Studies on Canine Gastric Motility Disorders and the Clinical Application of 5-HT4 Receptor Agonist Mosapride

（犬の胃運動障害および 5-HT4 受容体作動薬モサプリドの臨床応用に関する研究）

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General Introduction

Upper gastrointestinal (GI) clinical symptoms, such as anorexia, emesis, and vomiting are most common clinical problems which confronted in both human and veterinary medicine. The pathogenesis of these clinical symptoms mainly derived from the abnormal stimulation of vomiting or appetite center, the damage of GI mucosa, and GI motility disorders. Among the pathogenesis, GI motility disorder, particularly gastric motility disorder, constitutes an important part of clinical GI practice in humans (Ali et al., 2007). Gastric motility disorder is associated with various physiological, pathological, and pharmacological conditions, which may cause gastric regurgitation or emptying disorders, leading to the onset of upper GI clinical signs. Recent human studies enabled to assess the gastric motility on clinical setting and revealed the pathogenesis of gastric motility disorders and its relevance with upper GI clinical symptoms (Kusunoki et al., 2000; Schmidt et al., 2008). For example, gastric motility disorders are associated with GI clinical symptoms in patient with functional dyspepsia, diabetes, hypothyroidism, cancers, or patients received GI surgical intervention (Nelson et al., 1993; Maes et al., 1997; Ali et al., 2007; Sogabe et al., 2008). Gastric motility disorders are also associated with GI adverse events of drugs such as opioids, anti-cholinergic agents, or anti-cancer agents (Tomomasa et al., 1999; Greenwood-Van Meerveld, 2007). These gastric motility disorders are therapeutic target with prokinetic agents.

Prokinetic agents are drug which promotes or regulates the GI motor activity, and used for the management of GI clinical symptoms associated with GI motility disorders.

Several gastroprokinetic agents have been developed, which include dopamine receptor antagonists (eg; metoclopramide and itopride), motilin receptor agonists (eg; erythromycin and alemcinal), serotonergic agents (eg; cisapride, mosapride, and prucalopride) (Karamanolis et al., 2006) and opioid receptor antagonists (eg; alvimopan). In human medicine, the demand of gastroprokinetic agents is high because GI motility disorder is one of the major cause of GI clinical symptoms (Sturm et al., 1999). Therefore, many of novel prokinetic agents are still under developing (Karamanolis et al., 2006).

In veterinary field, the pathogenesis of gastric motility disorders and its relevance with upper GI clinical signs remained unclear, despite the high incidence of these clinical signs. Although some of prokinetic agents, like metoclopramide, have been applied to the management of GI clinical symptoms (Hall et al., 1999), there are few reports which demonstrates the efficacy of prokinetic agents on gastric motility disorders in dogs. One of the reasons for the lack of evidence is the methodological limitation for the assessment of gastric motility.

The second-generation 5-hydroxitriptamine-4 receptor (5-HT_4R) agonist mosapride is representative prokinetic agents in humans, which enhances the motility of smooth muscle by the promotion of acetylcholine in enteric nerve following serotonergic nerve stimulation (Karamanolis et al., 2006; Curran et al., 2008). Since first-generation 5-HT_4R agonist cisapride was withdrawn from market due to its cardiac adverse reaction, mosapride is used to the treatment of several GI motility disorders including functional

dyspepsia, irritable bowel syndrome, and gastric esophageal reflux disease in humans (Curran et al., 2008). As mosapride has high affinity on 5-HT_4R in gastrointestinal tract and no effect in the serotonergic receptor of central nervous system, mosapride mediates prokinetic action with low incidence of side effect (Yoshida et al., 1989). It is demonstrated that mosapride also mediates prokinetic action in dogs. Previous report indicated that intravenous administration of mosapride enhances the upper gastrointestinal motility in healthy dogs (Yoshida et al., 1991). However, its dose determination in dogs has not been accomplished, and like other prokinetic agents in dog, the efficacy on gastric motility disorders have not been investigated yet. Other than prokinetic action, recent experimental animal findings suggest the novel action of mosapride. Previous rodent studies indicated that mosapride shows anti-ulcerogenic and anti-inflammatory action on by activating the acetylcholine release following 5-HT_4R activation (Fujisawa et al., 2010; Tsuchida et al., 2011). These studies suggest the novel clinical application of mosapride in patients with gastric ulcer, or inflammatory diseases. However, to demonstrate the clinical significance of these actions, it is needed to investigate in larger species.

Studies on this thesis were carried out to assess the pathogenesis of gastric motility disorders and present the efficacy of mosapride in dogs. Study of chapter 1 in this thesis was aimed to establish the postprandial gastric motility assessment, which enable us to assess the gastric motility in real-time with minimum invasion. In chapter 2, the

prokinetic action of oral mosapride in dogs was investigated in terms of gastric antral motility and solid-phase gastric emptying. In chapter 3, I evaluated the association of gastric motility disorder in drug-induced adverse reaction of prednisolone and vincristine, which have clinical problems as the high incidence of GI adverse effect in both humans and dogs. The efficacy of mosapride was also investigated in these adverse effects. In chapter 4, I assessed anti-inflammatory action of mosapride which focused on the function of 5-HT_4R in immune cells. These works aimed to contribute on the assessment and treatment of GI clinical signs associated with gastric motility disorders in veterinary field. Because dog is appropriate experimental model of GI studies, present study is also aimed to be served as the preclinical study for the novel clinical application of mosapride not only in dogs but also in humans.

Chapter 1

Real-time Ultrasonographic Method of Canine Gastric Motility in Postprandial State

Abstract

Gastric motility is affected by several pathological conditions which may induce upper gastrointestinal (GI) clinical symptoms. The pathogenesis of canine gastric motility disorders is poorly understood because of methodological limitations. This study aimed at establishing a simple method of canine postprandial gastric motility using ultrasonography. Seven healthy beagles were used in this study. The motility index (MI), an indicator of gastric antral motility, was calculated by measuring the area of the gastric antrum in both a contracted and relaxed phase and by counting the number of contractions. The MI was measured every 30 min for 3 hr after feeding in the 7 dogs and compare the coefficient of variance (CV) in each time point. Gastric emptying analysis ^{13}C-octanoic acid breath test was concurrently performed for the comparison of MI and gastric emptying time. The ability of the ultrasonographic to detect gastric hypomotility was confirmed with atropine-induced gastric hypomotility model. The MI at 30 min had the lowest variability in the 7 dogs (mean ± SD, 9.77 ± 0.42; CV, 4.25%), and a significant correlation was observed with half-emptying time (R^2 = 0.654, P = 0.027) and gastric emptying coefficient (R^2 = 0.8126, P = 0.005). When atropine was administered, all dogs showed MI decrease (5.19 ± 0.22), compared with that of control (9.77 ± 0.42, P = 0.0003). In conclusion,

ultrasonographic evaluation of MI at 30 min served as simple method for the assessment of canine postprandial gastric motility.

Introduction

Gastric motility disorders are caused secondary by several diseases or drug administration and may associate with the pathogenesis of upper GI clinical symptoms such as anorexia, vomiting, abdominal pain, and emesis. Therefore, assessment of gastric motility function provides usable information for the management of these GI clinical symptoms. Several methods for evaluating gastric motility in both humans and dogs have been described (Wyse et al., 2003; Schmidt et al., 2008). Gastric motor activity has 2 characteristic phases: fasting and the postprandial phase. Many of the previous studies have focused on the postprandial phase. Postprandial gastric motility is assessed either by quantifying gastric emptying time and/or gastric antral motility. Gastric emptying can be evaluated using scintigraphy (Akkermans et al., 1994; Iwanaga et al., 1998), ^{13}C-octanoic acid breath test (Lester et al., 1999; Delbende et al., 2000; Wyse et al., 2001), or radiopaque markers (Lester et al., 1999; Stotzer et al., 1999), whereas gastric antral motility is examined using force transducers (Itoh, 1977) or abdominal ultrasonography (Fujimura et al., 1994; Choi et al., 2002; McLellan et al., 2004; Chalmers et al., 2005).

