The inhibition effect of lyophilized aspirin/trehalose on the aspirin-induced gastric mucosal injury

(アスピリン誘発性胃粘膜障害に対する凍結乾燥アスピリン/トレハロースの抑制効果)

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General Introduction

Section 1: Non-steroidal anti-inflammatory drugs (NSAIDs) and Aspirin

Thousands of years ago, our ancestors treated inflammation with the use of extracts of myrtle, willow bark and poplar tress, all of these contain salicylates.[1] To the modern times when the industry could succeed the synthesis of salicylic acid in the late 1800s permitted the widespread use of this drug for the treatment of fever, pain and the symptoms of rheumatoid arthritis.[2] However, salicylate was unpalatable and could cause gastric irritation, therefore an equally effective alternative was needed. In 1897, the young chemist Felix Hoffman of Bayer Pharmaceutical Manufacturer searched through the scientific literature and found a way of acetylating the hydroxyl group on the benzene ring of salicylic acid to form acetylsalicylic acid. The name "Aspirin" was given to the new drug which has better taste than the original salicylate and has the equal efficacy.

More than one hundred years have passed, and numerous nonsteroidal anti-inflammatory drugs (NSAIDs) were developed starting from the early 1970s. Now NSAIDs are one of the most widely used medication in daily practice over the world because of its efficacy in reducing pain and inflammation.[3] There are more than 70 million prescriptions and more than 30 billion over-the-counter tablets sold annually in the United States.[4] Although NSAIDs are generally well-tolerated, gastrointestinal

side effect occurs in a small but important percentage of patients, resulting in substantial morbidity and mortality.[3]

Probably because the types of NSAIDs of use were different in each country, estimates of the prevalence of adverse effects vary greatly. In general, it was estimated that at least 10 to 20 percent of patients have dyspepsia while taking NSAIDs, although the prevalence may range from 5 to 50 percent.[5, 6] The mortality rate among patients hospitalized for NSAIDs-induced gastrointestinal bleeding is about 5 to 10 percent.[7] Although the mortality is low, it must be emphasized that because a large population of patients are exposed to NSAIDs, often for extended periods, the risk over a lifetime is substantial. It has been estimated that over 107,000 hospitalizations and 16,500 deaths occur every year in the United States.[3, 8] The side effects of NSAIDs have also been considered to be financial burden for health insurance. According to the data of Arthritis, Rheumatism, and Aging Medical Information System (ARAMIS), suggesting an estimated cost of $15,000 to $20,000 per hospitalization, the annual costs of the serious gastrointestinal complications exceed $2 billion.[3] Therefore, to find out solutions for NSAIDs-induced gastropathy become one of the urgent topics of scientific research.

Section 2: Pathogenesis of Aspirin-induced Gastropathy

The possible mechanism of aspirin-induced gastropathy has been proposed by many researches, and multiple stage processes may be involved in this pathogenesis.

Cyclooxygenase (*COX)-dependent Mechanism*

The inhibition of COX enzymes and subsequent reduction of prostaglandin (PG) synthesis was firstly proposed to be the major mechanism of aspirin in 1971.[9] The secretion of both mucus and bicarbonate that could defense the gastric mucosa from the damage of acid is regulated by prostaglandin synthesis. Therefore, inhibition of prostaglandin synthesis caused by aspirin would affect the normal gastric protection system and induce gastric injuries.[10-14]

There are at least two isoforms of COX enzyme identified: constitutively active COX-1 primary present in the gastrointestinal tract and kidney; and the inducible COX-2 being expressed at inflammatory sites.[15-17] This discovery led to the development of selective COX-2 inhibitor NSAIDs, however some of the drugs were withdrawn from the market due to their adverse effect on the cardiovascular system.[16, 18-21] Recent studies found that both COX isoforms, COX-1 and COX-2, contribute to the mucosal defense. COX-1 inhibition results in reduced gastric blood flow, whereas COX-2 inhibition leads to increased leukocyte adherence to the vascular endothelium. Both inhibition processes contribute to the generation of mucosal injury.[22]

Inflammatory Mediator and Microcirculation

Among many potential mechanisms modulating gastric mucosal protection, one of the primary factors is blood flow in the gastric mucosa. The accumulation of activated neutrophils along the capillary walls and post capillary venules in the gastric microcirculation could contribute to aspirin-induced gastropathy by reducing the blood flow. [23] *In vitro*[24] and *In vivo*[25, 26] studies demonstrated that aspirin could promote leukocyte-endothelial cell adhesion via CD11/CD18-dependent interactions with intercellular adhesion molecule-1 (ICAM-1) in the microvasculation of the endothelium, thus exaggerate the mucosal inflammation and delay healing.

The involvement of proinflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), is suggested to induce hyper-adhesiveness of leukocytes to the endothelium and to stimulate the production of the other proinflammatory cytokines, such as IL-6 and IL-8, which also contribute to the recruitment of neutrophils to inflammation sites.[27-30]

Nitric oxide (NO)[31] and hydrogen sulfide (H_2S)[32] are also important factors to maintain normal function of the gastrointestinal mucosa, including mucus secretion, maintenance of microcirculation, response of leukocyte-endothelial interaction. This discovery further had resulted in the development of NO-derived aspirin[29] and H_2S-releasing aspirin.[33]

Direct Irritation

There are increasing evidences suggesting that COX-independent effects might also be important for aspirin-induced gastropathy. Several *in vivo* and *in vitro* data indicated that aspirin administration drives gastric epithelial cells to apoptosis through a mechanism involving activation of pro-apoptotic caspases.[29, 34, 35] Some inflammatory cytokines, for instance, TNF-α which is also a potent extracellular modulator of pro-apoptotic caspases in vitro, play an important role in regulating gastric epithelial cell apoptosis in aspirin-treated rats.[29] Although apoptosis represents an essential part in the regulation of gastric mucosal cell turnover, mediators involved in initiation and execution are still not well known.[36]

Besides apoptosis, there are still several hypotheses for NSAIDs-induced gastropathy, such as the detergent-like properties of NSAIDs,[37, 38] "ion trapping" effect,[39, 40] or pore formation characterization of membrane strength [41] are also hypothesis of direct damage caused by aspirin, but the contribution of these effects are still controversial.[42]

Strategies for Aspirin-induced Gastropathy

Numerous strategies have been proposed to prevent NSAIDs-induced gastropathy, including co-administration of protective substances such as sucralfate, proton pump inhibitors or prostaglandin analogues.[42] However, the protective effects

of these agents were not totally satisfied.[43] Except co-administration, investigators also tried to develop safer NSAIDs, for instance, selective cyclooxygenase-2 (COX-2) inhibitors,[44] but some of the drugs were withdrawn from the market because of the adverse effect on cardiovascular system.[16, 18-21]

Among the various strategies for prevention of NSAIDs-induced gastropathy, the combination of NSAIDs with small protective molecules such as nitric oxide,[45] hydrogen sulfide,[46] and phosphatidylcholine, which help to maintain mucosal integrity, has also been attempted.[47] Development of next generation NSAIDs, which is low gastrointestinal cytotoxic, low cardiovascular risky, and non-COX-2 selective, is warranted.[48]

Section 3: Trehalose and Its Cytoprotective Effect

Trehalose is a disaccharide formed by a 1,1- linkage of two D-glucose molecules and that is not easily hydrolyzed by acid.[49] This sugar is present in a wide variety of organisms, including bacteria, yeast, fungi, insects, invertebrates, and plants, in which it serves as a signaling molecule to direct or control certain metabolic pathways of even to affect growth.[50, 51] Elbein[52] summarized that trehalose can protect proteins and cellular membranes from inactivation of denaturation caused by a variety of stress conditions, including desiccation, dehydration, heat, cold and oxidation.

Trehalose is widely used as a food ingredient and in the cosmetics industry.[50, 51] Trehalose is an important protectant of protein integrity[53] and reduces oxidative damage to cells.[54, 55] It may also inhibit inflammatory cytokine production[56, 57] and suppress apoptosis.[58, 59] Therefore, trehalose is expected to be a potential powerful therapeutic material for various diseases.

For its cytoprotective effect, it has been adapted as preservation of tissues and blood cells for transplantation or transfusion.[60-62] Previous studies have also undergone several clinical trials on organ preservation during transplantation,[63] inhibition of dryness during dental treatment,[64] therapy of dry eye syndrome,[65] adhesion prevention after abdominal surgery[66] by its protective effect of cell membrane.

Section 4: Purpose

Our research group already successfully prevented post-operative adhesion and suppressed inflammation and vasospasm in experimental subarachnoid hemorrhage model by the usage of trehalose via its cytoprotective property.[55, 66] Hence, we further hypothesized that the combination usage of trehalose with aspirin may ameliotate aspirin-induced mucosal injury. In order to combine these two compounds, co-lyophilization of aspirin with trehalose was performed. Lyophilization is the freeze-drying procedure, which is regularly used to preserve vaccines, pharmaceuticals and proteins. Different aspirin preparations, including aspirin, lyophilized aspirin (Lyo A), mixture of aspirin and trehalose (Mix A/T), and lyophilized aspirin/trehalose (Lyo A/T) were utilized in this study.

In the first chapter, the author preliminarily investigated the direct effects of aspirin preparations on a human gastric cell line. In the second chapter, the author evaluated the effects of different aspirin preparations on gastric mucosa in a rat acute ulceration model. In the last chapter, the author further applied aspirin preparations to healthy dogs to evaluate the effects on canine gastric mucosa and to investigate the anti-inflammatory effects.

Chapter I

The *in vitro* Effects of Aspirin Preparations on Gastric Cells

Introduction

As I mentioned in the introduction, the anti-inflammatory effect of NSAID is mediated through their inhibitory effect on COX activity, leading to inhibition of PGs synthesis which induces inflammation. The inhibition of COX was believed to be the key role in the complications of NSAIDs, given that PGs exert a strong protective effect on gastric mucosa.[9, 67] However, there are increasing evidences suggesting that COX-independent effects might also be important for aspirin-induced gastropathy. The local effect of aspirin is an sole initiating factor in their toxicity.[42]

Activation of apoptosis on gastric mucosal cells or the dysfunction of mitochondria was considered to be one of the main mechanisms attributable to NSAIDs-induced local mucosal damage. Aspirin-induced apoptosis was initially studied in the colon carcinoma cell line HT-29.[68] Miller and his colleagues first indicated the relationship between apoptosis and aspirin-induced mucosal cell death using a human gastric cell line AGS,[69] and also proved that Bcl-2 protein family members and Smac regulate the apoptotic pathway in a caspase-dependent manner.[70] They further reported that caspase-independent pathway mediated by apoptosis inducing factor (AIF) might also be involved in an apoptosis-like programmed cell death.[71] In an *in vitro* model by utilizing guinea pig gastric mucosal cell primary culture, aspirin was suggested to induce both necrosis and apoptosis.[72]

Moreover, some inflammatory cytokines, such as TNF-α, which is also a potent extracellular modulator of pro-apoptotic caspases *in vitro*, play an important role in regulating gastric epithelial cell apoptosis in NSAIDs-treated rats. Chronic aspirin administration to rats also resulted in a persistent increase in plasma and mucosal TNF-α concentrations leading to an increased rate of gastric apoptosis.[29, 34, 35]

Although applied on different cell types, studies also indicated that salicylate potentiates both necrotic and apoptotic cell killing by promoting onset of mitochondrial permeability transition,[73] and that aspirin promotes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by down-regulation of Bcl-2 gene expression.[74] A study indicated that reactive oxygen species (ROS) plays a role in aspirin-induced apoptosis on hepatocellular carcinoma, however, the cause is still under investigation.[75]

Previous studies had found several anti-inflammatory effects and protection functions of trehalose. Among these protective functions, prevention of apoptosis was proposed as a possible mechanism. It is indicated that effective scavenging of ROS and stabilization of membrane leading to reduction in apoptosis during freezing/thawing enhanced cryopreservation of hematopoietic cells.[58] Another experiment also indicated that trehalose inhibits phagocytosis of refrigerated platelets via inhibition of caspase-dependent apoptosis.[62] Matsuo and co-workers had demonstrated trehalose solution was an effective and safe eye drop for the treatment

of dry eye syndrome,[65] and a recent experimental research further proved that suppression of apoptosis is the main effect against ocular desiccation.[59]

Base on these findings, I hypothesized the combined usage of aspirin and trehalose may protect the cell from direct cell damage (both necrosis and apoptosis). In this chapter, the AGS cell line was utilized to investigate the cytotoxicity and apoptosis-inducing potency of different aspirin preparations treatment. In this chapter, different preparation, including aspirin, lyophilized aspirin (Lyo A), mixture of aspirin/trehalose (Mix A/T), and lyophilized aspirin/trehalose (Lyo A/T) were employed. After exposure of AGS cells with each preparation for 24 hours, cytotoxicity was measured by tetrazolium dye colorimetric assay (MTT assay), and apoptosis occurrence was determined by DNA fragmentation analysis and quantified by Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay.

Section 1: Cytotoxicity of Aspirin Preparations on Gastric Cells

Materials and Methods

Cell and Culture

Human gastric cell line AGS was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured and maintained in Ham's F–12 (Invitrogen™, Carlsbad, CA, USA) culture medium supplemented with heat inactivated (56°C, 30 min) 10% fetal bovine serum (Gibco®, Carlsbad, CA, USA), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Chemicals and Reagents

Aspirin (acetylsalicylic acid) from Sigma (St. Louis, MO, USA), and trehalose [D(+)-trehalose dihydrate] from Wako (Osaka, Japan) were used in this experiment. Lyo A/T was obtained by lyophilization of the mixture of aspirin and trehalose at a molar ratio of 1:2. Aspirin and trehalose was dissolved in ethanol and distilled water, respectively, and then mixed in glass flask. After frozen with liquid nitrogen, the mixture was lyophilized during 3 days using a freeze dryer FDU-1100 (EYELA, Toyo, Japan). Lyo A was obtained using the same procedure without addition of trehalose.

Aspirin Preparations

Aspirin, Lyo A, and Lyo A/T were dissolved in dimethyl sulfoxide (DMSO) as stock solution. Trehalose was fresh prepared in culture medium prior to use.

Test compounds used in this chapter include aspirin, Lyo A, Mix A/T, and Lyo A/T which contained aspirin concentrations of 1, 3, 5, and 10 mM.

