were fasted for 24 h before being sacrificed.

Histological evaluation of the gastric mucosa in H pylori-infected gerbils

Samples of the gastric mucosa were excised from each gerbil stomach for the assessment of the presence of *H pylori* and gastric inflammation using Giemsa and hematoxylin-eosin (HE) staining for histological examination, respectively. The samples were fixed in 10% buffered formalin and embedded in paraffin[34]. The paraffin sections were cut at a thickness of 5 μm and stained. Two experienced pathologists, unaware of the treatment given, performed histological examinations blindly. Histological features of mucosal inflammation and intestinal metaplasia were evaluated for each specimen under a light microscope according to the classification of the Sydney system. The degree of inflammatory cell infiltration and the area of intestinal metaplasia were scored as follows: 0, normal; 1, mild; 2, moderate; 3, marked. For the evaluation of mucosal cell proliferation, the proportion of PCNA-positive cells per 1000 mucosal cells was assessed in the antrum and corpus as in a previous study by Suzuki *et al*[35]. We also recorded the size, depth and location of tumor.

Analysis of anti-proliferating cell nuclear antigen (PCNA) in gastric mucosa

Half of the excised stomachs were fixed in 10% neutral-

Table 1 Effect of Celecoxib treatment period on incidence of gastric cancer in Mongolian gerbils

Group	Regimen	Period of Celecoxib treatment (wk)	*n*	Rate of gastric cancer (%)	Type of cancer		
					Well	Poor	Sig
A	*HP*+MNNG	0	20	40 (8/20)	8	0	0
B	*HP*+MNNG+Celecoxib	30	20	0 (0/20)[a]	0	0	0
C	*HP*+MNNG+Celecoxib	20	20	0 (0/20)[a]	0	0	0
D	*HP*+MNNG+Celecoxib	20	20	20 (4/20)	4	0	0
E	*HP*+MNNG+Celecoxib	15	20	25 (5/20)	5	0	0
F	Vehicle	0	12	0	0	0	0

HP: *H pylori* (intra-gastric); MNNG: N′-methyl-N′-nitro-N-nitroso-guanidine; Well: Well-differentiated adenocarcinoma; Poor: Poorly differentiated adenocarcinoma; Sig: Signet-ring cell carcinoma. [a]$P < 0.05$ *vs* group A.

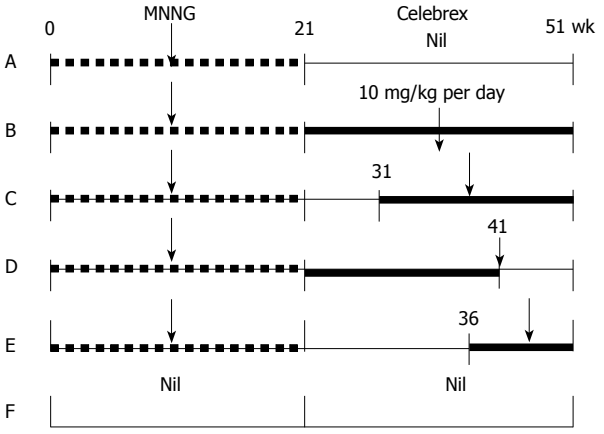

Figure 1 Design of the study. At the beginning of the experiment, gerbils were inoculated (i.g.) with *H pylori* (Grps A-E) or vehicle (Brucella broth; Grp F). Until the 21st week, the animals were given drinking water containing no Grp F (open bars) or 50 μg/mL MNNG (Grp A-E). All groups were then switched to distilled water and given a diet containing no drug (Grps A and F) or Celecoxib 10 mg/kg per day for 30 (Grp B), 20 (Grp C), 20 (Grp D) or 15 (Grp E) weeks. The gerbils were sacrificed at week 51.

buffered formalin and embedded in paraffin. Tissues sections were stained with HE and were analyzed by immunohistochemistry with anti-proliferating cell nuclear antigen (PCNA) serum (Dako).