Of these gastric motility methods, scintigraphy is considered the gold standard for evaluating gastric emptying in both humans and dogs (Parkman et al., 1995;

Wyse et al., 2003). However, it requires a specialized facility and exposure to radiation. The ^{13}C-octanoic acid breath test is an indirect method of assessing gastric emptying using a non-radioisotope-labeled substrate. The test involves monitoring the rate of $^{13}CO_2$ in expired air following ingestion of a test meal mixed with ^{13}C-octanoic acid. The ^{13}C-octanoic acid breath test has also been established as a method for assessing solid-phase gastric emptying in dogs (Wyse et al., 2001), cats (Peachey et al., 2000), and horses (Wyse et al., 2001). Although the ^{13}C-octanoic acid breath test has an advantage over scintigraphy in that it can be performed without exposure to radiation, it requires 6 hr of repeated collection of expired air samples. Because of the limitations in these methods, gastric motility disorders in dogs are poorly understood, and a more simple assessment method is required for use in clinical settings.

Ultrasonography is a noninvasive method that allows the veterinarian to assess gastric motility at the bedside. Ultrasonographic evaluation of gastric motility in humans has been applied in clinical settings, and several clinical trials have been performed to estimate the pathogenesis of gastric motility disorders in a large scale (Fujimura et al., 1994; Kusunoki et al., 2000; Sogabe et al., 2008; Kusunoki et al., 2010). Previous reports have described the ultrasonographic assessment of gastric motility or emptying in dogs (Choi et al., 2002; McLellan et al., 2004). In one of the studies, McLellan et al. determined the canine gastric emptying time by using

ultrasonography to measure the area of the stomach (McLellan et al., 2004). However, this requires making continued measurements over a period of 6 hr. Choi et al. used ultrasonography to investigate liquid-phase gastric motility by evaluating gastric contraction and area (Choi et al., 2002). However, liquid-phase gastric motility assessment may not be sensitive enough to detect the motility abnormality, compared with solid-phase gastric motility assessment, because solid-phase gastric motility reflects more physiological condition during feeding (Parkman et al., 1995; Wyse et al., 2003) In addition, they did not compare their results with those found by other methods (Choi et al., 2002). To assess the gastric motility in daily clinical practice, further simple solid-phase gastric motility assessment will be required.

The aim of the present study was to establish a novel method for assessing canine gastric motility in the postprandial state using ultrasonography. To determine the validity of the method, I investigated its correlation with gastric emptying as assessed by ^{13}C-octanoic acid breath test. I also used an atropine-induced gastric hypomotility model to examine the ability of this new method to detect gastric hypomotility.

Materials and Methods

Study design

First, ultrasonography and the ^{13}C-octanoic acid breath test were performed simultaneously on 7 healthy beagles. Gastric motility was ultrasonographically evaluated every 30 min for 3 hr after feeding. The motility index (MI), an indicator of gastric antral motility, was calculated at each time point. Variability in the MI at each time was estimated to determine the appropriate time of measurement. Correlation with gastric emptying parameters of the ^{13}C-octanoic acid breath test was then investigated. Next, ultrasonography was performed in the same 7 dogs by using an atropine-induced gastric hypomotility model to confirm the ability of the ultrasonographic method to detect gastric hypomotility.

Animals

Seven healthy beagles were used in this study. The dogs ranged in age from 3 to 5 years (mean: 3.8 years). The body weight of the dogs ranged from 9.4 to 13.0 kg (mean: 11.5 kg). Four dogs were male and 3 were female. No dog showed clinical signs of diarrhea, constipation, vomiting, anorexia, or weight loss. Blood tests (complete blood count, blood urea nitrogen, creatinine, alkaline phosphatase, and alanine aminotransferase) showed no abnormalities. All dogs were fasted for at least

12 hr prior to the study. Experiments and animal care procedures were approved by the Animal Use and Care Committee of the University of Tokyo.

Test meal

The test meal consisted of 10 g/kg body weight of commercial wet food (SPECIFIC® CIW, Intervet Schering-Plough Animal Health, Tokyo, Japan), 1 baked egg yolk, and 50 mg ^{13}C-sodium octanoate (Cambridge Isotope Laboratories, Inc., Andover, MA, USA). After the ^{13}C-octanoate was dissolved in the egg yolk, the wet food was mixed in. The test meal was baked in a microwave to increase retention in the solid phase.

Ultrasonography

Postprandial gastric antral motility was assessed according to a technique described previously in humans (Hausken et al., 1991; Kusunoki et al., 2000). Gastric antral motility was assessed using ultrasonography (ProSound SSD-5000 SV, Aloka Co., Ltd., Tokyo, Japan) with a 7.5-MHz phased array sector transducer. The ultrasonography was performed by a single operator. Dogs were restrained in the right recumbent position, and the probe position was adjusted to obtain maximum visualization of transverse image of the gastric antrum close to the left lobe of the liver. The cross-section of antral area was measured by tracing the serosal side of the antrum with the built-in caliper (Figure 1). The antral area was measured 3 times in

both the contracted and relaxed phases. The number of contractions in 3 min was counted. Amplitude (%) was calculated with following formula: (mean area relaxed - mean area contracted)/mean area relaxed. Frequency was defined as the number of antral contractions in 3 min. Motility index (MI), an indicator of gastric motility, was expressed as amplitude multiplied by frequency. Continuous calculation of the MI was performed before feeding and every 30 min after feeding for 3 hr.

^{13}C-octanoic acid breath test

The ^{13}C-octanoic acid breath test was performed according to previous reports in dogs (Wyse et al., 2001; McLellan et al., 2004). A breath bag (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was connected to a plastic mask, and expired air was collected from dogs through the mask. Breath samples were collected before giving the test meal as a baseline, every 15 min after feeding for 4 hr, and every 30 min for additional 2 hr. The breath samples were analyzed using a $^{13}CO_2$-infrared spectrophotometry analyzer (POC one, Otsuka Pharmaceutical), which was previously used to assess gastric emptying in horses (Okamura et al., 2008). The amount of ^{13}C in the samples was expressed as the change ($\triangle^{13}CO_2$, ‰) in the $^{13}CO_2/^{12}CO_2$ ratio before and after feeding of the test meal. Resting $^{13}CO_2$ production was assumed stable at 0.194 *l*/(m^2・min) (Wyse et al., 2001). Body surface area was calculated using the following formula: body surface area (m^2) = 10.1 × body weight

$(g)^{2/3}/10000$ (Orr et al., 1975). ^{13}C excretion rate (^{13}C %dose/hr) was calculated according to Ghoos (Ghoos et al., 1993) by fitting the following formula: $y = at^b e^{-ct}$ (y, %dose/hr; t, time; a, b, and c, constants; and e, exponential). Cumulative ^{13}C recovery in the breath (C%D, %dose) was determined using the following formula: $C\%D = m(1 - e^{-kt})^{\beta}$ (t, time; k, m, and ß, constants). Lag phase (t_{lag}), the gastric emptying coefficient (GEC), and half-emptying time ($t_{1/2}$) were regarded as gastric emptying parameters. t_{lag}, $t_{1/2}$, and GEC were calculated using the following formulae: $t_{lag} = b/c$, $t_{1/2} = (-1/k) \times \ln(1-2^{-1/\beta})$, and GEC = ln (a). The t_{lag} is time of the maximum emptying speed of the substrate. GEC is an index of gastric emptying rate. The $t_{1/2}$ is estimated time during which half of the total $^{13}CO_2$ is excreted. These parameters were calculated using excel program software.

