

ORIGINAL ARTICLE

위점막 손상 및 소장 손상으로 인한 만성 비스테로이드성 소염제 유발 위염증 백서 모델 수립의 어려움

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Difficult Establishment of a Chronic Nonsteroidal Anti-inflammatory Drugs Induced Gastric Inflammation Rat Model due to Gastric Adaptation and Small Bowel Damage

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Background/Aims: The prevalence of peptic ulcer disease has not decreased mainly due to an increase in the use of NSAIDs. This study was conducted in order to determine whether a chronic NSAID-induced gastric inflammation model could be established by repeated administration of NSAID.

Methods: Indomethacin (10 mg/kg) was administered once per week for six weeks in 8- and 26-week rats and animals were sacrificed every week after administration. Gross ulcer index, histologic damage index, myeloperoxidase (MPO) activity, and mucus (glucosamine) levels were measured. Small bowel damage was also evaluated.

Results: Gross gastric damage index showed a peak level at three weeks and then decreased slowly in the 26-week indomethacin group. Gastric mucosal glucosamine level increased in both the 8-week ($p=0.038$) and 26-week groups ($p=0.007$). In addition, gastric mucosal MPO level decreased in the 8-week group ($p=0.018$) but did not show a decrease in the 26-week group. Small bowel damage began to occur at three weeks during the schedule and eight of 36 rats (22.2%) died due to perforation or peritonitis of the small bowel in the 8- and 26-week indomethacin groups, respectively.

Conclusions: Due to gastric adaptation and small bowel damage, repeated administration of NSAID to experimental animals may not be an adequate method for establishment of the chronic gastric inflammation model. (Korean J Gastroenterol 2014;63:341-347)

Key Words: Adaptation; Glucosamine; Gastric; Nonsteroidal anti-inflammatory agents; Small intestine

INTRODUCTION

Peptic ulcer disease (PUD) has remained as one of the main problems in upper gastrointestinal tract diseases. As progressed to an aging society, complicated PUD was more prevalent, despite decreased prevalence of *Helicobacter py-*

lori infection.¹ The main cause of such a phenomenon was considered the result of increased usage of NSAID, particularly in the aged population.² Proton pump inhibitor (PPI) has been used for prevention of NSAID associated PUD³; however, several problems have been reported with chronic PPI use, including risk of hip fracture in aged individuals.⁴

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Therefore, more physiologic drugs other than PPI may be needed in chronic NSAID users.

To test the effect of gastroprotective drugs, many trials were conducted for establishment of a chronic ulcer animal model. Acetic acid model has been recommended as a chronic ulcer model mainly due to the consistency of ulcer formation and prolonged duration of ulcer maintenance.⁵ However, this model causes some artificial defaults, such as leakage peritonitis, subserosal necrosis containing nerves and vessels, which are not observed in human PUD.^{6,7} In addition, this model did not reflect the pathogenesis of NSAID-induced PUD, which occurs by repeated exposure to NSAID. To date, only a few studies regarding the formation of an NSAID-induced chronic gastric inflammation model have been reported.⁸ In addition, other reports have suggested the difficulty of this model due to adaptive mechanism.⁹

From this background we attempted to determine whether establishment of a chronic NSAID-induced gastric inflammation rat model is possible by repeated administration of NSAID. In addition, the effect of NSAID-induced damage on the small bowel was evaluated along with the gastric damage.

SUBJECTS AND METHODS

1. Animals

The animals used in this study were 6- and 24-week-old male Sprague-Dawley rats, which were purchased (Orient Co. Ltd., Seoul, Korea) and housed in wire-bottom cages. They were maintained at 20-26°C, with 35-75% humidity and in a 12/12 hour light/dark cycle (lights on, 8:00-20:00) under pathogen-free conditions. After two weeks of adaptation, 8-week-old (weighting 260-290 g), 26-week-old (weighting 600-800 g) rats were used for this study. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital (BA0903-040/013-02).

2. Experimental design

The time schedule of this study is shown in Fig. 1. A total of 72 rats (36 rats of 8-week-old and 26-week-old, respectively) were assigned to the indomethacin group, in which NSAIDs were administered on a weekly basis and were sacrificed at one week after administration. In each NSAID group, indomethacin (10 mg/kg body weight; Sigma-Aldrich Co., St. Louis, MO, USA) was administrated via gavage feeding through a metal tube attached to a 5- or 10-mL syringe ev-