Protein extraction and analysis of COX-2 expression in the gastric mucosa by Western blotting

Frozen gastric tissue was homogenized in lysis buffer (100 mmol Tris-HCl, pH 7.4, 15% glycerol, 2 mmol EDTA, 2% SDS, 100 mmol DDT) by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mmol PMSF as described in previous studies[32]. Approximately 100 μg of cellular protein extract was loaded into a well, separated electrophoretically on 13.5% SDS-polyacrylamide gel and transferred onto Sequi-Blot TMPVDF membrane (Bio-Rad, Hercules, CA, USA) by electroblotting. Western blotting was performed with specific primary rabbit polyclonal antibody against COX-2 (dilution 1:500, Santa Cruz, USA) or anti-β-actin rabbit polyclonal antibody as primary antibody, and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (dilution 1:2000, Santa Cruz, USA). Visualization of immune complexes was achieved by chemiluminescence using BM Chemiluminescence Blotting Substrate (Boehringer, Mannheim, Germany) and the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany). We did not perform Western blotting for group F.

Statistical analyses

We analyzed the collected data using the statistical software package STATA. An unpaired *t*-test or a Mann-Whitney *U* test was applied to determine the significance of differences between two groups. The incidence of cancer was assessed using χ^2 test. $P < 0.05$ was considered to be statistically significant.

RESULTS

In our study, all gerbils were alive till the end of this experiment; there was no significant difference in the survival rates among the various groups. Seventeen percent of *H pylori*-infected gerbils developed gastric cancer. All of these lesions were well-differentiated adenocarcinoma (Figure 2). The incidence of cancer in every experimental group is shown in Table 1. As a result of long-lasting infection with *H pylori*, 40% of the animals in group A developed cancer. There was no cancer found in groups B and C. However, the group treated with a shorter period of Celecoxib (group E) and the early-treatment group (group D) did not show obvious inhibitory effects on gastric carcinogenesis. Therefore, this meant that Celecoxib might have a chemopreventive effect on gastric carcinogenesis in some situations. Our data disclosed that the protective effect of Celecoxib might exist during long-term use or late-use of Celecoxib at a dose of 10 mg/kg per day (groups B and C).

We also evaluated the effect of Celecoxib on invasion of gastric cancer. Eight gerbils in the groups with no Celecoxib treatment and nine gerbils in groups with Celecoxib treatment developed gastric cancers. The incidence rate of advanced gastric cancer was higher in group A than groups D and E (75% *vs* 34%, $P < 0.001$).

In all of the *H pylori*-infected animals (groups A-E), different degrees of infiltration of inflammatory cells were observed in the lamina propria and submucosa. The infiltration was predominantly lymphocytes, although some macrophages and neutrophils were also observed. The histological examination also revealed hyperplasia of the epithelia accompanied by erosions, lymphoid follicle formation, and intestinal metaplasia. These findings are mimicked in *H pylori*-infected humans. The inflammatory