MTT Assay

To determine the cytotoxic effect of different treatments on AGS cells, MTT assay was performed. About 20,000 cells/well in a 96-well tissue culture plate were incubated overnight in 100 μl of culture medium. Cells were then treated with 1–10 mM of different aspirin preparations in serum-free media and incubated for 24 hr. Ten microliters of MTT (Roche, Basel, Switzerland) labeling reagent was added to each well and the cells were further incubated for another 4 hr at 37°C. One hundred microliters of solubilization solution in the kit were then added to each well. A microplate ELISA reader (Bio-Rad Model 680, Hercules, CA, USA) was used to measure the absorbency at wavelength between 550–600nm. The percentage of viable cell number in each treated group was compared to that of control (100%). The negative control well contained only culture medium without cells. The experiment was repeated at least 3 times.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism software (version 5.0). Data represented at least three independent experiments and were expressed as mean ± SE. One-way ANOVA followed by the Tukey's post hoc analysis was used to compare the results. A *P* value of less than 0.05 was considered statistically significant.

Results

MTT Assay

As shown in Fig. 1-1, all of the treatments caused gastric cell death in a concentration-dependent manner. The cell viability of aspirin-treated cells was 74.53 ± 0.72%, 55.35 ± 0.55%, and 30.09 ± 0.24% at the concentrations of 1, 3, 5 mM, respectively. The cell viability of Lyo A-treated cells was 72.15 ± 1.59%, 55.92 ± 0.41%, and 28.18 ± 0.55% at the concentrations of 1, 3, 5 mM, respectively. The cell viability of Mix A/T-treated cells was 71.19 ± 0.87%, 55.59 ± 0.55%, and 28.87 ± 0.71% at the concentrations of 1, 3, 5 mM, respectively. There were no significant differences in cell viability between in the aspirin, Lyo A, and Mix A/T groups at the same concentration. In contrast, the cell viability of Lyo A/T was 84.35 ± 1.28%, 61.58 ± 1.23%, and 35.48 ± 1.02% at the concentrations of 1, 3, 5 mM, respectively, and was significantly higher than those of the aspirin, Lyo A and Mix A/T groups at the same concentration ($P < .001$). The cells in any aspirin preparation groups at the concentration of 10 mM did not survive after 24 hours treatment.

Section 2: Apoptosis-inducing Potency of Aspirin Preparations on Gastric Cells

Materials and Methods

DNA Fragmentation Analysis

AGS cells were cultured as described in chapter 1-1. Cells were grown in a 15-cm petri dish to confluent in the medium, then treated with 1–10 mM of different aspirin preparations in the serum-free media and incubated for 24 hours. After the treatment, the cells were harvested and washed twice with ice-cold PBS. The final pellet obtained was lysed in 330 μl of 100 mM Tris-HCl (pH8.5) buffer containing 5 mM EDTA, 0.2 M NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K (Roche, Basel, Switzerland), and incubated in 37 °C overnight. Then 141 μl of 5M NaCl was added and centrifuged at 20,000 g for 15 minutes and the supernatant was obtained. An equal volume of 100% ethanol was added to the supernatant and centrifuged at 20,000 g for 15 minutes for DNA Extraction. The DNA pellet was then washed once with 70% ethanol, and dissolved in 20 μl TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). DNA samples were incubated with 10 μg/ml RNase A (Sigma, St. Louis, MO, USA). Equal amounts (5 μg/well) of DNA was electrophoresed in 1.5% agarose gels at 35V for 2 hours and visualized with ethidium bromide staining under UV light.

Determination of Apoptotic Cells by TUNEL Assay

AGS cells were cultured as described previously. Cells were grown in a 6-cm petri dish to confluent in the medium and then treated with 1–10 mM of different aspirin preparations in the serum-free media and incubated for 24 hr. After the treatment, the cells were harvested and fixed in 1% paraformaldehyde/PBS for 10 minutes. The fixed AGS cells were then washed twice with PBS and prepared as monolayer smear on glass slide.

In situ TUNEL assay kit (Millipore, Billerica, MA, USA) was employed to determine the yield of apoptotic cells according to the manufacturer's instructions. The number of TUNEL-positive cells was calculated under microscopic observation. One thousand cells were counted for one experiment, and at least three independent experiments were performed.

Statistical Analysis

Data represented at least three independent experiments and were expressed as mean ± SE. One-way ANOVA followed by the Tukey's post hoc analysis was used to compare the results at the same concentration treatment. A P value of less than 0.05 was considered statistically significant.

Results

DNA Fragmentation Analysis

Fig. 1-2 shows the agarose gel electrophoresis of genomic DNA to detect DNA fragmentation. Although there were no clear differences in pattern between each group under lower aspirin concentrations (1–5 mM) observed, DNA fragmentation in AGS cells treated with 10 mM aspirin, Lyo A or Mix A/T was shown as a ladder pattern on agarose gel, while the pattern in the Lyo A/T-treated cells showed much less fragmented DNA.

TUENL Assay

Fig. 1-3 shows the morphological changes of AGS cells treated with different test compounds for 24 hours at the concentration of 5 mM treatment. To distinguish apoptotic cells from necrotic cells, the nuclear morphology was observed at high maginification. Cells with condensed and shrinked nuclei or apoptotic bodies were characterized as positive cells.

Quantification of apoptosis determined by TUNEL assay is shown in Fig. 1-4. The apoptotic rate caused by aspirin was 31.67 ± 2.73, 58.67 ± 4.63, 130.00 ± 12.77, and 243.50 ± 11.50 apoptotic cells per thousand cells at the concentrations of 1, 3, 5, 10 mM treatment, respectively. The apoptotic rates caused by Lyo A and Mix A/T were almost similar to those of aspirin group. Aspirin concentrations ranging from 1

to 10 mM in these groups caused a significant, dose-dependent increase in the percentage of apoptotic cells after 24 hours treatment, and there were no significant differences between these three groups at the same concentration. On the contrary, the apoptotic rate caused by the Lyo A/T was 8.50 ± 0.50, 11.67 ± 1.20, 31.33 ± 2.65, and 41.00 ± 2.65 apoptotic cells per thousand cells at the concentration of 1, 3, 5, 10 mM treatment, respectively, and was significantly lower compared with other treatment groups at the same concentration ($P < .001$).

Discussion

AGS human gastric cell line, which had been characterized to be a proper model for estimating gastrointestinal toxicity of NSAIDs, was used in this chapter.[76] Tested preparations in this chapter included aspirin, Lyo A, Mix A/T, and Lyo A/T. According to the preliminary experiments, almost no AGS cells were alive after 24 hours exposure to aspirin at the concentration more than 10 mM (data not shown), thus NSAIDs treatment at the concentrations of 1, 3, 5, 10 mM were chosen in this study. The result of MTT assay showed that after 24 hours exposure to each preparation, Lyo A/T caused less cell death on AGS cells compared to other three test compounds at the same aspirin concentration.

NSAIDs-induced gastric cell death was suggested to be involved both with necrosis and apoptosis,[72] thus the experiments in the second section were designed to investigate apoptosis induced by different preparations.

Two approaches were used to evaluate apoptosis, DNA fragmentation analysis and TUNEL assay, both are late markers of apoptosis. Laddering represents the final dissolution of DNA, indicating that the cells have undergone suicide.[77, 78] Apoptotic cells then result via nuclear morphological changes, including shrinkage, condensation and margination, and fragmentation of chromatin.[79] The TUNEL assay allowed quantifying the percentage yield of apoptotic cells.

In this study, DNA fragmentation in AGS cells treated with aspirin was shown a ladder pattern on agarose gel only at 10 mM, and the same pattern was also observed in the Lyo A and Mix A/T-treated cells at the same concentration. On the contrary, there was no fragmented DNA pattern in any concentrations of Lyo A/T treatment. Aspirin treatment at lower concentration (under 5 mM) failed to induce DNA fragmentation on AGS cells was also noted in the previous study,[80] however another research[81] successfully induced this pattern on this cell line at low concentration treatment (1 mM). Therefore, I used the aspirin concentrations of 1 to 10 mM.

Although lower concentrations (1–5 mM) failed to demonstrate DNA fragmentation by aspirin exposure in this experiment as shown in Fig. 1-2, the results of TUNEL assay (Fig. 1-3 and Fig. 1-4) clearly presented the different apoptotic cell percentage of each treatment. Significantly lower percentage of apoptotic cells was noted when AGS cells were treated with Lyo A/T compared with those of other preparation groups at the same aspirin concentration. The apoptotic rate of AGS cells exposed to Lyo A/T was still quite low even at the highest treatment concentration (10 mM). This result was correlated with the absence of ladder pattern in the DNA fragmentation analysis.

Both cell death and apoptosis were observed in AGS cells treated with aspirin, Lyo A, or Mix A/T after 24 hours. This result is consistent with the previous study[72],

where the long-term treatment of aspirin (more than 16 hours) may decrease cell viability and induce apoptosis simultaneously *in vitro*.

A previous study[80] suggested that 20 mM aspirin concentration might be equivalent to the mucosal concentration when given a tablet of 325 mg aspirin, which is the most common dosage for headache control; and 3 mM was more consonant with the "baby aspirin" containing 81 mg aspirin, that is used for stroke and heart attack prevention. Hence this research team used 3–50 mM aspirin concentrations in their researches. However, they used aspirin suspension instead of completely dissolved solution, by which the actual concentration on gastric mucosa may be over evaluated. On the contrary, several previous reports assessing the role of apoptosis in aspirin-induced cell damage employed lower aspirin concentrations (0.1–10 mM), in which completely dissolved aspirin solutions were used.[72, 81, 82] In this experiment, completely dissolved aspirin preparations were applied, therefore 1–10 mM of aspirin concentrations might be more proper than higher concentrations.

In my study, both Lyo A and Mix A/T did not show any differences compared with aspirin treatment, which indicated the cytoprotective effect did not appear either by simple lyophilization of aspirin nor by mixture of trehalose with aspirin. The reason why co-lyophilization of aspirin with trehalose would enforce the protective characteristics was still not well elucidated. However, in a chemical analysis by

ramen spectroscopy performed by our research group showed the difference in structure between Mix A/T and Lyo A/T.

NSAIDs, especially aspirin which is an weak acid, are tend to be unionized in the gastric acidic environment and could easily diffuse across the cell membranes. Once inside the cell, the elevated intracellular pH promotes dissociation to the ionized form. Once ionized, aspirin remains water-soluble and trapped intracellularly. The nonionized form remains in the equilibrium across the cell membrane, and the total intracellular drug concentration will be much higher than outside the cell and damage the cells. This is so called "ion trapping phenomenon".[39, 40] When trehalose is co-lyophilized with a model compound, it would make a specific interaction mediated by hydrogen bonding with each other and form miscible amorphous solid solutions.[83] It is speculated that Lyo A/T would become more water-soluble than aspirin is through lyophilization procedure, and this amorphous property might avoid the occurrence of ion trapping phenomenon. However, further analysis is warranted.

In conclusion, the results of this chapter suggested that Lyo A/T exerted better cytoprotective effect on gastric cells, and this may be attributed to the inhibition of aspirin-induced apoptosis.

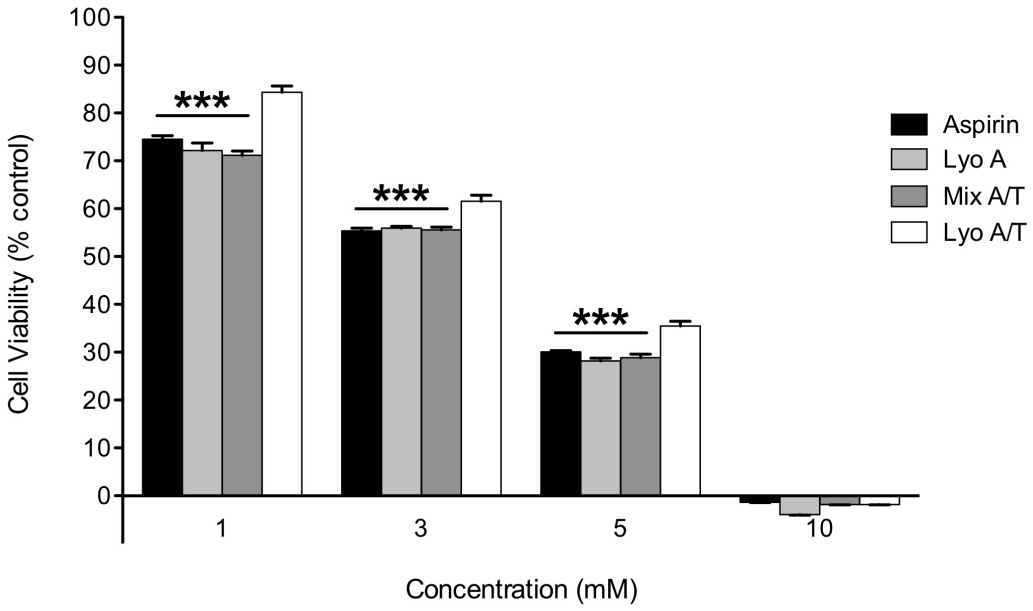

Fig. 1-1 The effects of different aspirin preparation treatments on AGS cell viability 24 hours after incubation. The cell viability in the Lyo A/T group was significantly higher than those in other groups at aspirin concentrations of 1, 3, 5 mM. The viability of the control group (1% DMSO) was set at 100%. Data represent the mean ± SE obtained from three independent experiments. *** $P< .001$, where the cell viability of the Lyo A/T group was significantly higher compared to other three groups.