Evaluation of atropine-induced gastric hypomotility by ultrasonography

Ultrasonography was performed on the same 7 dogs with atropine-induced gastric hypomotility. Atropine (atropine sulfate, Mitsubishi Tanabe Pharma, CO., Ltd., Osaka, Japan) was administered intramuscularly at a dose of 0.04 mg/kg just before giving the meal. The dose and route of administration were set according to a previous report that confirmed atropine-induced gastric antral hypomotility in healthy beagles (Burger et al., 2006). Ultrasonography was performed 30 min after

feeding, and the MI was calculated. The results were compared with those obtained without atropine administration (control).

Statistical analysis

The coefficient of variance (CV) was calculated for the MI at each time point to determine the time point at which the variance in the MI was lowest. The correlation between the MI and gastric emptying parameters (t_{lag}, $t_{1/2}$, and GEC) as determined by the ^{13}C-octanoic acid breath test was compared using Pearson's correlation coefficient. The MI of atropine-induced gastric hypomotility was compared with that of the control using paired *t*-test. All data were expressed as mean ± SD. Significance was set at $P < 0.05$.

Results

All dogs were able to take part in all studies. Cross-sectioning of the gastric antrum revealed a round shape close to the left lobe of the liver. Although there was a tendency for gastric gas to increase after 90 min of feeding, all gastric antrum were well visualized. The mean ± SD of amplitude (%) for the 7 dogs was 33 ± 11 before feeding and 61 ± 4 at 30 min, 59 ± 4 at 60 min, 61 ± 6 at 90 min, 51 ± 7 at 120 min, 47 ± 10 at 150 min, and 49 ± 11 at 180 min after feeding. The mean ± SD of frequency (number / 3 min) was 9.14 ± 4.81 before feeding and 16.57 ± 0.53 at 30 min, 16.86 ± 0.9 at 60 min, 16.29 ± 0.76 at 90 min, 15.57 ± 1.51 at 120 min, 15.14 ± 1.77 at 150 min, and 15.57 ± 1.4 at 180 min after feeding. The time course of the MI is shown in Figure 2. The mean ± SD of MI for the 7 dogs was 3.05 ± 2.77 before feeding and 9.77 ± 0.42 at 30 min, 9.90 ± 0.86 at 60 min, 9.85 ± 1.1 at 90 min, 7.92 ± 1.51 at 120 min, 7.32 ± 1.76 at 150 min, and 7.32 ± 1.76 at 180 min after feeding. The mean CV (%) of the MI for each time point was 90.92 before feeding and 4.25 at 30 min, 8.64 at 60 min, 11.14 at 90 min, 19.03 at 120 min, 25.41 at 150 min, and 24.6 at 180 min after feeding. The mean MI was at its peak at 30 min. The CV of the MI was the lowest at 30 min.

The mean ^{13}C-octanoic excretion curve for the 7 healthy dogs is shown in Figure 3. The mean ± SD of t_{lag}, $t_{1/2}$, and GEC were 81 ± 23.3 min, 110 ± 22.4 min, 4.28 ± 0.43

respectively. The MI tended to be negatively correlated with t_{lag} (R^2 = 0.472, P = 0.087; Figure 4A). The MI at 30 min after feeding was negatively correlated with $t_{1/2}$ (R^2 = 0.654, P = 0.027; Figure 4B). Furthermore, there was a positive correlation between the MI at 30 min and GEC (R^2 = 0.812, P = 0.005; Figure 4C).

MI was also evaluated in the 7 dogs with atropine-induced gastric hypomotility and compared with that of dogs that were not administered atropine (control). The mean ± SD of MI at 30 min after atropine administration was 5.19 ± 0.22. In all dogs, the MI decreased compared with control (9.77 ± 0.42) and this decrease was statistically significant (P=0.0003; Figure 5).

Discussion

In the present study, I established the ultrasonographic method for evaluating postprandial gastric motility in dogs. There was a significant correlation between the MI of antral contractions and gastric emptying as assessed by the ^{13}C-octanoic acid breath test. Furthermore, gastric hypomotility induced by atropine was detected by this method. These results indicate that the assessment of MI at 30 min after feeding is useful in screening postprandial gastric motility in dogs.

In the present method, gastric antral amplitude and frequency were evaluated ultrasonographically by observing real-time gastric movement. Amplitude, or the intensity of antral contraction, was estimated from changes in the antral area during contractions, whereas frequency was determined by the number of contractions. The MI, an indicator of gastric antral motility, reflects both the frequency and intensity of contractions. To reduce the degree of technical variability in the experiment, I measured antral area 3 times and defined the average as the amplitude. Although the use of force transducers or myograms provides more accurate information about antral motor activity, the ultrasonographic method can screen for antral motility with minimum invasion in dogs.

In this study, the MI was ultrasonographically measured every 30 min for 3 hr to compare variability at each time point. The variability of MI was lowest at 30 min

after feeding among the measurement time points. In a human report, difference in MI between cases with functional dyspepsia, which is known to cause gastric hypomotility, and healthy volunteers is prominent in the early postprandial state (Kusunoki et al., 2000). Therefore, 30 min after feeding is an appropriate time point at which to detect gastric antral motility abnormalities, because this time point is in the early postprandial phase and shows the lowest variability in healthy dogs. The method described here will enable clinicians to assess postprandial gastric antral motility more simply in a short time compared to previous methods described in dogs (Choi et al., 2002; Wyse et al., 2003; Chalmers et al., 2005).

The present study investigated the correlation between postprandial antral motility as evaluated by ultrasound and gastric emptying as assessed by the ^{13}C-octanoic acid breath test to confirm the validity of the ultrasonographic method. Gastric emptying is regulated by the tonic contraction of the proximal stomach, antral contraction, and the inhibitory forces of pyloric and duodenal contraction. Among them, gastric antral motility is regarded as one of the main regulators of gastric emptying of solid meals (Kelly, 1980). A previous report described that emptying rates for the entire gastric contents are correlated with antral motor activity during the active emptying phase for solids in humans (Camilleri et al., 1985). I adopted the ^{13}C-octanoic acid breath test as a comparative test because this method correlates with a gold standard of gastric emptying, scintigraphy, in humans

(Chen et al., 2003). The results showed that the MI at 30 min was negatively correlated with $t_{1/2}$, or the time of gastric emptying, and positively correlated with GEC, an indicator of gastric emptying rate (Ghoos et al., 1993). These results indicate that the MI of antral contraction is correlated with gastric emptying, demonstrating the validity of the ultrasonographic method.

In general, gastric hypomotility is clinically important and may be a therapeutic target of gastroprokinetic agents. Therefore, I confirmed the ability of the ultrasonographic method to detect atropine-induced gastric hypomotility. Atropine is a competitive antagonist for the muscarinic acethylcholine receptor, which acts on postganglionic parasympathetic neuroeffector sites and inhibits peristalsis in the GI tract in dogs (Burger et al., 2006). An obvious decrease in the MI was observed in all dogs after atropine administration, indicating the validity of the ultrasonographic method for detecting gastric hypomotility in dogs.