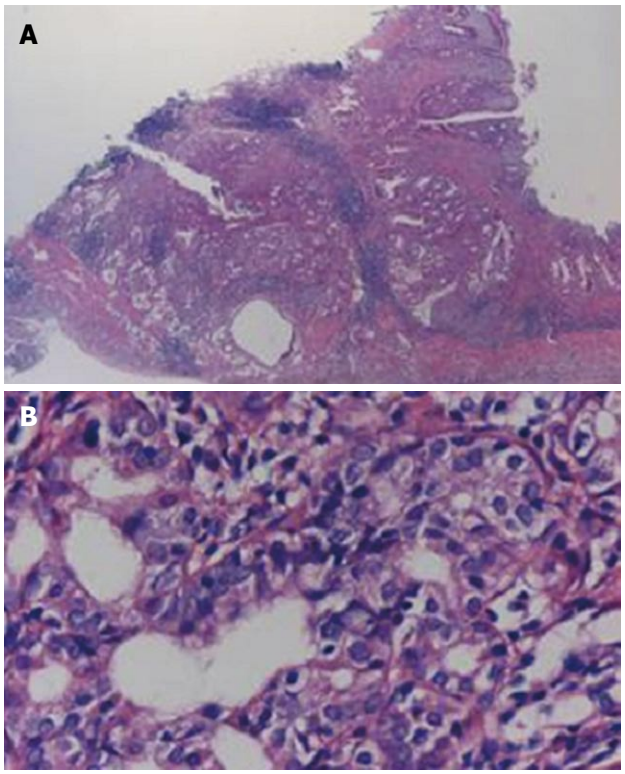

Figure 2 Typical adenocarcinoma in the pyloric mucosa of *H pylori*-infected MGs. Shown is a typical well-differentiated adenocarcinoma (A, B) stained with HE. Images were obtained at × 100 (A) and × 400 (B).

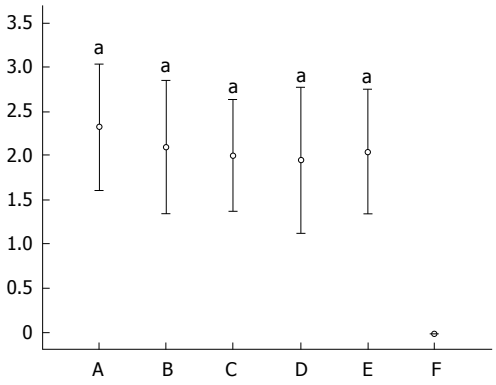

Figure 3 Effect of Celecoxib on inflammation of the stomach mucosa. No obvious inflammatory change was found in group F. Significantly obvious inflammation was shown in *H pylori*-infected groups (A-E) *vs* group F ($^aP < 0.05$), but there was no significant difference among *H pylori*-infected groups. Group B showed higher inflammatory response than group A, C, D, E.

score was higher in group B than other groups. However, there was no significant difference between groups A, B, C, D and E. No definite evidence of inflammatory response was found in group F (Figure 3).

Our data revealed that Celecoxib could repress the development of intestinal metaplasia. Intestinal metaplastic changes were observed after *H pylori* infection in our study (groups A-E) (Figure 4). These changes were significantly reduced in groups B and C. We did not find similar reductions in groups D and E. No intestinal metaplasia was found in group F.

In this study, we found that infection with

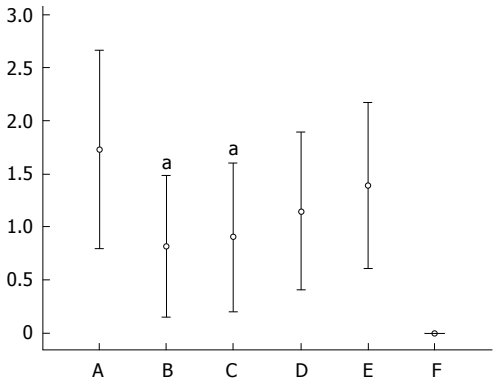

Figure 4 Effect of Celecoxib on the development of intestinal metaplasia (IM). Severe IM was found in group A. There was significantly lower rates of IM in groups B and C. A relatively lower rate of IM was found in groups D and E ($P > 0.05$ *vs* group A). There was no definite IM found in group F. $^aP < 0.05$ *vs* group A.

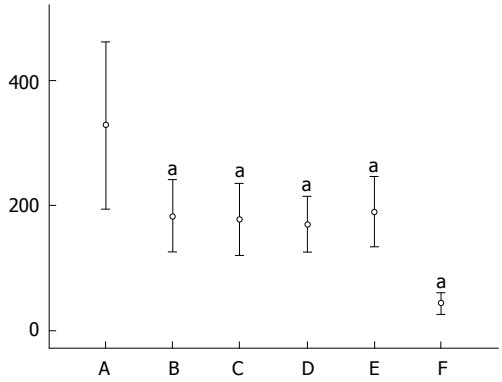

Figure 5 Effect of Celecoxib on the proliferation of gastric mucosal cells in gerbils. High PCNA positive ratio was found in group A, and the value decreased significantly after treatment with Celecoxib. The value was also lower in group F without *H pylori* infection. $^aP < 0.05$ *vs* groups A.