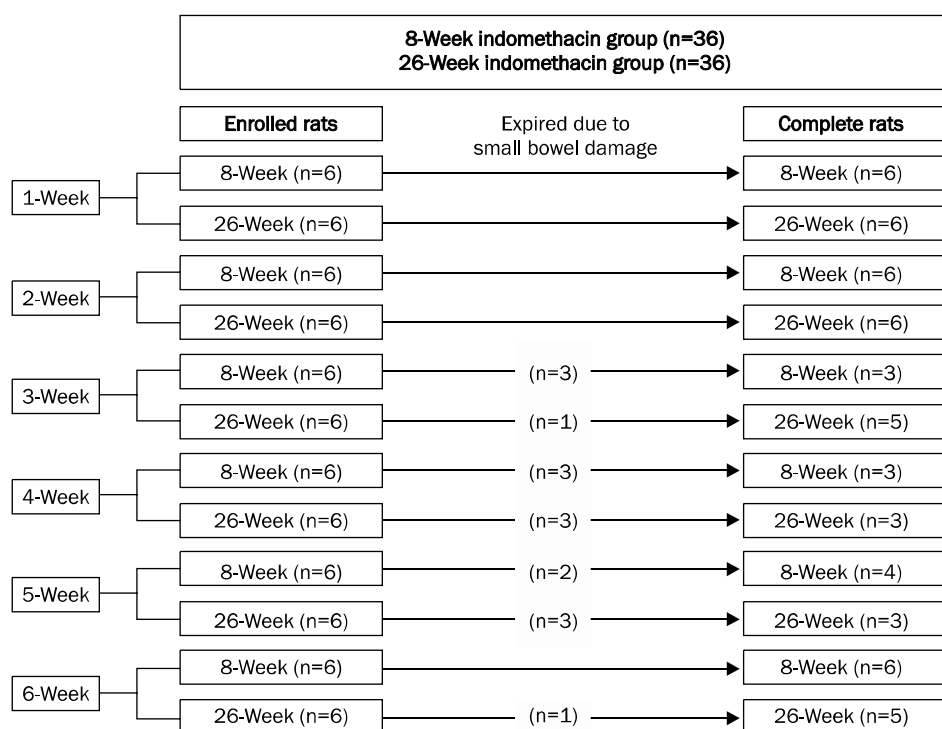


Fig. 1. Schematic diagram of study design.

ery week, respectively. The indomethacin solution was dissolved in 0.5% (wt/vol) carboxy methyl cellulose (CMC) with 10% ethanol, pH ranging from 1.3 to 1.5, mainly because of the solubility of the indomethacin. The 10% ethanol was tested on the control rat and no significant lesion was observed when compared to 0.5% CMC control rats.

3. Gross ulcer index and histological index

In this chronic experiment, starvation was not maintained due to the possibility of stress with recurrent starvation every week. After sacrifice, small bowel perforation or peritonitis was carefully evaluated in the indomethacin group. Next, the isolated stomach was cut open along the greater curvature and washed with ice-cold saline. To investigate the degree of gross mucosal damage, the mucosal sides of the stomachs were photographed, using a digital camera. Gross damage of the gastric mucosa was assessed by two experienced gastroenterologists, who were blinded to the experiment, using a previously described gross ulcer index¹⁰ defined as the sum of (number of type I lesions)+(number of type II lesions)×2+(number of type III lesions)×3. The lesion type was classified as follows: type I was defined as the presence of edema, hyperemia, or a single submucosal punctuated form of hemorrhage; type II was defined as the presence of submucosal hemorrhagic lesions with small erosions; and type III was defined as the presence of a deep ulcer with erosions.

The stomach was cut longitudinally to a width of 5 mm from the cardia to the pylorus in the anterior aspect after opening the stomach along the greater curvature, regardless of the damaged area or inflammation, and was fixed in 10% formalin. The tissue specimens were dehydrated and embedded in paraffin. The longest part of the specimen was sectioned into 6 µm fragments, and the sections were stained with hematoxylin and eosin. All tissues were examined by an experienced pathologist, who was blinded to the experiment. The mucosal damage then was graded by assigning a previously described index of histological injury¹¹ defined as (% type I damage)×1+(% type II damage)×2+(% type III damage)×3. The types of damage were defined as follows: type 0 damage meant that all gastric mucosal cells appeared intact and had a normal shape, location, appearance, and density; type I damage indicated that surface epithelial cells and the uppermost two or three cells lining the glands were damaged; type II damage described damage greater than type I

but involving < 50% of the thickness of the gastric mucosa; and type III damage indicated damage involving > 50% of the gastric mucosa depth.