Gastric motility is affected by many physiological, pharmacological, and pathological conditions, and abnormalities in gastric motility may be associated with upper GI clinical symptoms (Hall et al., 1999; Ali et al., 2007). In humans, many reports have investigated the effect of gastroprokinetic agents in the treatment of gastric motility disorders (Sturm et al., 1999; Karamanolis et al., 2006; Reddymasu et al., 2009). A better understanding of the pathogenesis of gastric motility will contribute to the management of upper GI clinical symptoms. Among the various

methods of assessing gastric motility or emptying, the advantage of ultrasonography is that it is non-invasive and is readily available in daily practice. Ultrasonography has already been used clinically on humans, and gastric motility has been investigated in patients with functional dyspepsia (Kusunoki et al., 2000), gastric ulcer (Fujimura et al., 1994), and diabetes with metabolic syndrome (Sogabe et al., 2008). In addition, the prokinetic effect of prokinetic agent mosapride was investigated using this ultrasonographic method (Kusunoki et al., 2010). In contrast, the significance of canine gastric motility is poorly understood because there have been limits to the use of previous assessment methods in clinical settings. Like in humans, this ultrasonographic method will be able to evaluate the canine gastric motility disorders and efficacy of gastroprokinetic agent clinically. A limitation of the present study is that gastric motility was assessed only in beagle. Further study is required to determine the normal range of motility index by performing the ultrasonographic method in various breeds and also ages. It is also be needed to compare the sensitivity and specificity of gastric motility abnormalities with other gastric motility or emptying methods.

In summary, I have established a simple ultrasonographic method to evaluate postprandial gastric antral motility in dogs. This method enables the screening of canine gastric motility simply in a short time. By using this method, further studies

are required for the better understanding of the pathogenesis of gastric motility disorders in dog

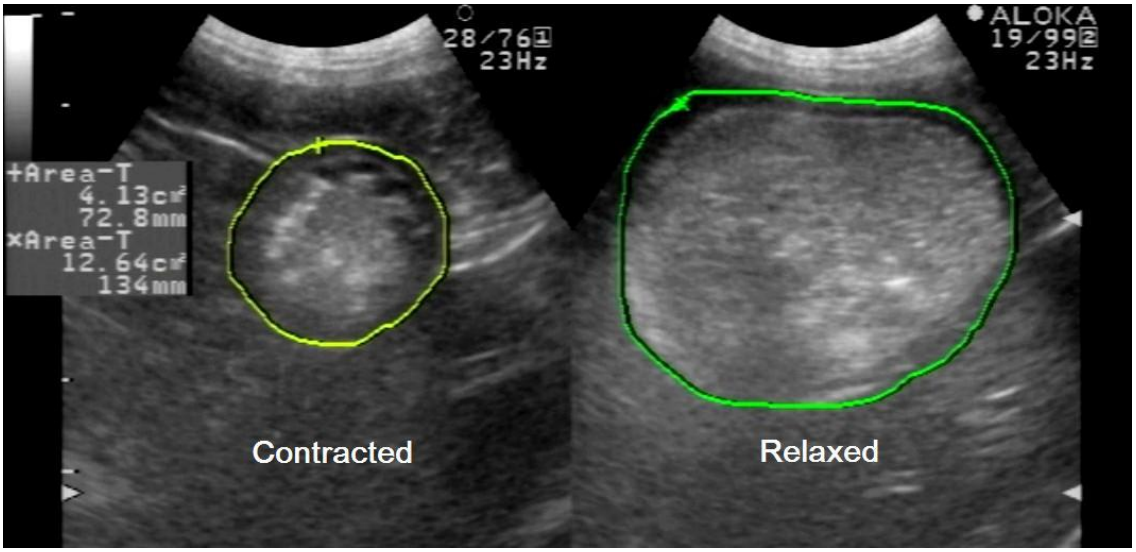

Figure 1. Ultrasonographic scanning of the cross-section of the gastric antrum in contracted (left) and relaxed (right) phase 30 min after having given the test meal.

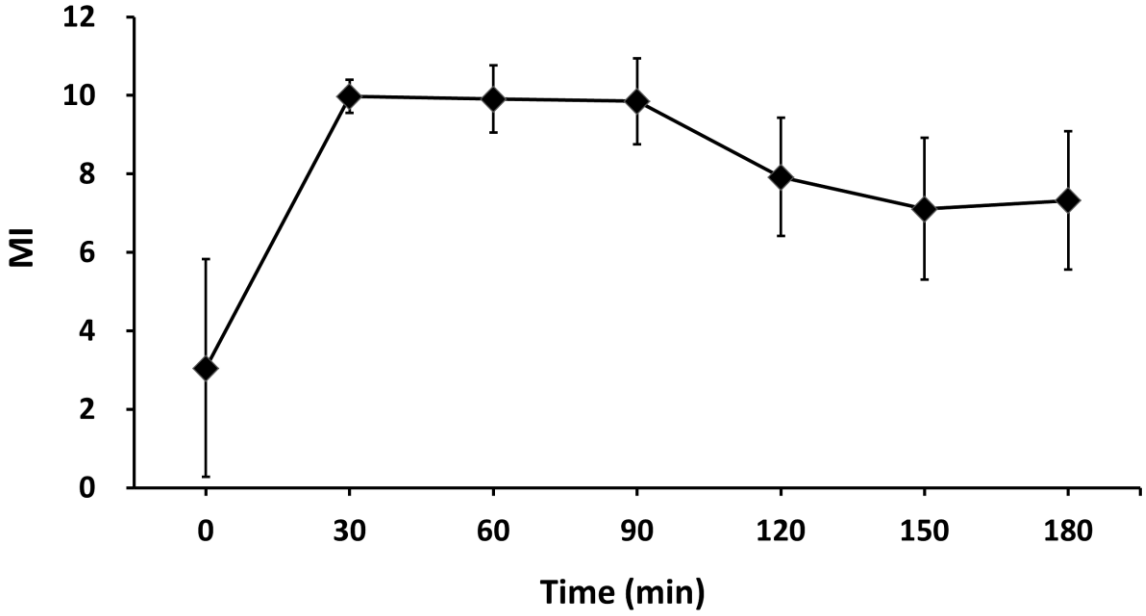

Figure 2. Time course of motility index after ingestion of the test meal in 7 dogs. Data are given as mean ± SD.

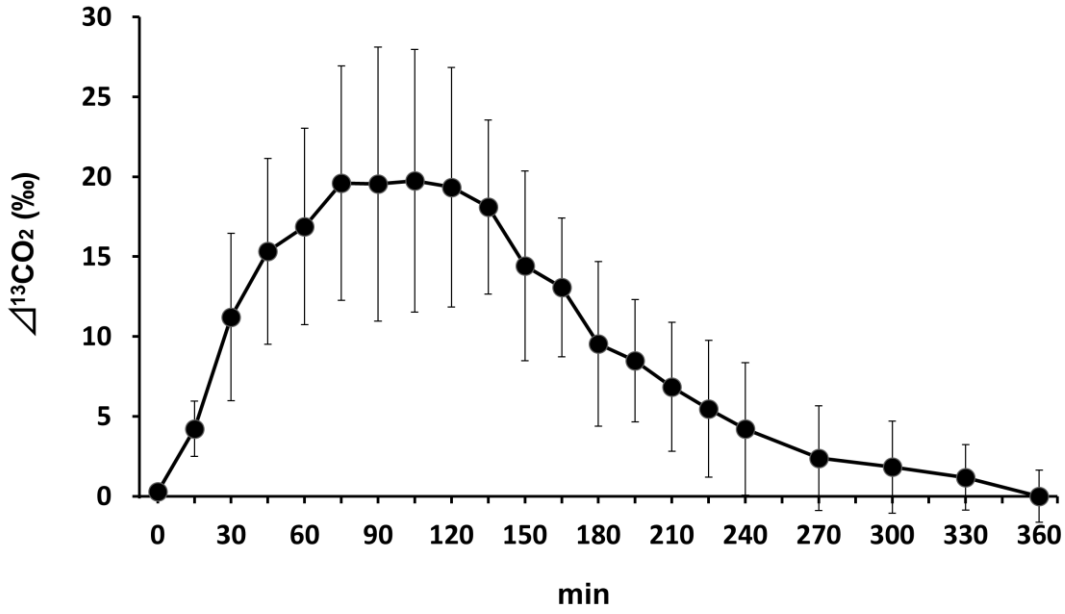

Figure 3. Mean $^{13}CO_2$ excretion curve in 7 healthy dogs as assessed with the ^{13}C-octanoic acid breath test. Data are given as mean ± SD.