H pylori greatly enhanced the density of anti-PCNA immunohistochemistry in mucosal cells (groups A-E). This showed that proliferation of mucosal cells was promoted after *H pylori* infection (Figures 5 and 6). Celecoxib treatment resulted in a significant reduction in the proliferation of *H pylori*-infected mucosal cells (groups B, C, D and E), ($P < 0.01$). The group F showed lowest proliferation indices.

Results showed strong expression of COX-2 protein in those gerbils inoculated with *H pylori*, but we did not detect any indication of this in gerbils treated with vehicle (Figure 7). Ratio of COX-2/β-actin protein was significantly increased in group A, and the signal for COX-2 protein was significantly attenuated in the Celecoxib-treated groups (B, C, D). The value of COX-2 protein/β-actin protein ratio was significantly lower in gerbils treated with long-term Celecoxib (groups B, C, D) compared to that treated with *H pylori* alone (group A) or short-term Celecoxib (group E).

DISCUSSION

In this study, we attempted to evaluate the effect of short-term treatment of Celecoxib on prevention of

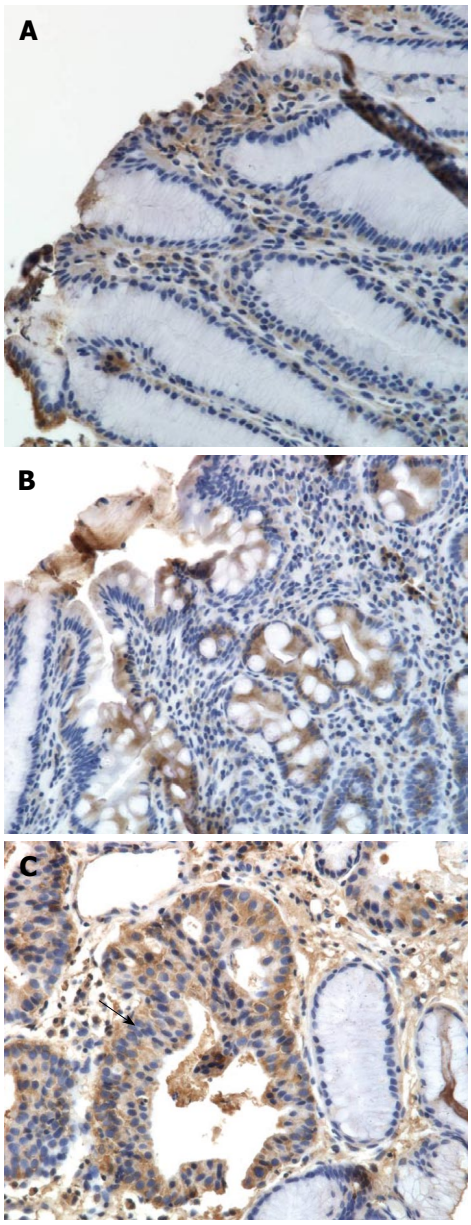

Figure 6 Expression of PCNA by immunohistochemistry method. A: Normal gastric mucosa; B: Intestinal metaplasia; C: Adenocarcinoma. Adenocarcinoma is pointed out by arrow. Images were obtained at × 400.

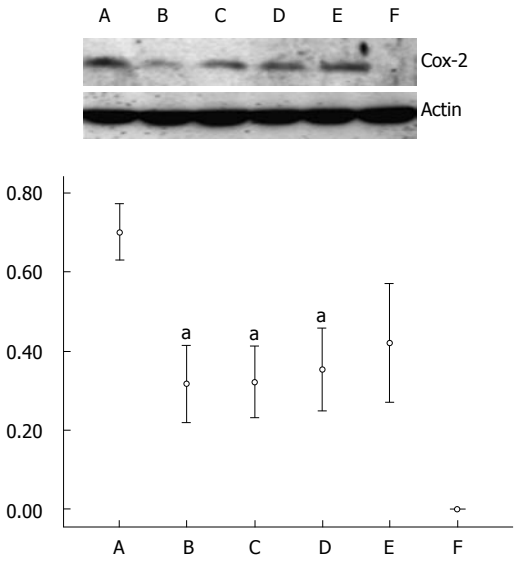

Figure 7 Analysis of COX-2 protein expression in gastric mucosa of gerbils. The expression of COX-2 protein in group A was high. There was no detectable expression in group F. The expression of COX-2 was inhibited significantly by Celecoxib. There was a significant decrease in groups B, C and D. $^{a}P < 0.05$ *vs* group A.