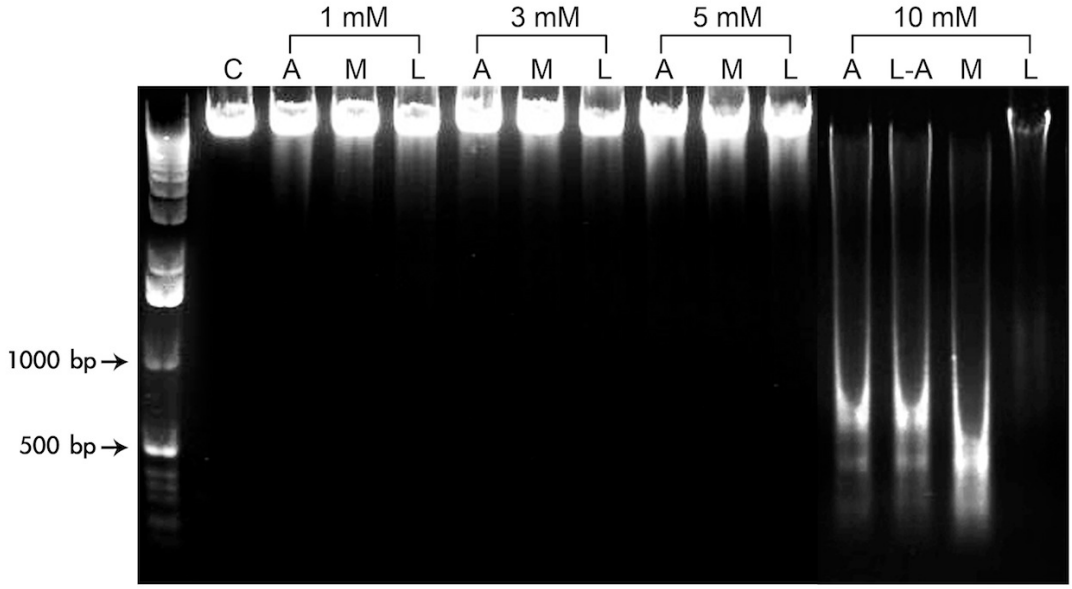

Fig. 1-2 Agarose gel electrophoresis patterns at different aspirin preparations treatments. The left lane is molecular mass marker; C, control DNA from untreated cells; A, aspirin; M, Mix A/T; L, Lyo A/T; L-A, Lyo A; bp, base pairs. The typical apoptotic laddering pattern on agarose gel was observed in the aspirin, Lyo A and Mix A/T groups at the aspirin concentration of 10 mM.

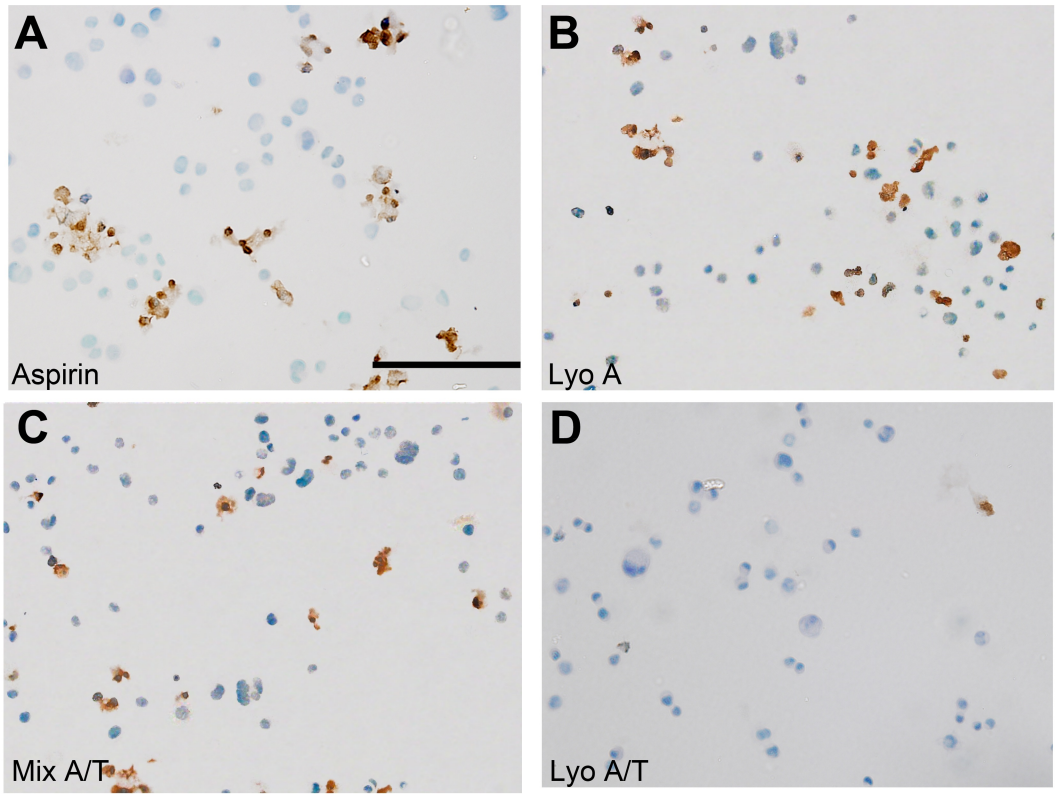

Fig. 1-3 Apoptosis of AGS cells caused by different treatments determined by TUNEL assay at aspirin concentration of 5 mM. Representative photographs of the mucosa were shown in aspirin (A), Lyo A (B), Mix A/T (C), or Lyo A/T (D). Less apoptotic cells were present in the Lyo A/T group compared to those of other groups. Scale bar: 200 μm.

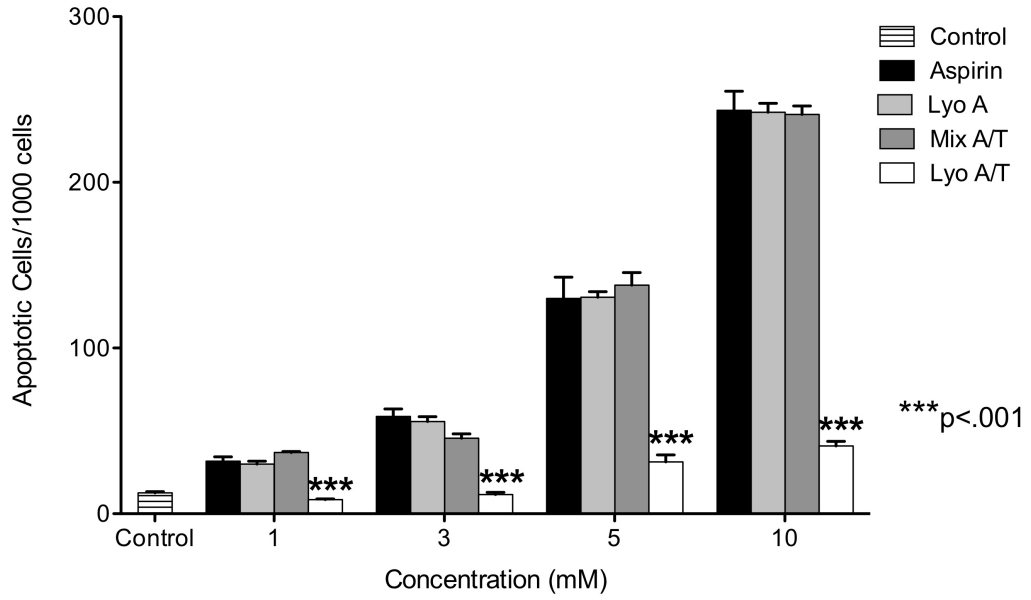

Fig. 1-4 Numbers of apoptotic cells in each group. The Lyo A/T induced significantly less apoptosis than aspirin, Lyo A, and Mix A/T did at any concentrations of aspirin. Data represent the mean ± SE obtained from three independent experiments. *** *P*< .001, where the apoptotic cell rate of the Lyo A/T group was significantly low compared with those of other three groups.

Chapter II

The *in vivo* Effects of Aspirin Preparations on Gastric Mucosa of Rats

Introduction

As described in the general introduction, multiple stage processes were thought to be involved in the mechanism of aspirin-induced gastropathy.

Inhibition of the COX enzymes and subsequent reduction of prostaglandin synthesis was first proposed to be the major mechanism of action of aspirin[9]. Recent studies found that both COX isoforms (COX-1 and COX-2) contribute to mucosal defense.

However, there have been increasing evidences suggesting that COX-independent effects might also be important for the NSAIDs-induced gastropathy. Fiorucci noted that the pathogenesis of NSAID-induced gastrointestinal damage is a consequence of COX inhibition and a topical, direct irritation effect of NSAIDs in his review artcicle.[36] The importance of direct irritation was emphasized by a previous study demonstrating that oral administration of aspirin caused more extensive gastric damage than parenteral administration did.[84]

As described in the introduction of chapter 1, activation of apoptosis on gastric mucosal cells or dysfunction of mitochondria was considered to be the main mechanism of NSAIDs-induced topical mucosal damage. Some studies have already demonstrated that NSAIDs induce *in vitro* cell death (apoptosis and necrosis) independent of COX inhibition and that both COX inhibition and NSAIDs-induced cell death are required to produce gastric lesions *in vivo*.[72, 85] Several animal

experiments and *in vitro* data indicated that aspirin administration drives gastric epithelial cells to apoptosis through a mechanism involving activation of pro-apoptotic caspases.[29, 34, 35] Although apoptosis represents an essential part in the regulation of gastric mucosal cell turnover, mediators involved in initiation and execution are still not well clarified.[36] Miller's group first indicated the relationship between apoptosis and aspirin-induced mucosal cell death on a human gastric cell line AGS,[69] and also proved that Bcl-2 protein family members and Smac regulated the apoptotic pathway in a caspase-dependent manner.[70] They further reported that caspase-independent pathway mediated by apoptosis inducing factor (AIF) may also be involved in an apoptosis-like programmed cell death.[71]

Although using different cell types, a study also indicated that aspirin promoted tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by down-regulation of Bcl-2 gene expression in human prostate adenocarcinoma and colorectal carcinoma cell lines.[74] Another study suggested reactive oxygen species (ROS) played a role in aspirin-induced apoptosis on hepatocellular carcinoma, however, the mechanism is still under investigation.[75]

Reduction of gastric blood flow and the induction of leukocyte adherence to the vascular endothelium also contribute to the aspirin-induced gastropathy,[23] and proinflammatory cytokines are speculated to be involved in this adherence phenomenon.[27] *In vitro*[24] and *in vivo*[25, 26] studies had further confirmed that aspirin

could promote leukocyte-endothelial cell adhesion via CD11/CD18-dependent interaction with ICAM-1 of microvascular endothelium, therefore exaggerated the mucosal inflammation and delayed healing. IL-1β and TNF-α induce hyper-adhesiveness of leukocytes to vascular endothelium and stimulate the production of the other proinflammatory cytokines, IL-6 and IL-8, which also contribute to the recruitment of neutrophils to the site of inflammation.[27-30]

Trehalose is a non-reducing disaccharide in which two glucose units are linked by an α, α-1glycosidic bond. Recent studies found several novel protective functions of trehalose, which distinguishes trehalose from other common disaccharides. Trehalose is present in wide variety of organisms, such as bacterias, yeast, fungi, insects, etc., but not in mammals. It is synthesized as a stress-responsive factor enable to protect proteins integrity[53] and cellular membranes from inactivation or denaturation caused by a variety of stress condition, including desiccation, dehydration, heat, cold, and oxidation.[52, 54, 55, 86]

Besides being as a food and cosmetic ingredient,[50, 51] trehalose is expected to be a potential powerful therapeutic material for various diseases, and has been used as preservation of tissue and blood cells for transplantation and transfusion.[60-62] *In vitro* study showed that trehalose could suppress NF-κB activity through inhibition of IκBα reduction induced by LPS, thus resulting in the inhibition of inflammatory cytokine production[56]. *In vivo* study also revealed that trehalose reduced NF-κB binding

activity, IκBα protein loss, TLR-4 activation, and TNF-α, IL-1, IL-6 levels, therefore trehalose blocked the inflammatory cascade triggered by endotoxin shock by stabilizing bio-membranes.[87]

Among the protective functions of trehalose, prevention of apoptosis was proposed as a possible mechanism for cell membrane integrity. A study indicated, by using trehalose, that effective scavenging of ROS and stabilization of the cell membrane lead to reduction in apoptosis during freezing/thawing in cryopreservation of hematopoietic cells.[58] Another experiment also indicated that trehalose inhibited phagocytosis of refrigerated platelets via inhibition of caspase-dependent apoptosis.[62] Matsuo and co-workers demonstrated trehalose solution was an effective and safe eye drop for the treatment of dry eye syndrome,[65] and a recent experimental research further proved that suppression of apoptosis was the main mechanism against ocular desiccation.[59]

The results in chapter 1 indicated that Lyo A/T had cytoprotective effect on AGS cells. In this chapter, I would further investigate the effects and the protective mechanism of Lyo A/T on gastric mucosa *in vivo*. According to the previous data, Lyo A showed similar effects to those treated with aspirin, therefore only aspirin, Mix A/T, and Lyo A/T were selected as test preparations in this chapter. An acute rat gastric ulceration model was used to evaluate the NSAID-induced gastropathy macroscopically and microscopically, followed by evaluation of apoptosis, which was

determined by *in situ* TUNEL assay and cleaved caspase-3 immunohistochemistry. The effects of treatments on mucosal prostaglandins synthesis and mRNA expression of inflammatory mediators were also evaluated.

Section 1: Macroscopic and Histopathological Evaluation of Aspirin Preparations-induced Gastropathy in an Acute Ulceration Model

Materials and Methods

Animals

Eight-week-old male Sprague Dawley rats weighing 260–300 g were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan) and maintained on a 12-hr light-12-hr dark cycle at constant temperature (22–24°C). The rats were fed with standard laboratory chow and tap water. All experiments were performed in accordance with the guidelines of the Committee for Animal Care, Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Chemicals and Reagents

Aspirin (acetylsalicylic acid), and trehalose [D(+)-trehalose dihydrate] were the same as used in chapter 1. Lyo A/T was prepared by lyophilization of the mixture of aspirin and trehalose at a molar ratio of 1:2 as described in chapter 1. In this chapter, aspirin, Mix A/T, and Lyo A/T were used.