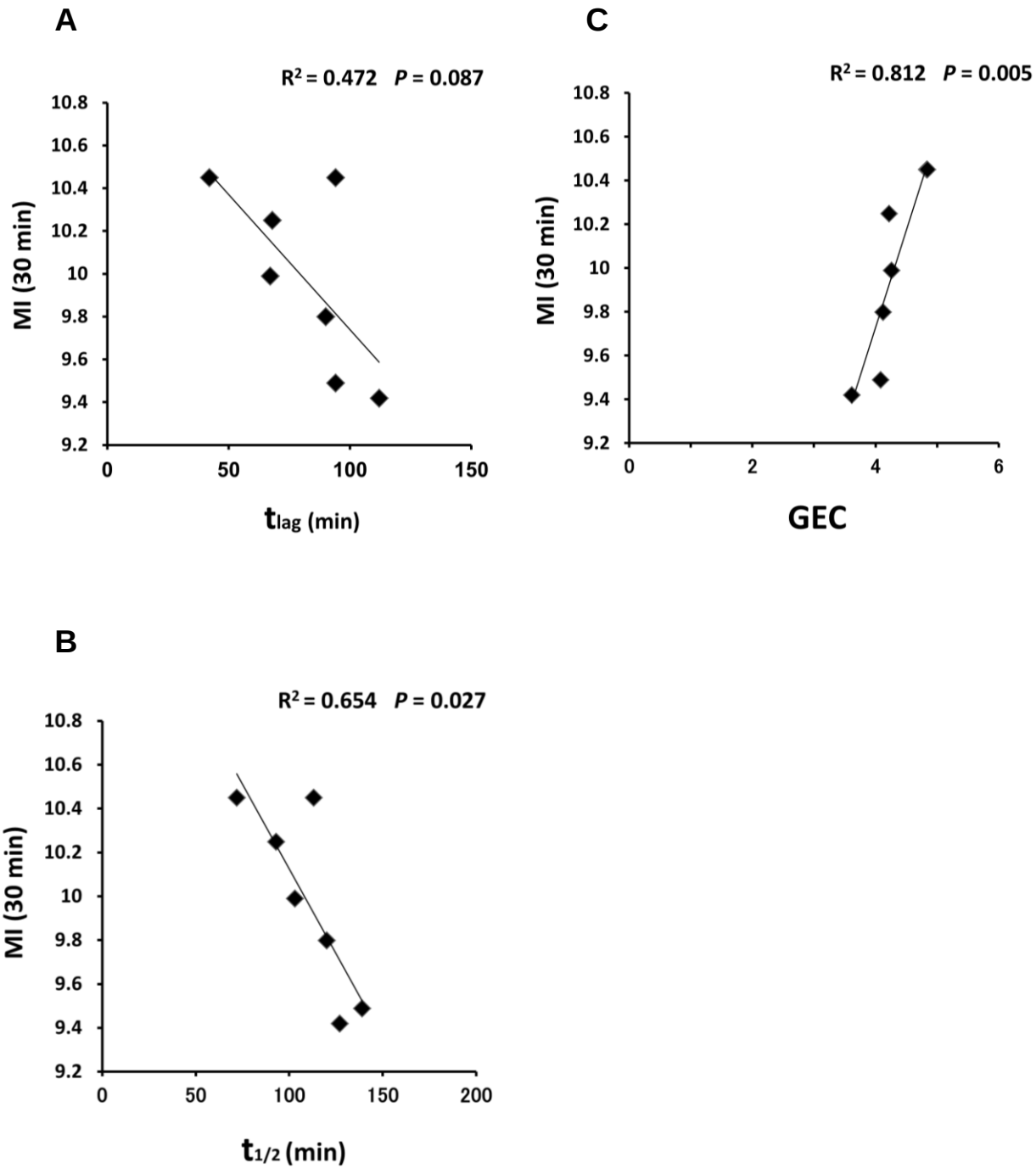

Figure 4. Correlations between the motility index (30 min) and gastric emptying parameters t_{lag} (A), $t_{1/2}$ (B), and GEC (C).

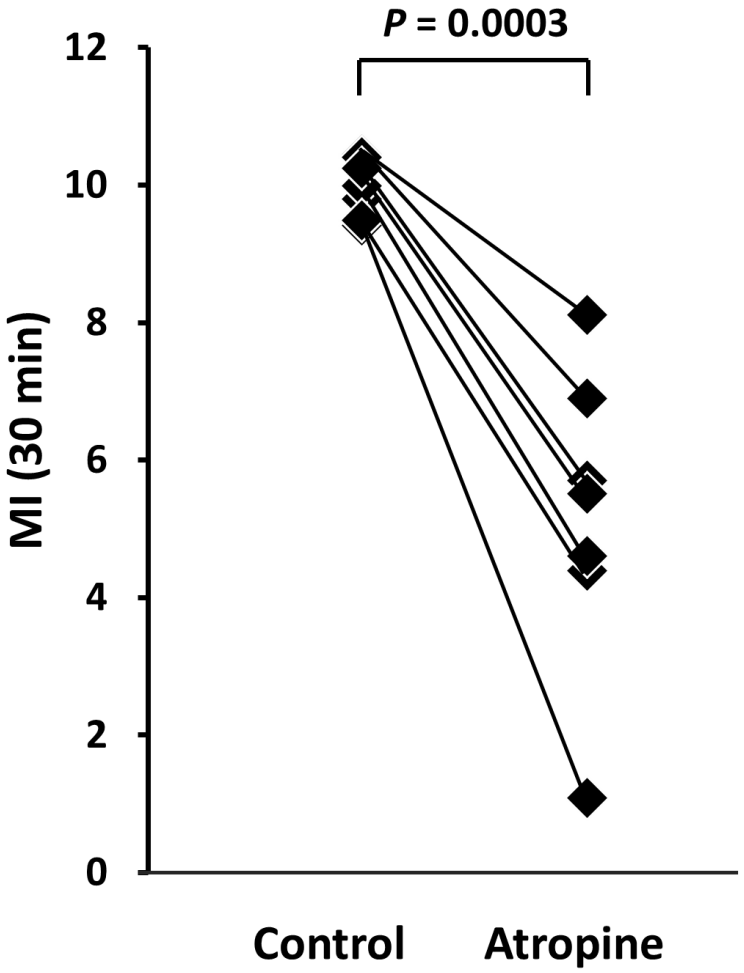

Figure 5. Comparison of the motility index value (30 min after feeding) with and without atropine administration in 7 dogs.