In the case of a rat that had expired or was in a dying condition before the sacrifice schedule, autopsy was performed quickly in order to determine the cause of death. If a small bowel perforation and/or peritonitis were detected without other definite cause of death then small bowel damage was interpreted as the cause of death. The small bowel, in whole, was extracted in non-peritonitis rats. In case of peritonitis, small bowel could not be obtained because of necrosis. The entire small bowel was grossly examined for the existence of perforation. For evaluation of myeloperoxidase (MPO), damaged small bowel segment was obtained. In case of no small bowel damage by gross finding, small bowel tissue within 5 cm from pylorus was used.

4. Measurement of mucosal myeloperoxidase

An assay of gastric mucosal MPO concentration was used to quantify the degree of neutrophil infiltration.^{12,13} Three hundred milligrams of scraped mucosa from the stomach and the small intestine were homogenized for 30 sec using a polytron homogenizer in 1.0 mL of ice-cold 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0). Hexadecyltrimethylammonium bromide was used to negate the pseudoperoxidase activity of hemoglobin and to solubilize membrane-bound MPO. The homogenate was sonicated for 10 sec, freeze-thawed three times, and centrifuged for 20 min at 18,000×g. The supernatant was isolated and the MPO concentration was determined using an ELISA kit (Immunodiagnostik AG, Bensheim, Germany).

5. Measurement of glucosamine

After the excised stomach was washed extensively with ice-cold saline, the gastric mucosa was scraped with a slide glass and then frozen at -70°C, until it was used. A stock solution of glucosamine hydrochloride (Fluca, Buchs, Switzerland) was prepared fresh, daily, and stored at 5°C; 0.3 mL (Sigma-Aldrich) of acetylacetone was added to 10 mL of carbonate buffer with pH 10 (8 g of sodium bicarbonate and 2.1 g of sodium bicarbonate in 100 mL); the solution was prepared immediately before use. Ehrlich's reagent was prepared immediately before use by dissolving 0.4 g of *p*-dimethylaminobenzaldehyde (Sigma-Aldrich) in 1.5 mL of con-

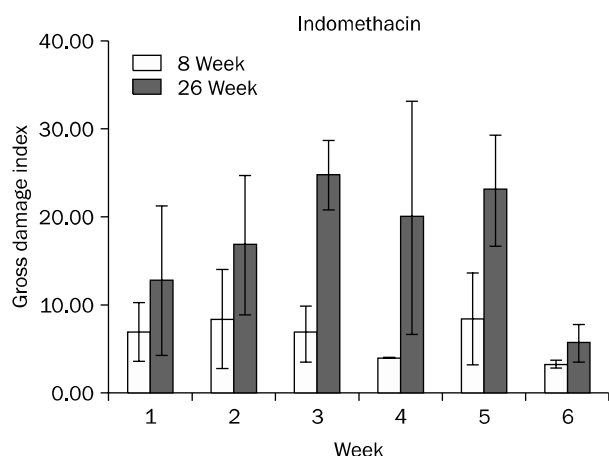


Fig. 2. Gross gastric damage index during the study period in the 8-week and 26-week indomethacin groups. Data are shown with mean±standard error.

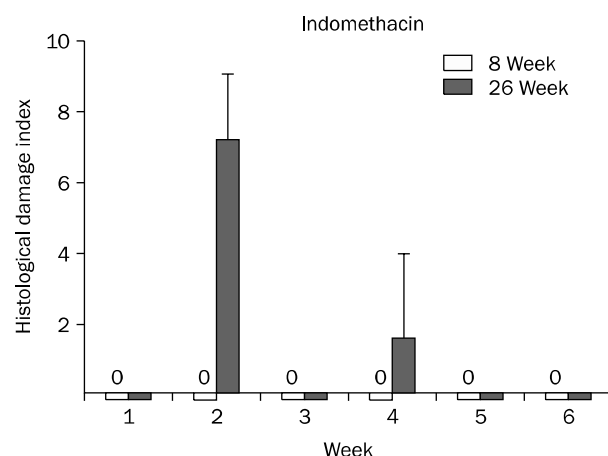


Fig. 3. Histological index of gastric mucosal damage during the study period in the 8-week and 26-week indomethacin groups. Data are shown with mean±standard error.