Preparation and Assessment of Aspirin Preparations-induced Acute Gastropathy

Rats were fasted for 24 hours and deprived of drinking water for 1 hour before

drug administration. Acute gastric ulceration was induced by a single oral administration of 200 mg/kg aspirin, 960 mg/kg Mix A/T (200 mg/kg aspirin), 960 mg/kg Lyo A/T (200 mg/kg aspirin), or vehicle (0.5% carboxymethylcellulose aqueous solution). All the treatment solutions were freshly prepared on each day of dosing. Rats were then euthanized with CO_2 inhalation 5 hours after administration. Macroscopic gastric damage was evaluated according to the method described by Yoshida et al.[26] with a slight modification. The stomach was removed and the pylorus of the stomach was ligated. The stomach was fixed in its entirety by injection of 6 ml of 10% neutral buffered formalin from the cardiac opening for 30 minutes. The fixed stomach was incised along the greater curvature, slightly rinsed with 0.9% physiological saline, and flattened on a corkboard, and photographed by a digital camera. The hemorrhagic erosions was determined upon the images and expressed as the ulcer area (mm^2). The ulceration area of the stomach was then excised, embedded in paraffin, sectioned in 3 μm thickness and stained with hematoxylin and eosin. Because the ulceration lesions tended to appear along the greater curvature tissue, samples of the vehicle group were also excised from the similar area of the stomach.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism software (version 5.0). Data were expressed as mean ± SE. Each group consisted of 6–8 rats. One-way

ANOVA followed by the Tukey's post hoc analysis was used to compare the results. A *P* value of less than 0.05 was considered statistically significant.

Results

Macroscopic Evaluation

Multiple linear hemorrhagic lesions were noted in the aspirin and Mix A/T groups (Fig. 2-1 B and C), while less extent of gastric injuries were present in the Lyo A/T group (Fig. 2-1 D).

Quantified comparison of macroscopic injury was shown in Fig. 2-2. The ulcer areas caused by aspirin, Mix A/T and Lyo A/T were 11.43 ± 1.76, 7.95 ± 1.36, 3.13 ± 0.68 mm^2, respectively, and no ulceration was observed in the vehicle group. The ulcer areas in the aspirin and Mix A/T groups were significantly larger than that in the vehicle groups ($P< .001$), while the ulcer area in the Lyo A/T group did not significantly differ from that in the vehicle group. The ulcer area of the Lyo A/T group was significantly smaller than those in the aspirin ($P< .001$) and the Mix A/T ($P< .05$) group.

Fig. 2-3 shows the histological findings after administration. In the aspirin and Mix A/T groups, almost complete ablation of the superficial epithelium and gastric glands necrosis were observed with cellular debris, compared with the intact gastric mucosal tissue in the vehicle group. (Fig. 2-3 A, B, and C). On the contrary, only mild focal superficial epithelial damage was observed, and the gastric pits and the muscular layer were almost intact in the Lyo A/T group (Fig. 2-3 D). These results coincided to those of gross appearances.

Section 2: Apoptosis-inducing Potency of Aspirin Preparations on Gastric Mucosa

Materials and Methods

Animals and Induction of Acute Gastropathy

Gastric tissue samples obtained in chapter 2-1 were used in this section. Eight-week-old male Sprague Dawley rats weighing 260–300 g were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan) and maintained as described in chapter 2-1. Rats were orally treated with different aspirin preparations and sacrificed as described in chapter 2-1. The stomach was removed and entirely fixed by 10% neutral buffered for 30 minutes. The fixed stomachs were incised along the greater curvature, and areas of the stomach with ulcer were excised, embedded in paraffin, and sectioned in 3 μm thickness.

In situ Detection and Quantification of Apoptotic Cells

Apoptotic cells were detected using *in situ* TUNEL assay kit (Millipore, Billerica, MA, USA) according to the manufacturer's instruction. Nuclei that stained dark brown and showed morphological signs indicative of apoptosis, including condensation of chromatin and/or fragmentation of nuclei, were considered positive. Apoptotic cells were counted under microscopic observation at 400X magnification.

The epithelial and glandular tissue layers were included in the calculation, while the submucosal connective tissue and the muscular tissue were excluded. Ten areas per total gastric tissue were counted and expressed as the number of apoptotic cells per 10^6 μm^2. Only grossly intact epithelial surface areas were included in the assessment of apoptosis, while areas with obviously ill lesion were omitted due to the increased density of staining and difficulty of accurately determining the indicative apoptosis characteristics of nuclei. Cells detached from the epithelium were not counted.

Immunohistochemistry and Quantification of Cleaved Caspase-3

Deparaffinization and the antigen retrieval were performed in 1 mM EDTA solution by autoclaving at 100°C for 10 minutes. Endogenous peroxidase activity was blocked by 10 minutes incubation with 3% hydrogen peroxide. Slides were incubated overnight with a polyclonal cleaved caspase-3 antibody (Cell Signalling #9664, Beverly, MA, USA) at 4°C, that specifically recognizes the large fragment (17/19 kDa) of activated but not full length caspase-3, at a working dilution of 1:200. The slides were subsequently treated with the horseradish-peroxidase labelled anti-rabbit immunoglobulin secondary antibody (EnVision+System-HRP Labelled Polymer, Dako, Osaka, Japan) for 30 minutes. The immunoreactive cells were visualized with DAB chromogen (Dako, Osaka, Japan) followed by counterstaining with hematoxylin.

The cleaved caspase-3-positive cells were counted under microscopic observation at 400X magnification. The quantified assessment was performed as described in the quantification of TUNEL-positive cells.

Statistical Analysis

Data were expressed as mean ± SE. Each groups consisted of 6–8 rats. One-way ANOVA followed by the Tukey's post hoc analysis was used to compare the results. A *P* value of less than 0.05 was considered statistically significant.

Results

In situ TUNEL Assay and Quantification of Apoptotic Cells

Fig. 2-4 shows the histological findings in *in situ* TUNEL assay in each group. Nuclei that stained dark brown and showed morphological signs indicative of apoptosis, including condensation of chromatin and/or fragmentation of nuclei, were considered positive. There were less TUNEL-positive cells in the vehicle and Lyo A/T groups compared to the aspirin and Mix A/T groups. In any sample groups, the majority of apoptotic cells were located in the surface epithelial compartment of the gastric mucosa.

Fig. 2-5 shows the quantification of apoptosis. The numbers of apoptotic cells (per 10^6 μm^2) in the vehicle, aspirin, Mix A/T, and Lyo A/T groups were 7.24 ± 1.67, 25.34 ± 2.20, 18.80 ± 1.94, and 7.01 ± 1.55, respectively. Positive cells in the aspirin and Mix A/T groups were significantly more than those of the vehicle group ($P< .001$, $P< .05$, respectively). There was no significant difference between the vehicle and Lyo A/T groups. Positive cells in the Lyo A/T group were significantly less than those in the aspirin and Mix A/T groups ($P< .001$, $P< .01$, respectively).

Immunohistochemistry and Quantification of Cleaved Caspase-3

Fig. 2-6 shows the immunohistochemical findings of cleaved caspase-3. Cleaved caspase-3 was present in the cytoplasm of cells with morphology consistent with apoptosis, as well as in some morphologically healthy appearance cells. The number of these positive cells seemed less in the vehicle and Lyo A/T groups compared to those of other two groups.

Quantification of the cleaved caspase-3 protein expression is shown in Fig. 2-7. The numbers of positive cells (per 10^6 μm^2) in the vehicle, aspirin, Mix A/T, and Lyo A/T groups were 6.85 ± 1.15, 39.77 ± 7.05, 20.22 ± 7.23, and 8.17 ± 1.28, respectively. The number of positive cells in the aspirin was significantly higher than those of the Lyo A/T and vehicle groups ($P< .01$, $P< .05$, respectively). There was no significant difference between the vehicle and Lyo A/T groups. The number of positive cells in the Mix A/T group was higher than those in the vehicle and Lyo A/T groups, however there were no significant changes.

Section 3: Effects of Aspirin Preparations on Gastric Expression of Inflammatory Mediators and Mucosal PGE_2 Synthesis in an Acute Ulceration Model

Materials and Methods

Animals

Sprague Dawley rats used in this section were similar to those in chapter 2-1. All experiments were performed in accordance with the guidelines of the Committee for Animal Care, Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Induction of Acute Gastropathy

Rats were fasted, deprived of drinking water, and orally treated with different aspirin preparations as described in chapter 2-1. Rats were then sacrificed by bleeding under deep isoflurane anesthesia. The stomach was removed and incised along the greater curvature, and the gastric corpus mucosa was scraped off on ice using surgical blade. A half of the mucosal sample of each stomach was preserved in RNA*later*® tissue stabilization solution (Life Technologies, Carlsbad, California, USA), and the rest of the mucosal sample was immediately snapped frozen in liquid nitrogen, and then stored at -80°C for the determination of PGE_2 concentration.

Messenger RNA (mRNA) Expression of Inflammatory Mediators

RNeasy Mini Kit (QIAGEN, Hilden, Germany) and DNase I (QIAGEN) digestion were used to extract total RNA according to manufacturer's instructions. Complementary DNA (cDNA) was generated from 2 μg of RNA and Superscript III reverse transcriptase (Invitrogen™, Carlsbad, CA, USA) was used. Resulting cDNA was subjected to expression analysis by using real-time PCR. Real-time PCR was performed by using Thunderbird SYBR qPCR mix kit (Toyobo, Osaka, Japan) at 95°C for 1 minute, 40 cycles at 95°C for 15 seconds and 60°C for 45 seconds, and then dissociation curve was also analyzed after reaction. The expression of mRNA of intercellular adhesion molecule-1 (ICAM-1), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were analyzed and expressed relatively to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used were listed in Table 2-1.

Table 2-1 Primers for real-time PCR of inflammatory mediators.

Gene name		5'-3' primer sequence
ICAM-1[46]	FW	CAAGGGCTGTCACTGTTCAA
	RW	CTTCAGAGGCAGGAAACAGG
IL-1β[88]	FW	CACCTCTCAAGCAGAGCACAG
	RW	GGGTTCCATGGTGAAGTCAAC
IL-6[88]	FW	TCCTACCCCAACTTCCAATGCTC
	RW	TTGGATGGTCTTGGTCCTTAGCC
GAPDH	FW	TAAAGGGCATCCTGGGCTACACT
	RW	TTACTCCTTGGAGGCCATGTAGG
TNF-α[46]	FW	TGATCCGAGATGTGGAACTG
	RW	CGAGCAGGAATGAGAAGAGG

Sample Preparation and Measurement of Mucosal PGE_2 Concentration

The mucosal samples were thawed and added to a tube containing homogenization buffer (0.1M phosphate buffer, pH 7.4, containing 1 mM EDTA and 10 μM indomethacin), where the indomethacin could prevent further synthesis of prostaglandins. Then samples were homogenized and centrifuged at 8,000g for 10 min at 4°C. The supernatant of each sample was collected and the total protein concentration of each sample was determined using BCA protein assay reagent kit (Thermo Scientific, IL, USA) according to the manufacturer's instructions.

The concentration of PGE_2 was measured using an enzyme immunoassay (Cayman Chemical, MI, USA) according to the manufacturer's protocol.

The mucosal PGE_2 concentration was calculated and expressed as pg/mg protein.

Statistical Analysis

Data were expressed as mean ± SE. Each groups consisted of 6–8 rats. One-way ANOVA followed by the Tukey's post hoc analysis was used to compare the results. A *P* value of less than 0.05 was considered statistically significant.

Results

mRNA Expression of Inflammatory Mediators on Gastric Mucosa

Fig. 2-8 shows the mRNA expressions of inflammatory mediators after the treatments with various aspirin preparations. The relative mRNA expression of IL-1β of the vehicle, aspirin, Mix A/T, and Lyo A/T groups were 0.52 ± 0.14, 3.48 ± 0.80, 2.67 ± 0.58, and 2.21 ± 0.66, respectively (Fig. 2-8 A). The expression of IL-1β mRNA tended to increase 5 hours after the treatments compared with the vehicle group, however there was a significant difference only between the vehicle and the aspirin treated group. Although the expression in the Lyo A/T group tended to be lower than that of the aspirin and Mix A/T groups, no significant differences were observed.

The relative mRNA expressions of IL-6 of the vehicle, aspirin, Mix A/T, and Lyo A/T groups were 0.18 ± 0.04, 0.32 ± 0.06, 0.35 ± 0.06, and 0.24 ± 0.07, respectively (Fig. 2-8 B). There were no significant differences between each group.

The relative mRNA expressions of TNF-α of the vehicle, aspirin, Mix A/T, and Lyo A/T groups were 4.83 ± 1.13, 7.09 ± 0.83, 4.50 ± 0.54, and 7.93 ± 0.66, respectively (Fig. 2-8 C). There were no significant differences between each group.

The relative mRNA expressions of ICAM-1 of the vehicle, aspirin, Mix A/T, and Lyo A/T groups were 6.16 ± 1.57, 12.49 ± 1.92, 13.21 ± 4.32, and 11.35 ± 2.93, respectively (Fig. 2-8 D). Although the mRNA expression was higher in the aspirin,

Mix A/T, and Lyo A/T groups compared to the vehicle group, there were no significant differences.

Mucosal PGE_2 Concentration

Fig. 2-9 shows PGE_2 synthesis on the gastric mucosa treated with aspirin preparations. The PGE_2 concentrations in the vehicle, aspirin, Mix A/T, and Lyo A/T treated gastric mucosa were 291.00 ± 53.46, 26.02 ± 15.28, 34.40 ± 11.80, and 31.01 ± 8.69, respectively. The gastric PGE_2 synthesis was profoundly decreased in these treatment groups compared to that in the vehicle group.

Discussion

In this *in vivo* rat model, acute gastric ulceration was induced by oral administration of aspirin, Mix A/T, or Lyo A/T. The gastropathy was reduced by Lyo A/T both macroscopically and histologically, despite profound suppression of gastric PGE_2 synthesis. The inhibition of PGE_2 synthesis elicited by the Lyo A/T was comparable with that elicited by aspirin, thus indicated the lyophilization procedure did not interfere with the ability to inhibit COX activity by aspirin. Moreover, lack of ulceration in the Lyo A/T group with the presence of profound suppression of gastric PG synthesis suggested that Lyo A/T exerted protective effects that counteracted the potential damaging effects of COX inhibition.

In our previous experiments showed that Lyo A/T suppressed inflammation as well as aspirin alone in a carrageenan-induced paw edema model (under submission). This also indicated that the co-lyophilization aspirin with trehalose procedure did not alter the anti-inflammatory effect of aspirin.