Chapter 2

Prokinetic action of 5-HT_4R Agonist Mosapride in Dogs

Abstract

The object of this study was to assess the prokinetic action of 5-hydroxitriptamine-4 receptor (5-HT_4R) agonist mosapride in dogs. Firstly, effect of oral mosapride administration (0.5, 0.75, 1, and 2 mg/kg, *SID*) on canine gastric antral motility was ultrasonographically evaluated with randomized-cross over trial. Six healthy beagles were administered single oral mosapride at doses of 0.5, 0.75, 1, and 2 mg/kg, followed by 1-week interval. The motility index (MI) of gastric contraction was ultrasonographically evaluated. Administration of mosapride increase MI in dose-dependent manner. Significant increases ($P < 0.05$) in MI were observed at doses of 0.75 mg/kg (mean ± SEM, 11.11 ± 0.19), 1 mg/kg (11.65 ± 0.34), and 2 mg/kg (12.04 ± 0.34), compared with that of the control (9.37 ± 0.51). Next, the effect of mosapride (1 mg/kg, *PO*) on solid phase gastric emptying was investigated under high-energy density meal feeding. As a result, mosapride (1 mg/kg, *PO*) significantly promoted the gastric emptying compared with that of control. Finally, the safety of repeated administration of mosapride was confirmed according to clinical assessment and blood testing. As the result, mosapride administration (2.0 mg/kg, *BID, PO*) for 1 week did not cause any adverse effects. The results of the present study will be served as basic study for the application of mosapride as canine prokinetic agent.

Introduction

Prokinetic agent is a drug that promotes gastrointestinal (GI) motor activity and is used for symptomatic treatment of GI motility disorders. In humans, the clinical demand on gastroprokinetic agent is high, and routinely prescribed for the treatment of GI clinical symptoms. Several prokinetic agents have been developed in humans and some of them are utilized in dogs (Washabau, 2003; Karamanolis et al., 2006). The prokinetic agents which have been used in dogs include dopamine D_2 receptor antagonist metoclopramide, motilin receptor agonist erythromycin, and 5-HT_4 receptor agonist cisapride. Among the prokinetic agents used in veterinary field, metoclopamide is representataive prokinetic agent and has been empirically used in dogs. However, previous study in dogs demonstrated that metoclopramide does not promote solid-phase gastric emptying, while it accelerates gastric antral motility (Gue et al., 1988; Orihata et al., 1994; Burger et al., 2006). This drug may cause may cause sedation through dopamine receptors in the central nervous system (Agostinucci et al., 1986; Donald et al., 2008). 5-hydroxytryptamine-4 receptor (5-HT_4R) agonist cisapride had also been used as canine prokinetic agent. However, this drug was withdrawn from market due to its cardiac side effects in humans (Washabau et al., 1995; Dinarello, 2000).

Mosapride, a second generation 5-HT_4R agonist, promotes the release of acetylcholine from enteric nerves by activating 5-HT_4R, thereby enhancing GI motility (Curran et al., 2008). This drug is widely used in humans as a prokinetic agent for the control of dyspeptic symptoms of GI disorders, including chronic gastritis, functional dyspepsia, irritable bowel syndrome (IBS), and gastroesophageal reflux disease (GERD) (Curran et al., 2008). Since mosapride has high affinity on the 5-HT_4R on gastrointestinal tract, this drugs mediates no action on central nervous systems or myocardium (Yoshida et al., 1989; Curran et al., 2008). Prokinetic action of mosapride was also confirmed in dogs and expected as novel prokinetic agent for the treatment of GI clinical signs. Previous reports demonstrated that intravenous administration of mosapride as drug substance (AS4370) enhanced gastric antral motility in dogs (Yoshida et al., 1991). However, the appropriate oral dose which enhances gastric motility has not been determined in dogs. In addition, effect of mosapride on canine gastric emptying has not been confirmed. The purpose of the present study was to assess the dose of mosapride as oral drug that had gastric prokinetic action in dogs.

Materials and Methods

Animals

Six healthy beagles were used in this study. The age of the dogs ranged from 5 to 6 years (median, 5.1 years) and body weight ranged from 10.5 to 15.0 kg (median, 12.5 kg). Three dogs were male and 3 were female. None of the dogs displayed any clinical symptoms prior to the experiments. Food was offered twice daily. Experiments and animal care complied with the policies outlined in the Guide to Animal Use and Care of the University of Tokyo.

Effect of mosapride on gastric antral motility in healthy beagle

This study was performed with open-label cross-over study design. Six dogs received mosapride at doses of 0.5, 0.75, 1, and 2 mg/kg of mosapride (Pronamid; DS Pharma Animal Health Co., Ltd., Japan) followed by 1-week washout period. The gastric antral motility was ultrasonographically assessed after single administration of mosapride using the method established in Chapter 1. The tablet drug was ground into powder and dissolved in 15 ml of distilled water. Administration of distilled water (15 ml) was used as control. Gastric motility after the single administration of mosapride at each dose was compared with that of control. The order of the assessment in the 6 dogs was randomly assigned and a washout period of at least 1 week was set

between each experiment. Thirty minutes after mosapride was administered, 10 g/kg of commercial wet food (CIW; Intervet Schering-Plough Animal Health, Japan) was offered. Gastric antral motility was evaluated by using an ultrasound (Aplio 80 SSA-770A; Toshiba Medical Systems Corporation, Co., Ltd., Japan) with a 7.5-MHz phased array sector transducer as described in chapter 1. Briefly, dogs were restrained in the right recumbent position, and the probe was adjusted for maximum visualization of the transverse image of the gastric antrum close to the left lobe of the liver. The cross section of the antral area was measured by tracing the serosal margin of the antrum by using the built-in caliper. The antral area was measured 3 times in both the contracted and relaxed phases, and amplitude was calculated with the following formula: (mean area relaxed - mean area contracted)/mean area relaxed. Next, frequency was determined by counting the number of antral contractions in 3 min. Motility index (MI), an indicator of gastric motility, was determined by the multiplication of amplitude and frequency. The MI was evaluated in each dog at 30 min after feeding. The measurement time point of MI was determined according to the study on chapter 1. The same investigator performed all ultrasonographic examinations.

Effect of mosapride on gastric emptying under high energy density meal feeding

Prokinetic effect of mosapride was assessed from the standpoint of solid phase gastric emptying using ^{13}C-octaonoic acid breath test. Effect of conventional dopamine D_2 receptor antagonist prokinetic agent, metoclopramide (Primperan Fine Granules 2 %; Astellas Pharma Inc. Japan) on gastric emptying was concurrently evaluated. Cross-over study design was employed to examine the gastric emptying in mosapride treatment (1 mg/kg, *PO SID*), metoclopramide treatment (1 mg/kg, *PO SID*), and no treatment. Mosapride and Metoclopramide were diluted in 15 ml of distilled water and orally administered 30 minutes before feeding.

The test meal was consisted of 200 g of commercial wet food (CIW; Intervet Schering-Plough Animal Health), 1 egg yolk, 15 cc of corn oil, and 50 mg ^{13}C-sodium octanoate (Cambridge Isotope Laboratories, Inc., Andover, MA, USA).

Gastric emptying was assessed with ^{13}C-octanoic acid breath test according to previous report (Wyse et al., 2001; McLellan et al., 2004). A breath bag (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) which was connected to a plastic mask was used for the collection of air samples. Expired air was collected prior to feeding, every 15 min after feeding for 4 hr, and every 30 min for additional 2 hr. $^{13}CO_2$-infrared spectrophotometry analyzer (POC one, Otsuka Pharmaceutical) was used to assess the $^{13}CO_2$ excretion rate. The amount of ^{13}C in the samples was expressed as the change ($\triangle^{13}CO_2$, ‰) in the $^{13}CO_2/^{12}CO_2$ ratio before and after feeding of the test meal. Resting $^{13}CO_2$ production was assumed stable at 0.194 *l*/(m^2・min) (Wyse et al., 2001). Body

surface area was determined by using the following formula: body surface area (m^2) = 10.1 × body weight (g)$^{2/3}$/10000 (Orr et al., 1975). ^{13}C excretion rate (^{13}C %dose/hr) was calculated according to Ghoos (Ghoos et al., 1993) by fitting the following formula: $y = at^b e^{-ct}$ (y, %dose/hr; t, time; a, b, and c, constants; and e, exponential). Cumulative ^{13}C recovery in the breath (C%D, %dose) was determined using the following formula: $C\%D = m(1 - e^{-kt})^{\beta}$ (t, time; k, m, and ß, constants). Lag phase (t_{lag}), half-emptying time ($t_{1/2}$), and gastric emptying coefficient (GEC) were calculated were calculated using the following formulae: $t_{lag} = b/c$, $t_{1/2} = (-1/k) \times \ln(1-2^{-1/\beta})$, and GEC = ln (a). t_{lag} is the time of the maximum emptying speed of the substrate. GEC is an index of gastric emptying rate. $t_{1/2}$ is the estimated time during which half of the total $^{13}CO_2$ is excreted. All calculation was performed with Excel program software.