gastric carcinogenesis in a Mongolian gerbil model. Our data support the concept that Celecoxib has a chemopreventive effect on *H pylori*-associated stomach carcinogenesis. Besides this, we also showed that a short-term treatment period (20 wk) in the late infection phase could provide a similar chemoprevention effect as a longer treatment period (30 wk) reported in a previous study[26]. According to this finding, we postulate that a COX-2 inhibitor could be used as chemoprevention for people older than about forty years old. However, this suggestion is not strongly definite due to the small scale of our study. The exact time-point of Celecoxib intervention needs further investigation.

This chemoprevention may play an important role for some people. For example, subjects with extensive metaplastic gastritis have the highest risk for the development of gastric cancer (annual cancer incidence, 0.87%), but they cannot be treated with therapy to eradicate *H pylori* because the infection is no longer present[36]. In this small population, inhibition of COX-2 expression may be useful for the prevention of gastric cancer. Our study supports the fact that a COX-2 inhibitor has a chemopreventive effect by inhibiting expression of COX-2.

Eradication of *H pylori* is a well-known effective prevention of gastric cancer[37]. COX-2 inhibitors are expensive and less cost-effective than therapy aimed at eradicating *H pylori*. However, there are still people with refractory infection despite rescue eradicating regimens. So this chemoprevention is very important for those patients with refractory *H pylori* infections at high risk of gastric cancer.

Various mechanisms have been proposed to explain the anti-tumor action of NSAIDs, including cell growth suppression, inhibition of angiogenesis and metastasis, and NSAID-induced apoptosis in cancer cells[38,39]. Persistent activation of COX-2 is associated with oncogenesis as well as with increased invasive potential of tumor cells[40-42]. In our study, we found that Celecoxib could inhibit the tumor invasion even with short-term use. According to our results, Celecoxib may have effects including anti-oncogenic effect, inhibition of angiogenesis and metastasis, and it was shown that these effects were obtained by both late and short-term use of Celecoxib. In our study, the treatment began at 30-40 wk of age of the gerbils which is equivalent to 35 to 45-years old in humans.

With regard to PCNA results in our data; these disclosed that the inhibitory effect of Celecoxib was related to the inhibition of cell proliferation. This was comparable with previous studies' findings[43,44]. It is well-known that carcinogenesis in the *H pylori*-infected stomach also results from an inflammation-mediated

sequence. Chronic inflammation is thought to play a cardinal role in the accumulation of genetic damages leading to transformation and cancer by inducing the proliferation of target cells[45-47]. Consequently, it is important to find the suitable time-point at which COX-2 inhibitors prevent the carcinogenesis.

In our study, group D had a higher incidence rate of gastric cancer than group C (20% *vs* 0%). This finding supports the theory that the protective effect of Celecoxib is involved in an early oncogenic phase not in an early inflammation phase. This was comparable with findings of previous studies[14-17]. According to our findings, Celecoxib could be used latterly and short term for refractory *H pylori* infection in a clinical situation; a point which has been seldom discussed in previous reports. This short term use is very important for decreasing the possible side effects of COX-2 inhibitors.

In our study, different periods of treatment with Celecoxib produce different results in inflammatory score. The longer treatment period results in more obvious gastric mucosal damages. Previous studies which used long-term treatment indicated that COX-2 inhibitors were not a placebo and had toxic effects. Accordingly, COX-2 inhibitors should not be used too long for chemoprevention of gastric cancer. Although we did not find any sudden death resulting from cardiovascular events in the gerbils, this may be due to the small sampling size. However, we found that the average ventricular size was large in group B (data not shown). Therefore, subclinical cardiovascular events may occur. The above findings show that the treatment period of Celecoxib should be as short as possible.