In this chapter, I investigated whether Lyo A/T inhibited gastric mucosal apoptosis or not, using *in situ* TUNEL assay and cleaved caspase-3 immunohistochemistry, which indicated the late stage and early apoptotic responses respectively. Lyo A/T induced significantly less extent of apoptosis than aspirin and Mix A/T did. Quantification of TUNEL positive-cells was carried out on randomly chosen regions of multiple sections. However, the areas of obvious mucosal lesions

were excluded due to the difficulty in distinguishing between cells undergoing programmed cell death and necrosis, and due to that apoptotic cells were likely to detach from eroded surface.

Programmed cell death requires the activation of the intracellular protease family of caspases, which are activated by proteolytic cleavage from inactive procaspases and consist of heterotetramers with two large subunits and two small subunits.[89] These proteolytic enzymes participate in a cascade in which upstream members cleave and activate those immediate downstream, which in turn cleave substrate proteins, leading to the biochemical and morphological changes characteristic of apoptosis.[90] Caspase-3 is one of the more downstream caspases of the apoptotic cascade and is activated by the cleavage of an inert 32 kDa precursor to yield a 17 kDa active enzyme (cleaved caspase-3). Increased expression of the active enzyme correlates with an elevated incidence of apoptotic cell death.

In this study, less protein expression of active caspase-3 was observed in the Lyo A/T group, and which was almost equivalent to the vehicle group. Although not significant, the treatment with Mix A/T tended to induce less apoptosis than aspirin but more than Lyo A/T did. This may suggest that combined use of trehalose, at least to some extent, protects the mucosal cells against apoptosis; however, lyophilization of aspirin with trehalose would offer the best inhibition for apoptosis of the gastric mucosa.

The relative mRNA expression of inflammatory mediators, including IL-1β, IL-6, TNF-α, and ICAM-1 were analyzed using real-time PCR in this study. The up-regulation of IL-1β mRNA 5 hours after the treatment with each aspirin preparation was observed, however the significance was only noted between the vehicle and the aspirin group. Although the expression in the Lyo A/T group tended to be lower than that of the aspirin and Mix A/T groups, no significant differences were observed. In addition, no significances were noted in the relative mRNA expression of IL-6, TNF-α, and ICAM-1 between each group, including the vehicle group, This result differs from previous study,[33] which may be due to the different time point of sampling. However, the lack of up-regulation of inflammatory mediators may be consistent with the results that there was few neutrophils infiltration in the inflammatory sites, even in the aspirin group.

Taking all these findings together, the inhibition of caspase-dependent apoptosis of Lyo A/T was suggested to be the most potential, at least partially, protective effect against aspirin-induced gastropathy.

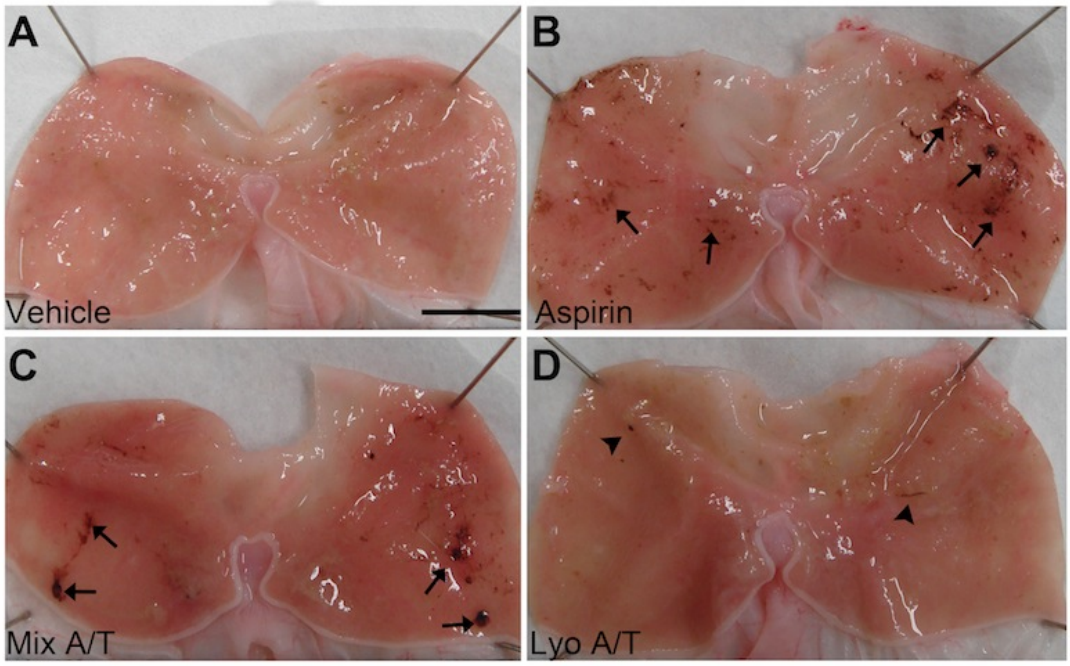

Fig. 2-1 Macroscopic appearance of acute gastropathy. Rats were orally administered with each aspirin preparations as described in the materials and methods. Multiple hemorrhagic lesions (arrows) were present in the aspirin and Mix A/T groups, while less extent of hemorrhage (arrow heads) was present in the Lyo A/T group. Representative photographs of the mucosa were shown in vehicle (A), aspirin (B), Mix A/T (C), or Lyo A/T (D). Scale bar: 1cm.

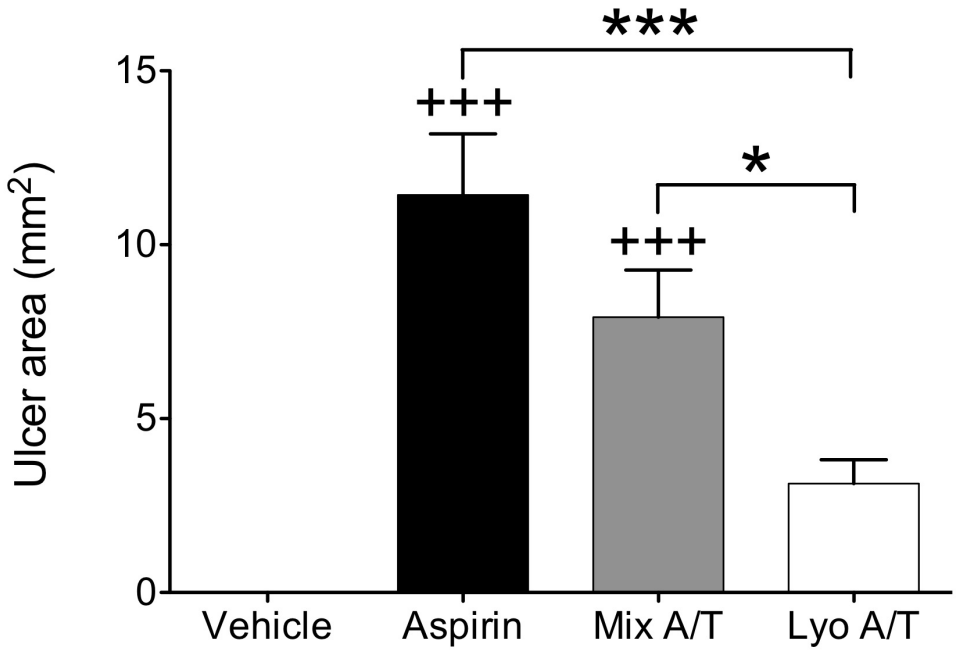

Fig. 2-2 Comparison of macroscopic injury of acute gastropathy in the rat 5 hours after the administration. The ulcer area of Lyo A/T group was significantly smaller than those of the aspirin and Mix A/T groups. Data represent the mean ± SE. *P< .05 and ***P< .001, as compared with the Lyo A/T group. +++P< .001, as compared with the vehicle group (ANOVA).

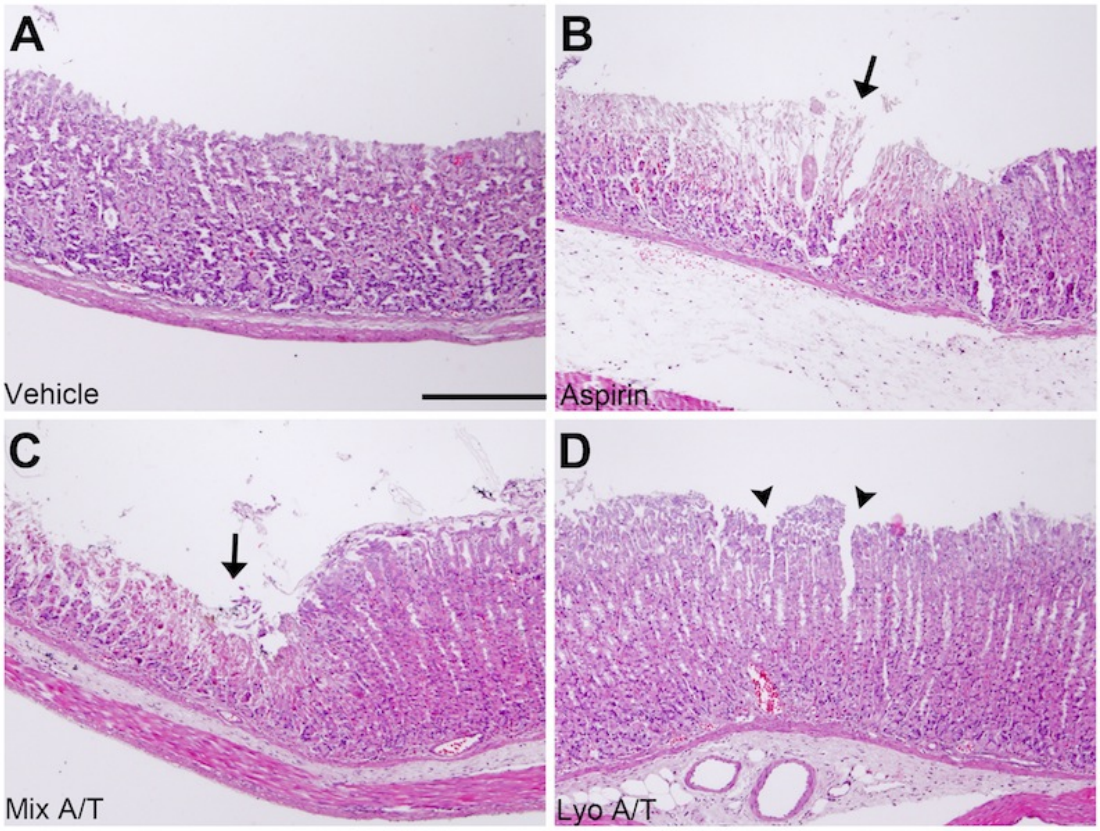

Fig. 2-3 Histological findings of rat gastric mucosa 5 hours after the administration. Representative photographs of the mucosa were shown in vehicle (A), aspirin (B), Mix A/T (C), or Lyo A/T (D). Extensive superficial epithelial damage and necrosis of gastric glands (arrows) were noted in the aspirin and Mix A/T groups, while mild superficial damage (arrow heads) was present in the Lyo A/T group. HE staining. Scale bar: 200μm.

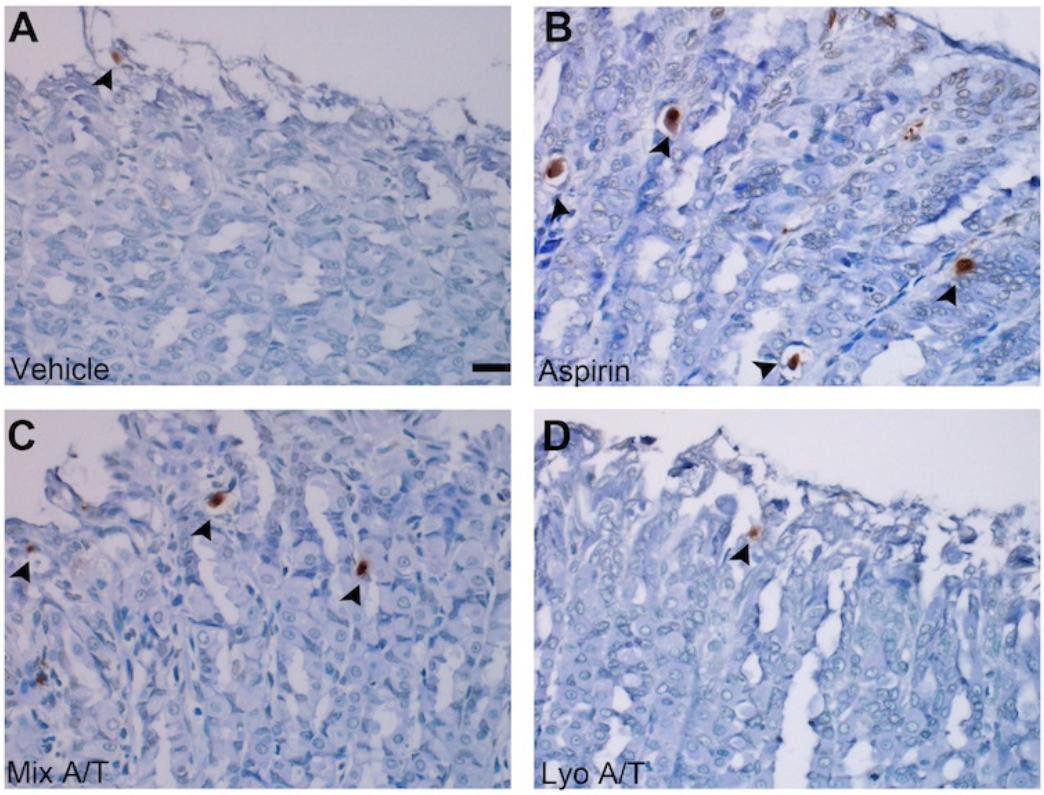

Fig. 2-4 Histological findings of rat gastric mucosa with staining of TUNEL assay counterstained with methyl green. Representative photographs of the mucosa were shown in vehicle (A), aspirin (B), Mix A/T (C), or Lyo A/T (D). TUNEL-positive cells (arrow heads) exhibited morphological characteristics indicative of apoptosis, including condensed of chromatin or shrinkage of nuclei. Scale bar: 20 μm.