Safety evaluation

Safety evaluation in repeated mosapride administration was performed according to the result of blood tests and clinical assessments. Six dogs were administered mosapride at a dose of 2 mg/kg twice daily for 1 week. Blood testing was performed before and after 1-week administration of mosapride. Venous blood samples were collected into EDTA tubes for the measurement of red blood cell (RBC) counts, white blood cell (WBC) counts, differential WBC counts, hematocrit, hemoglobin concentration and platelet counts. Additional blood samples were collected into

heparin tubes for analysis of blood chemistry (blood urea nitrogen, creatinine, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, total bilirubin, glucose, total cholesterol, triglyceride, calcium, phosphate, sodium, chloride, potassium, albumin, and total protein levels). During the medication period, the dogs were assessed for clinical symptoms 3 times a day, and physical examination was also performed after the 1-week period. The clinical assessments were performed by a veterinarian.

Statistical analysis

The difference in MI for each treatment was analyzed by one-way repeated measures of ANOVA. When significant, multiple comparisons were adjusted using Dunnett's *t* test. In the gastric emptying analysis, paired *t* test was performed for the comparison of gastric emptying parameters (t_{lag}, $t_{1/2}$, and GEC) in each treatment. Results were expressed as mean ± SEM. $P < 0.05$ was considered as a significant difference.

Results

Firstly, the prokinetic action of mosapride in each dose was investigated with gastric antral motility assessment. The difference in MI after administration of mosapride at each dose is shown in Figure 6. The mean ± SEM values of MI were 9.37 ± 0.51 in the control, 9.99 ± 0.53 at 0.5 mg/kg, 11.11 ± 0.19 at 0.75 mg/kg, 11.65 ± 0.34 at 1 mg/kg, and 12.04 ± 0.34 at 2 mg/kg. The MI increased dose-dependently and significant increases in MI were observed at the doses of 0.75, 1, and 2 mg/kg, compared with that of the control. During the trials, none of dogs showed any adverse reactions after single administration of mosapride at each dose.

Next, the prokinetic action of mosapride was confirmed from the standpoint of solid phase gastric emptying. The mean ^{13}C-octanoic excretion and cumulative curve for the 6 dogs in each treatment was shown in Figure 7. Mean ^{13}C-excretion curve without medication showed shallow curve with 2 peaks. In contrast, when mosapride or metoclopramide was administered, both ^{13}C-excretion curves showed enhancement of $^{13}CO_2$ excretion rate in early postprandial phase with 1 peak. Mean ± SEM of gastric emptying parameters t_{lag}, $t_{1/2}$, and GEC in each treatment were shown in Table 1. Administration of mosapride significantly decreased the $t_{1/2}$ and increased the GEC, compared with those in no medication, while significant differences were not observed in any parameters between metoclopramide treatment and no treatment.

As a result of safety assessment, none of the dogs had any blood test abnormalities before or after the 1-week administration of mosapride. Clinical adverse events were not observed in any of the dogs, and there were no abnormalities on the physical examinations.

Discussion

Present study demonstrated that oral administration of mosapride increases postprandial antral motility in a dose-dependent fashion within the dose of 0.75 to 2 mg/kg in dogs. However, significant increases in gastric motility were not observed when 0.5 mg/kg of mosapride was orally administered to the dogs. This result is consistent with that of a previous report that evaluated the intravenous administration of AS-4370, drug substance of mosapride in dogs (Yoshida et al., 1991). In humans, mosapride is usually prescribed at 5 mg TID for the treatment of GI discomfort (Curran et al., 2008). The dose of mosapride appears to be higher in dogs than that in humans. This dose difference may be associated with dissimilar bioavailability or metabolism. A previous report indicated that the bioavailability of mosapride in dogs was lower than that in primate species (Sakashita et al., 1993).

Following the antral motility assessment, prokinetic action of mosapride on solid phase-gastric emptying was confirmed under high energy meal feeding. Previous report described that high energy density meal feeding causes gastric emptying delay in dogs (Chalmers et al., 2005), which may be associated with cholecystokinin secretion (Liddle et al., 1986; Kleibeuker et al., 1988). The ^{13}C-excretion curve with no medication showed shallow curve with 2 peaks, suggesting the gastric emptying delay. Mosapride significantly shorten the gastric emptying times compared with that of no

medication, indicating that mosapride has prokinetic action on solid phase-gastric emptying. In contrast, there were no significant differences on gastric emptying parameters, between metoclopramide treatment and no medication. Metoclopramide has long been used in dogs and be the one of representative prokinetic agent in veterinary field. The prokinetic action of metoclopramide in dogs was previously indicated by gastric motor activity and liquid phase gastric emptying assessment (Gue et al., 1988; Burger et al., 2006). However, it was also demonstrated that metoclopramide did not promote solid phase gastric emptying in healthy dogs by using radiolabeled techniques (Gue et al., 1988; Orihata et al., 1994), which is consistent with the result obtained here. Following the present study, definitive comparison for the prokinetic action of metoclopramide and mosapride may be warranted with further large clinical investigation.

In the present study, repeated administration of mosapride in dogs for 1 week did not cause any adverse clinical effects or blood test abnormalities. Previous safety assessment in dogs also showed no adverse reactions after oral administration of mosapride at a dose of 12.5 mg/kg (Yatera, 1993). The advantage of mosapride over several prokinetic agents in humans is its safety. Some gastroprokinetic agents, like metoclopramide, may cause sedation through dopamine receptors in the central nervous system (Agostinucci et al., 1986; Donald et al., 2008). Mosapride has a high affinity for the 5-HT_4R in the GI tract and does not have affinity for the

5-hydroxytryptamine-1 (5-HT_1), 5-hydroxytryptamine-2 (5-HT_2), or Dopamine-2 (D_2) receptors in the central nervous system (Yoshida et al., 1989). The pharmacological action of mosapride is similar to that of cisapride, a first-generation 5-HT_4R agonist prokinetic agent. Both agents enhance GI motility by triggering acetylcholine release from enteric nerves subsequent to 5-HT_4R stimulation. Cisapride had been used for the treatment of GI motility disorders in humans and dogs (Bedford et al., 1996; Washabau, 2003). However, cisapride has potent action on cardiac tissue, which may inhibit the K^+ channel, leading to cardiac adverse effects such as QT prolongation (Wysowski et al., 1996; Potet et al., 2001). Due to the cardiac adverse effects in human, cisapride was withdrawn from market and only available in experimental use (Wysowski et al., 1996; Karamanolis et al., 2006). In contrast, it has been confirmed that mosapride has no effect on cardiac K^+ channel and do not cause any cardiac adverse effects in both humans and dogs (Yatera, 1993; Potet et al., 2001; Curran et al., 2008). Mosapride can be an alternative prokinetic agent to cisapride for the treatment of GI discomfort associated with upper GI motility disorders in dogs.