Table 1. Occurrence of Small Bowel Damage in the Indomethacin Group during Six Weeks in 8- or 26-Week Rats

Rats' age (week)	Perforation or peritonitis ^a						Total
	1 Week	2 Week	3 Week	4 Week	5 Week	6 Week	
8	0/6	2/6	2/3	2/3	1/4	0/6	7/28 (25.0%)
16	0/6	0/6	2/5	2/3	0/3	0/5	4/28 (14.3%)

^aPerforation or peritonitis number/total sacrifice rat number.

centrated hydrochloric acid (HCl) and 13.5 mL of isoamyl alcohol. Gastric mucosal mucin extracted with Triton X-100, was hydrolyzed with 1 mL of 3 N HCl. Finally, hexosamine obtained from hydrolyzed mucin was assayed.¹⁴

6. Statistical analysis

All statistical calculations were performed using SPSS software (version 12.0; SPSS Inc., Chicago, IL, USA). The time trend of the results was compared using regression analysis. Statistical significance was set at $p < 0.05$

RESULTS

1. Gastric gross and histological damage index

In 26-week rats, gross gastric damage index showed a peak level at three weeks and then showed a slow decrease in the indomethacin gavaged group. However, gross damage index was very small and no tendency was observed in 8-week rats in the indomethacin group (Fig. 2). Histological damage index was rather small and no constant trend was observed (Fig. 3). This could be explained by the fact that

NSAID-induced gross damage was usually not even and 5 mm width gastric tissue for histologic examination was collected from the anterior aspect of the antrum regardless of gross gastric damage.

2. Small bowel damage

Perforation began to occur from three weeks in the 8-week-rat and 26-week-rat groups. Death rate was 22.2% (8/36) in both the 8-week and the 26-week indomethacin groups (Fig. 1). In addition, small bowel perforation or peritonitis was observed in the 8-week group (25%, 7/28) compared with the 26-week indomethacin group (14.3%, 4/28) in survived rats observed during sacrifice (Table 1). In cadaver autopsy, all rats showed peritonitis, thus, small bowel damage was considered the cause of death.

3. Gastric mucosal and intestinal mucosal myeloperoxidase levels during the course of study

Gastric mucosal MPO showed a decreasing trend after four weeks with repeated administration in the 8-week indomethacin group ($p=0.018$). However, this was not a sig-

Table 2. Time Trend of Gastric Mucosal Myeloperoxidase (MPO) and Glucosamine Levels in the Indomethacin Groups

		1 Week	2 Week	3 Week	4 Week	5 Week	6 Week	p-value ^a
Indomethacin	MPO (ng/mg protein)	0.68±0.19	0.54±0.09	0.64±0.12	0.26±0.03	0.31±0.06	0.22±0.05	0.018
8-week-rat group	Glucosamine (mg/g mucosa)	8.36±1.09	8.18±0.59	8.01±1.15	9.31±1.35	9.59±2.10	12.24±0.91	0.038
Indomethacin	MPO (ng/mg protein)	0.51±0.07	0.72±0.11	0.71±0.07	0.36±0.06	0.31±0.02	0.48±0.11	0.270
26-week-rat group	Glucosamine (mg/g mucosa)	7.74±1.35	6.49±1.70	10.54±1.31	11.19±1.07	11.73±2.53	14.06±1.95	0.007

Data are presented as mean±standard error.

^aBy regression analysis.

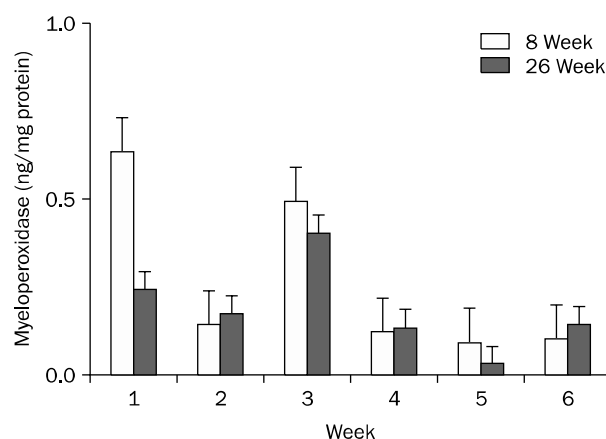


Fig. 4. Myeloperoxidase levels in the small bowel during the study period in 8- and 26-week groups. No statistically significant trend was observed. Data are shown with mean±standard error.

nificant trend in the 26-week indomethacin group ($p=0.270$). This trend could be matched with less significant inflammation in the 8-week indomethacin group than in the 26-week indomethacin group (Table 2). In the small bowel, MPO did not show any specific trend in both the 8-week and the 26-week indomethacin groups (Fig. 4).

4. Gastric mucosal glucosamine levels during the course of study

In both groups, the levels of glucosamine showed a steady increase from four weeks after administration of indomethacin in the 8-week group ($p=0.038$) and three weeks after in the 26-week group ($p=0.007$) (Table 2).