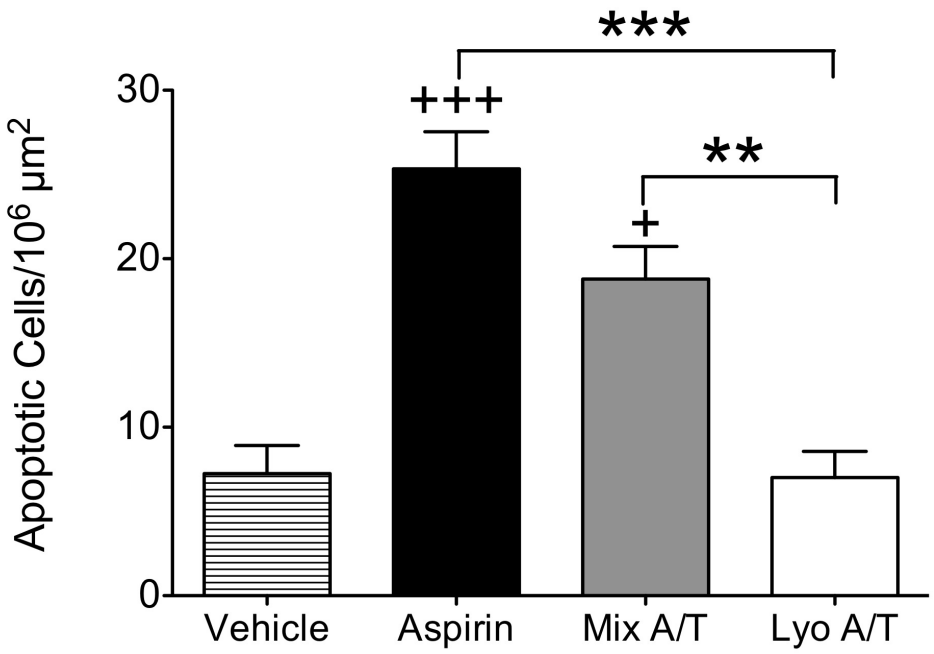

Fig. 2-5 Quantification of apoptosis in the rats 5 hours after the administration. The apoptotic cells (TUNEL-positive cells) of Lyo A/T were significantly less than those of the aspirin and Mix A/T groups. Data represent the mean ± SE. ***P*< .01 and ****P*< .001, as compared with the Lyo A/T group. +*P*< .05, +++*P*< .001 as compared with the vehicle group (ANOVA).

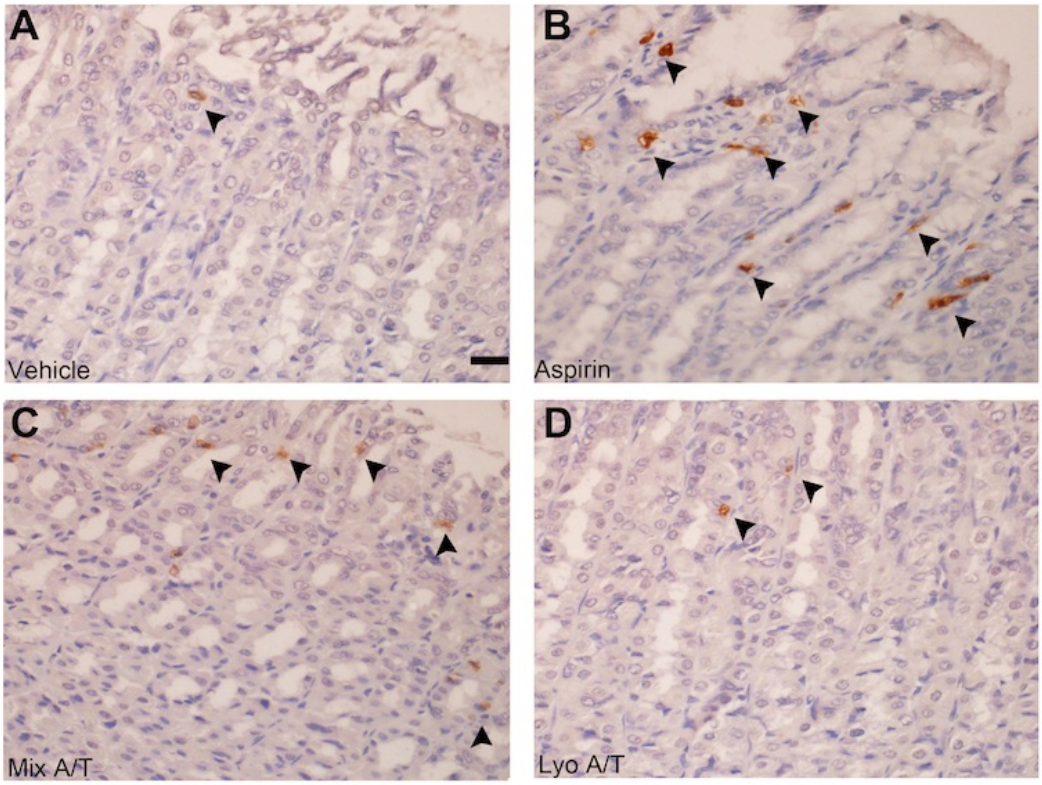

Fig. 2-6 Sections of rat gastric mucosa on cleaved caspase-3 immunohistochemistry. Cleaved caspase-3 (activated caspase-3) was present in the cytoplasm of cells with morphology consistent to apoptosis, as well as in the cytoplasm of morphologically healthy cells. Representative photographs of the mucosa were shown in vehicle (A), aspirin (B), Mix A/T (C), or Lyo A/T (D). The positive cells were indicated by arrow heads. Scale bar: 20 μm.

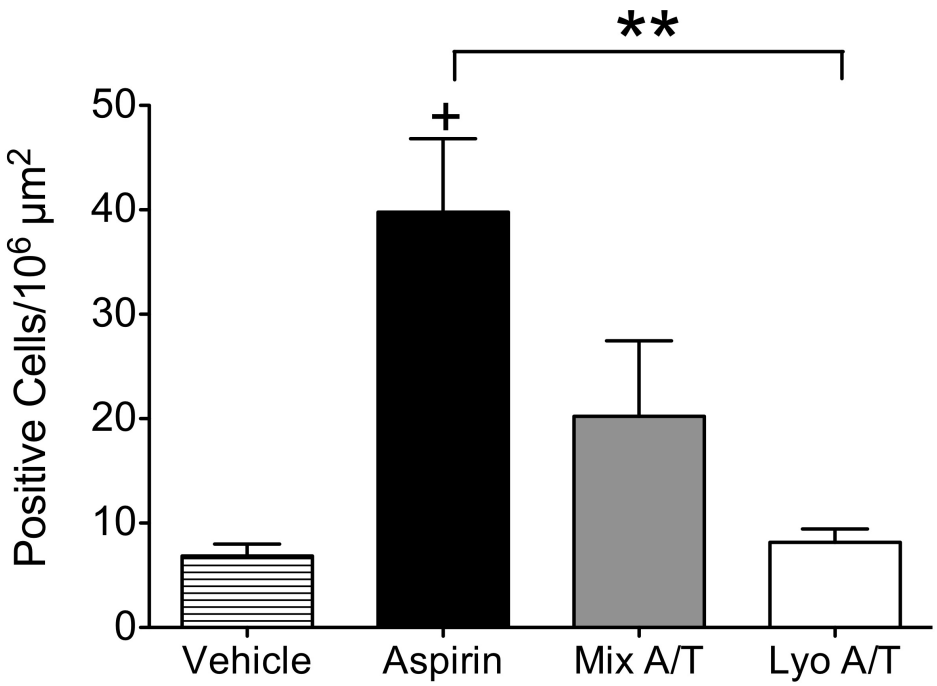

Fig. 2-7 Quantification of gastric cleaved-caspase-3 immunohistochemistry in the rat acute ulceration model 5 hours after administration. The cleaved-caspase-3-positive cells in the Lyo A/T were significantly less than those of the aspirin group. Data represent the mean ± SE. ***P*< .01, as compared with the Lyo A/T group. +*P*< .05, as compared with the vehicle group (ANOVA).

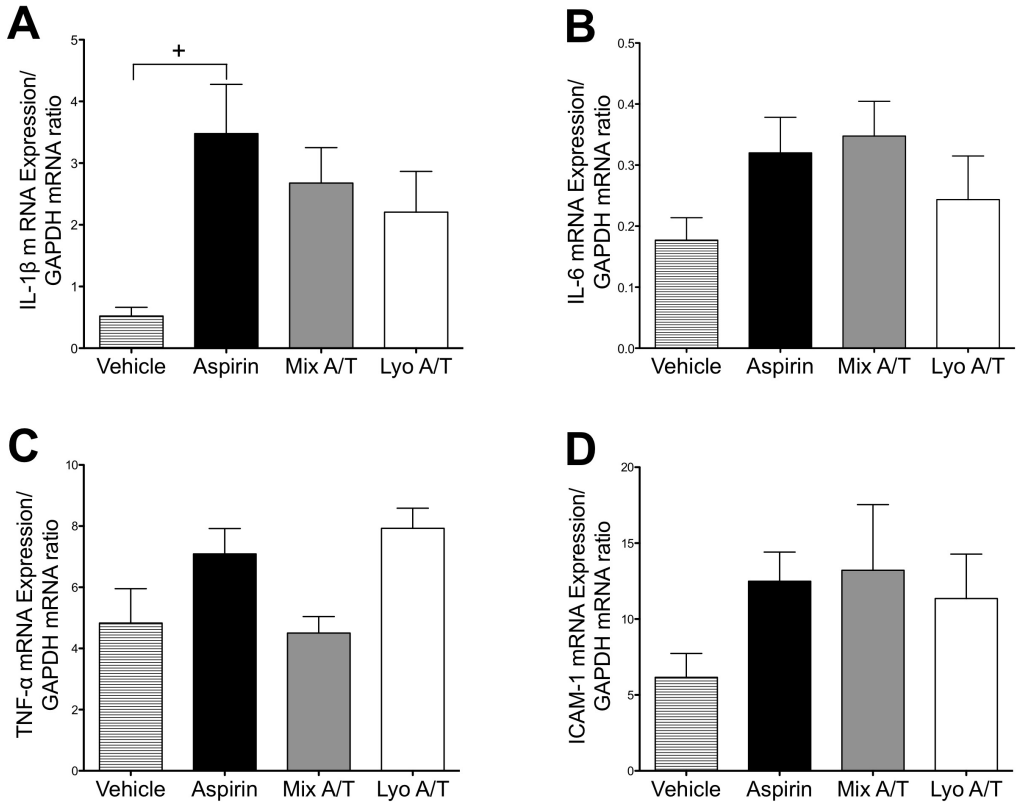

Fig. 2-8 Effects of different preparations on expressions of mRNA for (A) IL-1β, (B) IL-6, (C) TNF-α, and (D) ICAM-1 on the gastric mucosa 5 hours after administration. Data represent the mean ± SE. +$P<$ .05, as compared with the vehicle group (ANOVA).

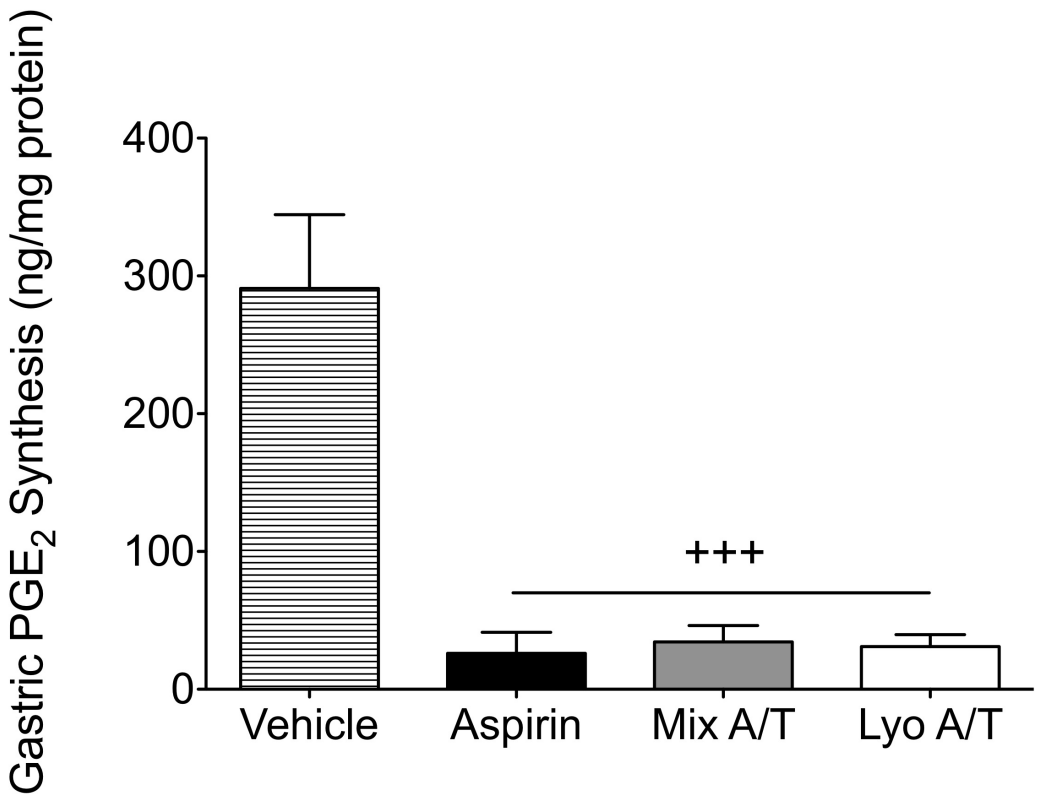

Fig. 2-9 Mucosal PGE_2 synthesis was profoundly suppressed in the aspirin, Mix A/T, and Lyo A/T groups compare to that of the vehicle group. Data represent the mean ± SE. +++P< .001, as compared with the vehicle group (ANOVA).