It should be noted that there could be other COX-2-independent mechanisms involved in stomach carcinogenesis because a relatively high dose of Celecoxib is needed for the anti-carcinogenic effect. Further studies are required to survey these mechanisms.

In conclusion, our study supports the hypothesis that Celecoxib has a potent anti-carcinogenic effect, and that short-term use could result in an almost equal effect to longer term use with less side effects. The protective effect of Celecoxib could be involved in the early oncogenic phase not in the early inflammation phase. This chemoprevention may be suitable for subjects with high risk for the development of gastric cancer: such as people with extensive metaplastic gastritis or refractory *H pylori* infection. Thus we suggest that Celecoxib could be used short-term for high-risk patients.

COMMENTS

Background

Long-term high dose cyclooxygenase-2 (COX-2) inhibitors can inhibit gastric carcinogenesis in animal models, but the possible life-threatening cardiovascular events limit its popular application. Therefore, in the present study, we wished to evaluate the optimal intervention point of a selective COX-2 inhibitor, Celecoxib, for inhibiting *Helicobacter pylori* (*H pylori*)-associated gastric carcinogenesis in Mongolian gerbils (MGs).

Research frontiers

COX-2 is a prostaglandin-synthesizing enzyme. Elevated expression of COX-2 is observed in a wide variety of human malignancies, including gastric cancer. *H pylori*-induced chronic gastritis also shows elevated levels of COX-2 expression in the stomach mucosa. The selective COX-2 inhibitors have been reported as preventing chemical carcinogen-induced carcinogenesis in C57/BL6 mice and Mongolian gerbils. It is important to evaluate the optimal therapeutic period of COX-2 inhibitors to prevent possible severe adverse events.

Innovations and breakthroughs

Chronic inflammation is thought to play a cardinal role in the accumulation of genetic damages leading to transformation and cancer by inducing the proliferation of target cells. Therefore, it is important to find the suitable time-point at which COX-2 inhibitors prevent the carcinogenesis. Previous studies used relatively long-term periods of chemopreventive treatment. However, COX-2 inhibitors were not a placebo and had a toxic effect, so COX-2 inhibitors should not be used too long for chemoprevention of gastric cancer. According to our results, Celecoxib may have effects including anti-oncogenic effect, inhibition of angiogenesis and metastasis, and it was shown that these effects were obtained by both late and short-term use of Celecoxib. In our study, the treatment began at 30-40 wk of age of the gerbils which is equivalent to 35 to 45-years old in humans. In our study, we found that the protective effect of Celecoxib could be involved in an early oncogenic phase not in an early inflammation phase. The short-term use also resulted in less severe inflammation and inhibited the invasion degree of gastric cancer. According to our findings, Celecoxib could be used latterly and short-term for refractory *H pylori* infection in a clinical situation; a point which has been seldom discussed in previous reports. This is very important for decreasing the possible side effects of COX-2 inhibitors.

Applications

Our study supports the theory that short-term treatment with Celecoxib has an anti-carcinogenic effect. Consequently, Celecoxib could be used in the later stages of *H pylori* infection to achieve safe and effective chemoprevention of gastric adenocarcinoma. In addition to this finding, The authors would like to suggest that COX-2 inhibitor should be used as chemoprevention for people older than about forty years old. This chemoprevention may play an important role for people who have extensive metaplastic gastritis with the highest risk for the development of gastric cancer, and it is also very important for those patients with refractory *H pylori* infections at high risk of gastric cancer.

Peer review

In the present study, the author investigated the optimal intervention point of Celecoxib for inhibiting *H pylori*-associated gastric carcinogenesis in MGs. They found the animals with extensive metaplastic gastritis or refratory *H pylori* infection may be suitable for celecoxib chemoprevention. This result provided us with some new information about personalized therapy for gastric cancer prevention and will prove beneficial for clinical application in the future.

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