DISCUSSION

Chronic NSAID induced gastric inflammation model was not well-established in previous animal studies. In addition, the causes of difficulty in chronic NSAID induced gastric damage formation have not been clearly identified. A few reports have suggested that an adaptation mechanism, with re-

peated NSAID exposure, could be one of the causes in the chronic NSAID induced gastric damage model.^{9,15,16} The mechanisms of such adaptation were suggested as an up-regulation of COX-2 enzyme¹⁷ or enhancement of a defense mechanism.⁹ In the current study, no increases of the gross damage scale and MPO in 8-week rats also suggest the possibility of an adaptation to repeated NSAID exposure. Gastric glucosamine, which reflects secreted mucin as a strong mucosal protecting marker, was increased in the indomethacin rat groups during the study period. In addition, gastric glucosamine level showed an increasing trend, which is quite a contrast with the decrease of MPO in the 8-week indomethacin group. In the 26-week group, the gross ulcer scale showed a peak level at three weeks and then a slow decrease, and an increasing trend was observed in gastric glucosamine level but not in MPO level. These results suggest enhanced mucosal protection as an adaptive mechanism in case of repeated administration of indomethacin.

Another difficult point in formation of a chronic NSAID-induced gastric inflammation model was found to originate from the small bowel damage. Small bowel damage could not be avoided if a sufficient dose of NSAID was administered in order to produce gastric damage. We thought that adult rats may have thicker small bowel wall and greater resistance to NSAID than young rats. Therefore, we compared small bowel damage between 8-week young rats and 26-week adult rats. In this study, critical small bowel damage was also observed in the indomethacin groups, as death occurred in 16/72 (22.2%) and perforation or peritonitis in 11/56 (19.6%), when the rate was calculated in the total rats regardless of 8- or 26-week rats. As a result, no significant difference in small bowel damage was observed between the 8- and 26-week age groups. Furthermore, in our preliminary study, small bowel damage was not avoidable with repeated administration of 5 mg/kg indomethacin daily (data not shown). Previous reports also demonstrated that 7.5-15 mg/kg was

a sufficient dose in the indomethacin-induced acute enteritis rat model.¹⁸⁻²⁰ This low dose of indomethacin might be insufficient to induce a satisfactory gastric damage overwhelming adaptation mechanism. Taken together, small bowel damage may be the most serious problem in establishment of a chronic NSAID-induced gastric inflammation model.

Acetic acid model, with maintenance dose of NSAID, may be an alternative method in formation of a chronic NSAID-associated ulcer model and a few studies have demonstrated this possibility.²¹⁻²³ However, this method is somewhat artificial and it may induce leakage peritonitis and subserosal necrosis. A recent study suggested that calcitonin gene-related peptide was an important mechanism in gastric protection.⁷ Therefore, subserosal and muscular damage in an acetic acid model may differ from those in the human gastric ulcer induced by chronic NSAID usage. Furthermore, an adaptive mechanism might also have an effect on the maintenance dose of NSAID. Refed NSAID model could be possible as a chronic NSAID-induced antral ulcer model.^{24,25} Diffuse antral ulcer was documented in these study models. However, 25-30 mg/kg indomethacin used in this study could induce small bowel perforation. Therefore, another method is required for maintenance of a chronic model. As a result, the model in the combination of refed NSAID and inducer, such as HCl, could be attempted in the next study. In this model, NSAID dosage must be lowered as much as possible in order to minimize damage to the small bowel.

The limitations of our study could be that we administered only one dose of indomethacin (10 mg/kg) once per week. Polat et al.,⁹ who administered various doses of indomethacin, found a decrease of ulcerous area at the dose of 1-3 mg/kg/day, whereas the ulcerous area started to increase at the dose of 4-5 mg/kg/day. They concluded that the best adaptation was observed in rats administered 3 mg/kg/day. We speculated that reduced dosing and longer administration would not cause small bowel perforation, fibrosis, or stenosis as well as chronic strictures in the NSAID-induced chronic model. Contrary to our expectation, we found perforation of the small intestine in approximately 20% of cases. In addition, it is quite puzzling that intestinal MPO levels in the survived rats did not show any relationships with small bowel damage. The possibility that severe inflammatory response occurred in some rats, resulting in death,

could not be excluded; on the other hand, the other rats that survived indomethacin-induced damage were not affected by NSAIDs. In the future, these issues could be explained when we succeed in establishment of this chronic NSAID-induced gastric damage rat model with several doses and administration intervals of several kinds of NSAID such as indomethacin, diclofenac, and aspirin.

In conclusion, establishment of a chronic NSAID-induced gastric damage model was difficult due to an adaptive mechanism of the stomach and small bowel damage. Gastric mucus, a defensive mechanism, could be suggested as a cause of adaptive mechanism. Conduct of further studies will be needed in order to establish a chronic NSAID-induced gastric damage model.

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