Chapter III

The *in vivo* Effects of Aspirin Preparations on Gastric Mucosa of Dogs

Introduction

Although NSAIDs have been frequently used for the treatment of musculoskeletal diseases in veterinary medicines, the prevalence of NSAID-induced gastrointestinal lesion in veterinary medicine has not been well clarified. Adverse effects of NSAID therapy recognized in dogs include gastrointestinal bleeding, ulceration, hepatopathy, and possible nephrotoxicity.[91, 92] Dogs are suggested to be more susceptible to NSAID-induced gastrointestinal toxicoses than humans. However, gastric ulcer formation resulting from NSAID administration in dogs is still controversial.[91, 93, 94]

More than ten years ago, aspirin was recorded as the most commonly used anti-inflammatory drug in companion animals,[95] however, it is not approved for use in the dog in US FDA.[96] A study also suggested that gastrointestinal erosion, ulceration, and hemorrhage should be considered as common sequela of therapeutic aspirin administration in dogs similar to the complications as in human.[93] Aspirin has been applied in many canine experimental research models, and results had shown the possibility to cause gastrointestinal bleeding endoscopically at a dosage of 25–35 mg/kg PO q8h.[97-100]

In cat, in one complete and in-depth review of NSAIDs was published by Lascelles and co-workers. Though some of NSAIDs have been used safely, cats are known to be more susceptible to the toxicity of NSAIDs because of slow clearance

and dose-dependent elimination.[101] Aspirin has been used at doses of 10 mg/kg every other day in cats.

Lyo A/T was proved to be lower cytotoxic and lower apoptosis-inductive *in vitro* in chapter 1, which was further confirmed in an acute rat gastric ulceration model in chapter 2. In this chapter, a canine ulceration model was employed to evaluate its effects on the gastric mucosa by a relatively long-term administration. Plasma concentrations of salicylic acid after a single administration were measured to evaluate whether the Lyo A/T had the same equivalent anti-inflammatory effect as aspirin alone.

Endoscopic Evaluation of Aspirin Preparations-induced Gastropathy and Anti-inflammatory Effects in Canine Ulceration Model

Materials and Methods

Chemicals and Reagents

All aspirin preparations were the same as in chapters 1 and 2. All preparations were prepared as a form of oil capsule to administer to dog. Aspirin preparations used in this chapter included aspirin and Lyo A/T.

Dogs for Ulceration Model

Thirteen healthy beagles (7 males and 6 females) aged from 1 to 3 years old were used in this experiment. They were on the basis of a normal physical examination, complete blood count (CBC), and serum biochemistry panel before the experiment. Commercial dry food for adult dogs (Hill's^{TM}, USA) was given twice daily and water was available *ad libitum* throughout the study.

They were randomly assigned to each of 3 treatment groups: 3 dogs in the control group, 5 dogs in the aspirin group, and 5 dogs in the Lyo A/T group. The dogs received oral administration of the following drugs every 12 hours before feeding for 28 consecutive days: placebo (oil capsules only) in the vehicle group, 25 mg/kg aspirin in the aspirin group, and 125 mg/kg (25 mg/kg aspirin) Lyo A/T in the Lyo

A/T group. Dogs were anesthetized for gastroscopy, and the photos of the gastric mucosa were taken and scored 7 days before drug administration (day -7), and 5, 14, 28 days after initiation of drug administration. CBC and serum biochemical analysis were also performed on days –7, 5, 14, and 28.

Clinical signs including appetite, activity, vomiting, and diarrhea were observed daily and recorded. One dog-day of vomiting was defined as 1 dog was vomiting on a certain day. Diarrhea or anorexia was also recorded similarly.

All experiments were performed in accordance with the guidelines of the Committee for Animal Care, Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Gastroscopy and Evaluation

After atropine sulfate (0.025mg/kg, SC) was premedicated, an intravenous catheter was placed and anesthesia was induced with propofol (6–8mg/kg, IV to effect). After intubation, anesthesia was maintained with isoflurane and oxygen. Routine gastroscopy was performed in left recumbency.

Upon entry into the stomach, air was insufflated and the endoscope was advanced along the greater curvature to reach the pyloric antrum. After examination of the pyloric antrum, the incisura angularis extending along the lesser curvature was examined by partially retroflexing the tip of the endoscope. Then total retroflexion

allowed visualization of the cardia, extending from the greater curvature to the portion of the lesser curvature, not including the incisura angularis. The tip of the endoscope was then straightened to observe the fundus. Each region of the stomach (pyloric antrum, incisura angularis, cardia, and fundus) was photographed by an endoscopic camera (Olympus CL-VU40D/VO-2A/VQ-8143A, Tokyo, Japan) and the gastric lesions were scored by an observer unaware of the treatment groups. Each gastric region was assessed and scored separately based on a previously used adaptation of the modified Lanza scale (Table 3-1).[97, 102, 103]

Submucosal hemorrhage was defined as small, reddened hemorrhagic area with intact mucosa. Erosion was defined as lesions with defect of the mucosal epithelium. Ulcer was defined as lesions with wide and volcano-like shape defects of the mucosa. If lesions were noted near the border of 2 contiguous regions, the lesion was assigned to the most appropriate region and care was taken not to score the lesion twice when evaluating the adjacent region. The scores for each region were summed and a total lesion score was assigned to each dog for each day of gastroscopy. Median scores were calculated for each group on each day of gastroscopy.

Dogs for Plasma Salicylic Acid Concentration Measurement

Another six healthy beagles (3 males and 3 females) from 1 to 3 years of age were randomly assigned into 2 groups of 3 dogs each. A single oral dose of 25 mg/kg

aspirin and 125 mg/kg (25 mg/kg aspirin) Lyo A/T was administered. Food was withheld for 12 hr before drug administration. Blood was collected from the cephalic vein at 1, 2, 4, 6, 8, 12, 16, 20, and 24 hours after the drug administration. To avoid further conversion from aspirin to salicylic acid, sample preparation for the measurement was performed within 1 hour after venipuncture.

Plasma Salicylic Acid Concentration Measured by High Performance Liquid Chromatography (HPLC)

Plasma salicylic acid levels were determined using reversed-phase HPLC according to the method described by Cham et al.[104] with a slight modification. The *p*-hydroxybenzoic acid methyl ester as an internal standard and an aqueous solution of hydrochloric acid and chloroform were added to the sample, which was then centrifuged at 800 g for 5 min. The lower organic phase was collected and evaporated. HPLC was conducted using a unit (JASCO DG-2080-53/PU-2080/UV-2075, Tokyo, Japan) with a Develosil Ph column (250 × 4 mm i.d., 5 μm particle size; Nomura Chemical Co., Aichi, Japan). The detection wavelength was 270 nm. The mobile phase consisted of methanol and phosphate buffer (1:3 v/v). The flow rate was 1 ml/min. Salicylic acid (Sigma Co., St Louis, MO, USA) was used as standard.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism software (version 5.0). Lesion scores were represented as median ± range and analyzed using a Kruskal-Wallis rank-sum test. Post hoc pairwise comparisons were made using a multiple comparison test based on Kruskal-Wallis rank sums. Lesion scores for the antrum, incisura angularis, cardia, and fundus within groups at each time point were compared similarly.

The Friedman test was used to compare the scores within a treatment group among each time points, and post hoc comparisons were made using a multiple comparison test.

The Kruskal-Wallis test was used to compare the number of dog-days of vomiting among groups. The dog-days of diarrhea and reduced appetite were not evaluated statistically because no days of diarrhea and reduced appetite were noted in any groups. A P value < .05 was considered significant for all statistical tests.

The Mann–Whitney U test was used to compare the peak plasma salicylic acid concentration and the area under curve (AUC) in the aspirin and Lyo A/T groups. Data represented as mean ± SE. A P value < .05 was considered statistically significant.

Results

Gastroscopy and Evaluation

Fig. 3-1 shows typical gastroscopic findings of 4 regions in the aspirin and Lyo A/T groups 5 days after administration. Multiple erosions (arrows) were present in the fundus, pyloric antrum, and cardia region (Fig. 3-1 A, C, and G, respectively) of a dog in the aspirin group. Several linear submucosal hemorrhages (arrow heads) were also present in the incisura angularis region in the aspirin group (Fig. 3-1 E). A linear submucosal (arrow head) hemorrhage was present in the fundus and pyloric antrum region (Fig. 3-1 B and D) of a dog in the Lyo A/T group. Less extent injury was observed in the Lyo A/T group compared to the aspirin group.

Fig. 3-2 shows the total lesion scores of three groups. No gastropathy was observed on day –7 in all three groups, with the median total lesion score of 4. The median total lesion scores of the aspirin group rapidly increased after administration to 21 (range 17–25) on day 5 and maintained high on day 14 (median 21, range 17–22). On the contrary, median total lesion scores of the Lyo A/T group were significantly lower than those of aspirin group on day 5 (median 6, range 4–15) and on day 14 (median 7, range 6–13). On day 28, the median total lesion score of the aspirin group (median 19, range 6–20) was still higher than that of the Lyo A/T group (median 8, range 4–9), however there was no significant differences between the two.

Fig. 3-3 shows the change in median lesion scores at each anatomical region. Scores of the aspirin group were significantly higher than those of the Lyo A/T group at the fundus and pyloric antrum during the whole treatment period (days 5, 14, and 28). Significant differences were also noted at the incisura angularis (days 5 and 14) and cardia (days 5 and 28) between the two groups. No significant differences were observed between the vehicle and Lyo A/T groups at any regions during the study (data not shown).

Only 3 dog-days of vomiting were noted in the aspirin group (1 dog accounted for 2 dog-days of vomiting), and the vomiting was self-limiting. No vomiting was noted in the dogs of the Lyo A/T and vehicle groups. There were no differences among groups in the number of dog-days of vomiting. No diarrhea, anorexia, or abnormal blood chemical profiles were noted in any of the dogs during the experimental period.

Plasma Salicylic Acid Concentration

The change in plasma salicylic acid concentration after oral administration of 25 mg/kg aspirin is shown in Fig. 3-4. Plasma salicylic acid level increased rapidly after administration and peaked 2 hours after administration. The mean peak plasma salicylic acid concentration was 55.6 ± 7.3 μg/ml in the aspirin group and 56.3 ± 1.5 μg/ml in the Lyo A/T group, and there was no significant difference between the two

groups. The area under curve (AUC) was 506.6 ± 31.8 in the aspirin group and 574.4 ± 87.9 in the Lyo A/T group, and there was no significant difference between the groups.

Discussion

In this *in vivo* canine model, a 28 day-designed canine ulceration model was employed to evaluate the effects of aspirin and Lyo A/T on gastric mucosa. Moreover, plasma concentrations of salicylic acid after a single administration of these two preparations were also measured to compare the anti-inflammatory effect between two.

In this study, total lesion scores in dogs receiving Lyo A/T were significantly lower than those in the aspirin group on days 5 and 14. However, on day 28, there was no significant difference between the two groups, though the score of the aspirin group was higher. This indicated that Lyo A/T might cause less gastric damage in the first two weeks of treatment.

Although there were no significant differences in lesion score at each time point within the aspirin group, lesion scores tended to decrease according to the period after administration. One possible explanation for the initial increase followed by a decrease in lesion scores is gastric adaptation to aspirin, in which gastric mucosa becomes more resistant to the cytotoxic effect of aspirin[105]. There have been reports on natural resolution of gastric lesions in dogs,[106, 107] while conflicting studies have reported that dogs receiving aspirin treatment for up to 28 days did not show this effect.[97, 102, 103]

In this study, I did not include the dogs receiving Mix A/T. This was because the previous studies using rats showed that the effect of Lyo A/T, and that Mix A/T did not decrease the severity of gastropathy. In addition, I would like to reduce the number of experimental dogs in this study.

Duodenal lesions were not evaluated in this study. NSAIDs have been shown to cause the development of duodenal erosions or ulcers in humans and dogs after aspirin administration.[3, 100, 102, 108, 109] In the preliminary experiment in which the duodenum was evaluated, minimal lesions were observed in this region after oral administration of 25 mg/kg aspirin every 12 hours. Furthermore, attempting entry of the endoscope into the duodenum might increase the risk of iatrogenic lesions and prolong experimental procedures. Therefore, I did not evaluate duodenal lesions in this study.

Examining 4 anatomical region separately, a predilection site for gastric lesions was not identified, and this observation is consistent with previous studies.[100, 102, 103, 109] However, a predilection site for NSAID-induced injury is controversial. Certain human studies,[110] veterinary clinical reports,[99, 111] and endoscopic studies in dogs[93] have reported that the pylorus is the region most prone to NSAID-induced gastric mucosal damage. However, there is a report[102] in which the highest lesion scores were obtained in the fundus and the lowest lesion scores were obtained in the pyloric antrum region.

Despite the significant differences in lesion scores, there were no corresponding clinical signs or abnormal laboratory data. Only few dogs in the aspirin group vomited but most of them did not show any severe gastrointestinal signs. Similar to other studies in which the investigators found gastrointestinal injuries without clinical consequences in a limited period (28 days), the significance of the lesions for clinical signs is unclear. Although vomiting was noted in certain dogs of the aspirin group in this study, appetite was unaffected and the signs did not correlate to the lesion scores. However, clinical signs might have been produced over a longer treatment period.

A plasma salicylic acid concentration of about 50 µg/ml is adequate for producing analgesic and antipyretic effects.[112, 113] In this study, the plasma salicylic acid concentrations in both the aspirin and Lyo A/T groups exceeded the therapeutic concentration (>50 µg/ml), and there was no significant difference between the groups. This result indicates that lyophilization of aspirin with trehalose does not alter the absorption of aspirin and that comparable analgesic and anti-inflammatory effects are expected in dogs. Although further clinical trials are warranted, the protective function of Lyo A/T might be speculated to be also effective on human gastric mucosa.

Table 3-1 Numerical value assigned to each stomach regions based on the following criteria as recognized on gastroscopy.

Score	Description
1	Normal
2	1 Submucosal hemorrhage
3	2-5 Submucosal hemorrhages
4	>5 Submucosal hemorrhages
5	1 Erosion
6	2-5 Erosions
7	>5 Erosions
8	1 Ulcer
9	2 Ulcers
10	3 Ulcers
11	Perforating ulcer

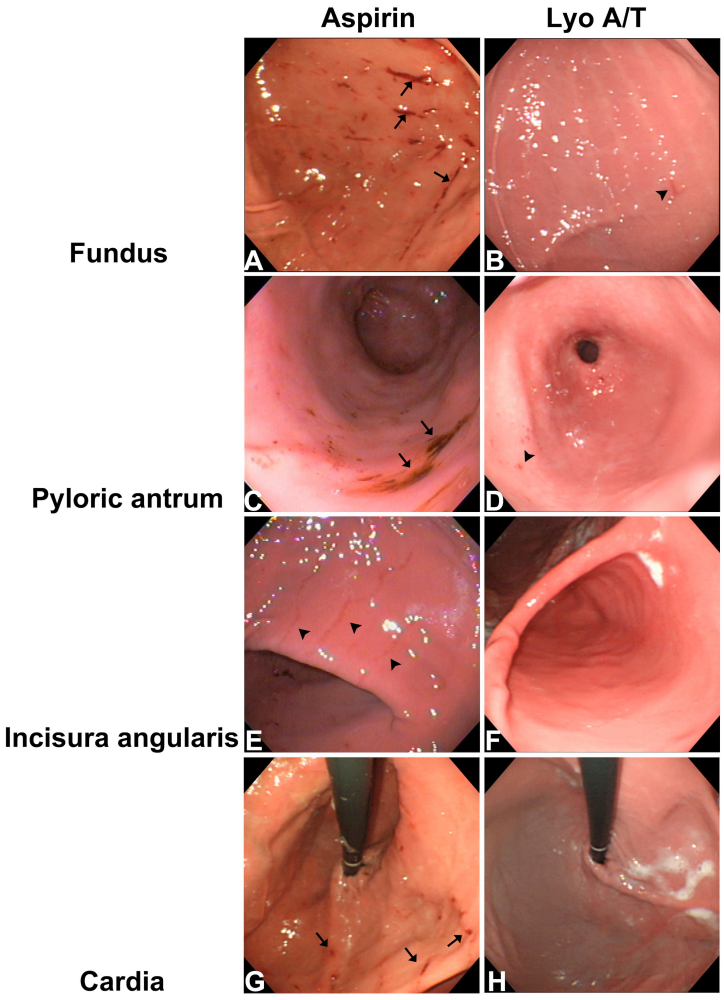

Fig. 3-1 Representative gastroscopic findings of the aspirin group (A, C, E, and G) and the Lyo A/T (B, D, F, and H) group 5 days after drug administration. Multiple erosions (arrows) were present in the fundus (A), pyloric antrum (C), and cardia region (G) of a dog in the aspirin group. Several linear submucosal hemorrhages (arrow heads) were also present in the incisura angularis region in the aspirin group (E). A linear submucosal hemorrhage (arrow head) was present in the fundus (B) and pyloric antrum region (D) of a dog in the Lyo A/T group.

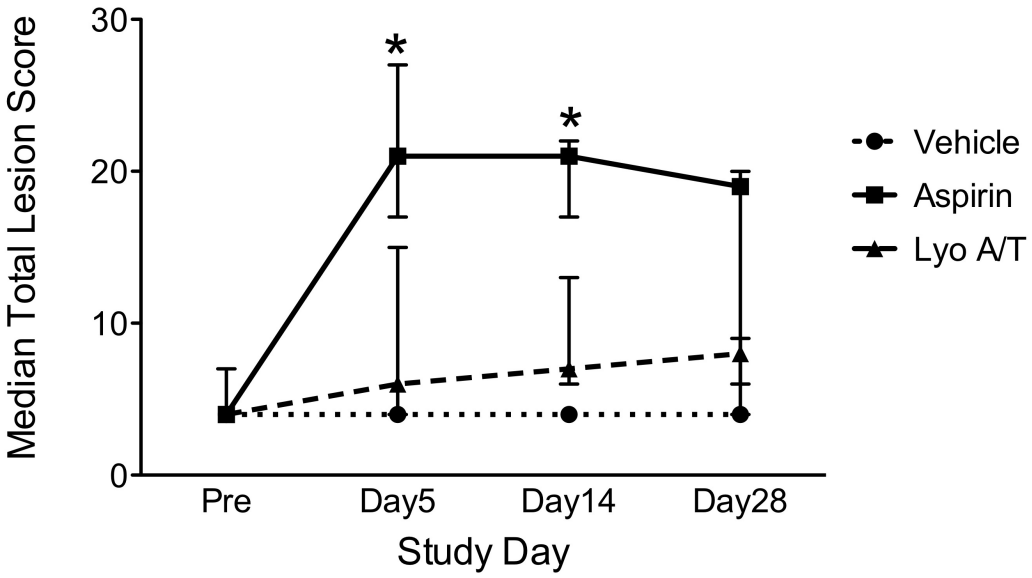

Fig. 3-2 Total lesion scores of the vehicle, aspirin and Lyo A/T groups. The scores of aspirin group were significantly higher than those of the vehicle and Lyo A/T groups on day 5 and 14. *P< .05, where the scores of the aspirin group were significantly higher compared with the Lyo A/T.

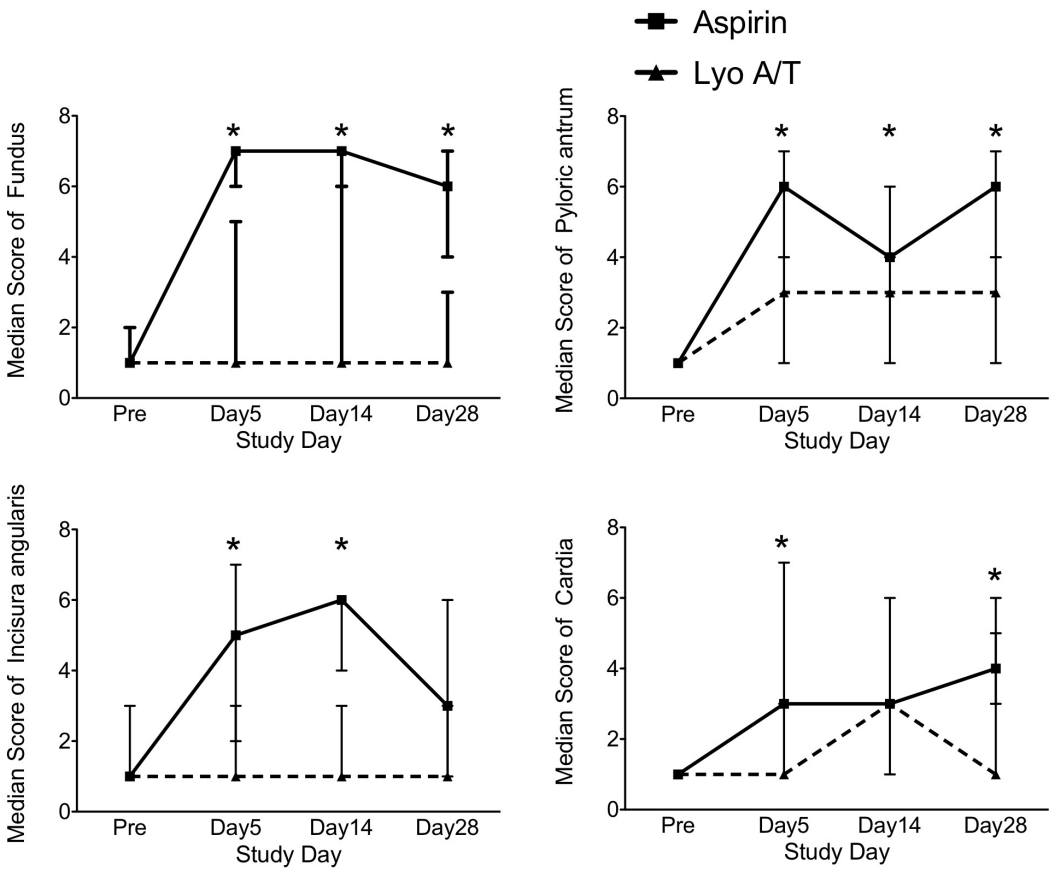

Fig. 3-3 Lesion scores of 4 regions. *P< .05, aspirin group compared with Lyo A/T group.

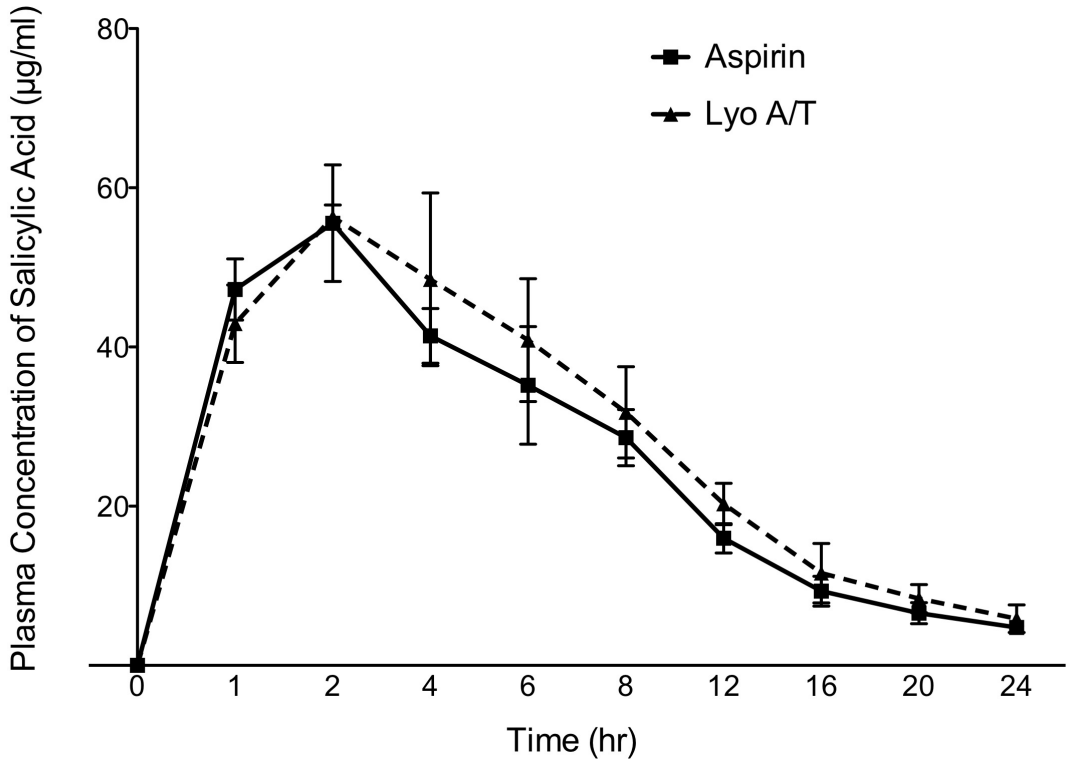

Fig. 3-4 Changes in plasma salicylic acid concentrations after 25 mg/kg aspirin preparations administration. There was no significant difference in the peak plasma salicylic acid concentration and area under curve (AUC) between the aspirin and Lyo A/T groups.

Conclusion

Aspirin might be the most popular NSAID worldwide for its anti-inflammatory and analgesic effect, however, the gastrointestinal injury remains a major complication of aspirin. Aspirin has long been considered to cause gastric injuries through inhibition of COX enzymes; however, studies have increasingly shown that not only COX inhibition but also direct irritation to the mucosa are required for the production of gastric lesions. Both in *in vitro* and *in vivo* models[48, 85, 114], membrane permeabilization activity of drugs may be involved in the production of necrosis and apoptosis of gastric mucosal cells. Thus, molecules that protect the integrity of mucosal cells and have anti-apoptotic properties were applied to prevent aspirin-induced gastric injuries.

According to our previous reports and other, we found that trehalose exerts cell protective and anti-inflammatory effects against many stress condition, including desiccation, dehydration, heat, cold, and oxidation. Hence, it was considered to be a potential therapeutic material for various diseases. We hypothesized that a combination usage of aspirin and trehalose by co-lyophilization (Lyo A/T), which may alter the hydrogen bond interaction, might decrease the incidence of aspirin-induced gastric injury.

The author first examined the effects of different aspirin preparations on gastric cells by utilizing AGS cells. Aspirin, Lyo A, Mix A/T and Lyo A/T were employed in the first experiment. After exposure of AGS cells to different

preparations, low cytotoxicity and low apopotosis-inducing potency of Lyo A/T compared with other preparations was determined. Neither mixture of aspirin with trehalose nor lyophilization of aspirin did alter the cytotoxicity and apoptotic rate than that of aspirin. This indicated that only co-lyophilization of aspirin with trehalose enabled the protective capacity.

In chapter 2, the effects of different aspirin preparations on gastric mucosa were evaluated in a rat acute gastric ulceration model. Lyo A/T significantly induced less gastropathy than aspirin alone and Mix A/T, and maintained the inhibition ability of mucosal prostaglandins synthesis. This indicated 1): Lyo A/T has the equivalent anti-inflammatory effect with aspirin, and 2): Lyo A/T does counteract mucosal injuries caused by decreased prostaglandins synthesis through inhibition of other pathogenesis of aspirin-induced gastropathy. Further experiment of the rat model, in which *in situ* TUNEL assay and cleaved caspase-3 immunohistochemistry were utilized as apoptosis indicator, proved that Lyo A/T exhibited low apoptosis-inducing potency as well as discovered previously in the *in vitro* study. However, there were no specific differences of inflammatory mediator genetic expression between each treatment.

Combining the *in vitro* and *in vivo* data together, it was speculated that Lyo A/T exhibited less gastric mucosal injuries might mainly through the inhibition of caspase-mediated apoptosis pathway.

In chapter 3, the author investigated the effects of aspirin and Lyo A/T on gastric mucosa in a canine ulceration model. Twenty-eight days consecutive administration and gastroscopic evaluation revealed that Lyo A/T caused less gastropathy than aspirin did. Moreover, the plasma concentration of salicylic acid revealed both aspirin and Lyo A/T could reach effective therapeutic concentration after administration. This result indicated that Lyo A/T might also be safer NSAID than traditional aspirin for companion animals, and suggested the possible usage in human medicine.

In summary, Lyo A/T clearly reduced the gastric mucosal damage induced by aspirin without losing anti-inflammatory effects. Trehalose is a natural disaccharide and is a widely used food ingredient due to low price and minimal side effects. Hence, the production of co-lyophilized aspirin with trehalose would be offered at a lower price than other therapeutic materials employed for the reduction of gastropathy. Although further research to clarify the anti-apoptotic mechanism and structural analysis are warranted, Lyo A/T might be a solution to decrease aspirin-induced gastropathy in both veterinary and human medicine.

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