In summary, oral mosapride administration dose-dependently increased canine postprandial gastric motility without causing adverse effects within the dose of 0.75 to 2 mg/kg. Mosapride also promotes solid-phase gastric emptying. The present study will be served as a basic study of mosapride as canine prokinetic agent, and contribute to the appropriate prescription for the treatment of upper GI clinical signs in dogs.

Table 1. Effect of mosapride and metclopramide on gastric emptying t_{lag}, $t_{1/2}$, and GEC assessed by ^{13}C-octanoic acid breath test.

	No medication	Mosapride (1 mg/kg *PO*)	Metclopramide (1 mg/kg *PO*)
t_{lag}	167 ± 24	143 ± 23	157 ± 44
$t_{1/2}$	241 ± 46	*203 ± 40	222 ± 80
GEC	2.60 ± 0.52	*2.80 ± 0.47	2.82 ± 0.62

Data are expressed as mean ± SEM.
*Significant difference ($P < 0.05$) with no medication.

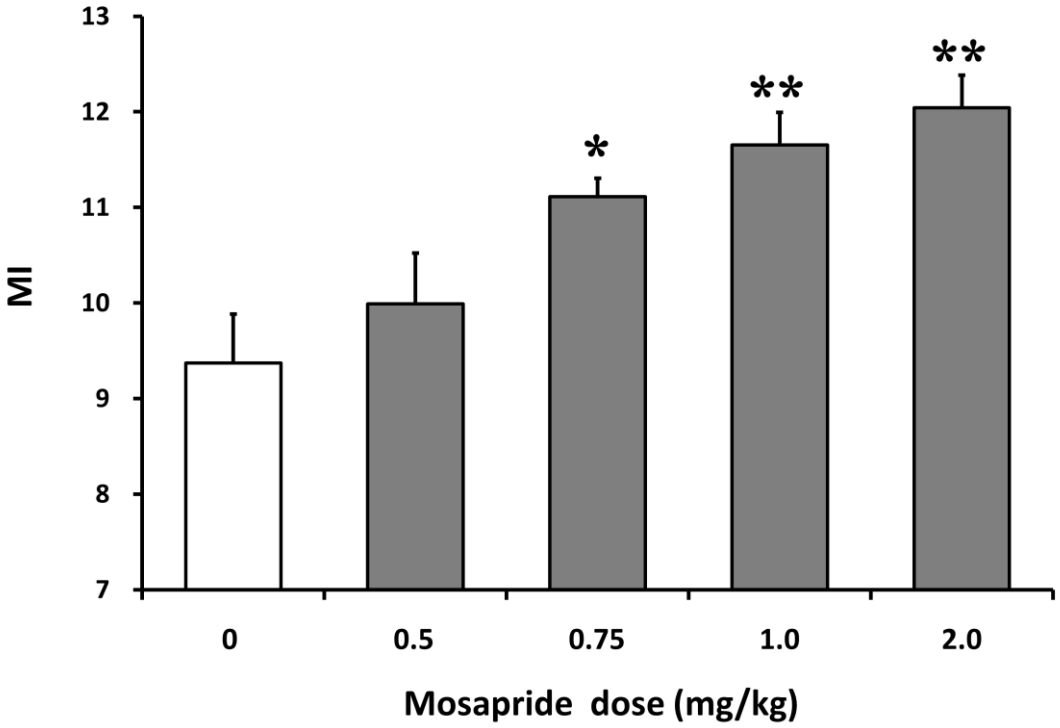

Figure 6. Difference in motility index (MI) after administration of mosapride at several doses in 6 dogs. Data are expressed as mean ± SEM. * and ** indicate significant difference from the control at $P < 0.05$ and $P < 0.01$, respectively.

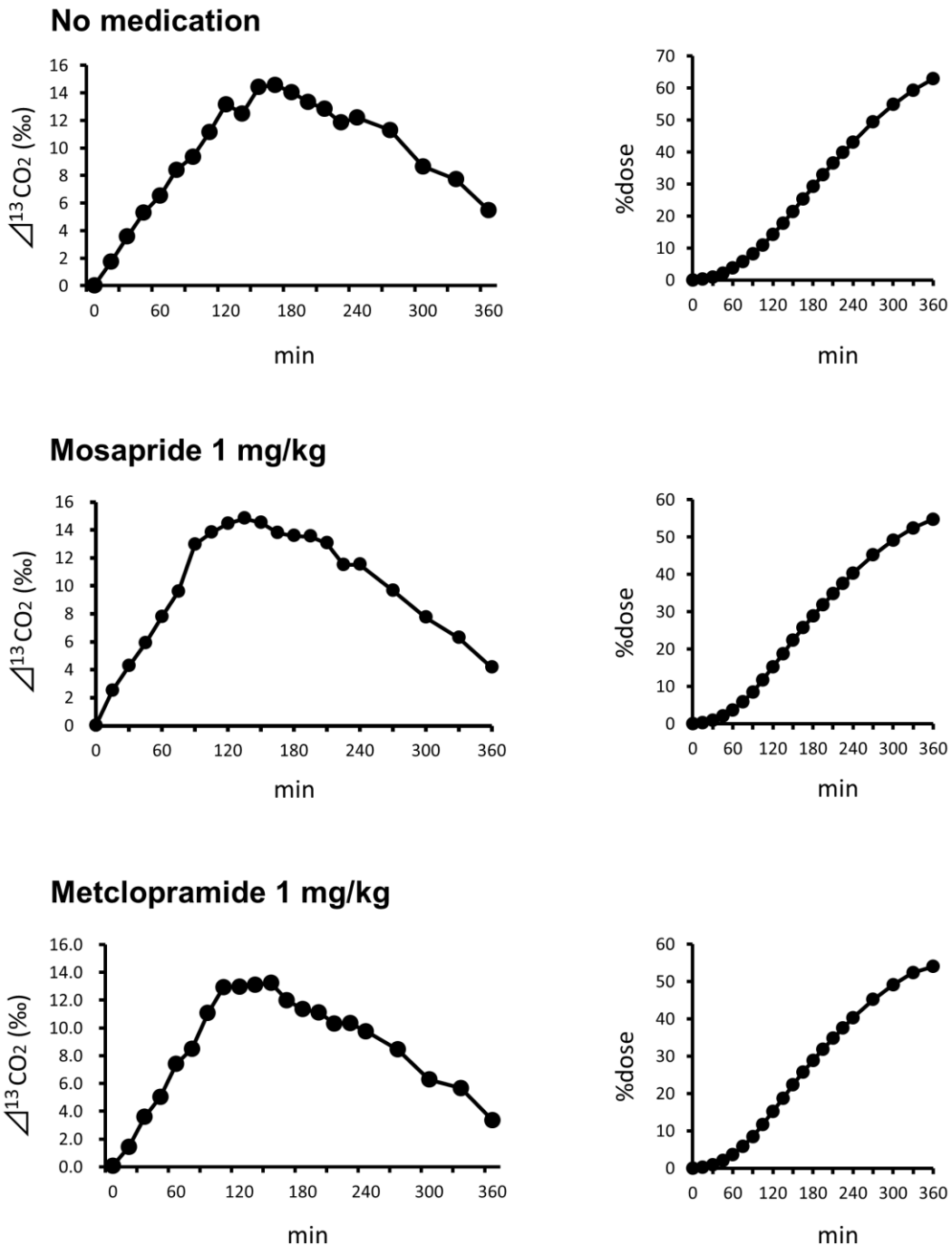

Figure 7. Mean $^{13}CO_2$ excretion and cumulative curves in 7 healthy dogs as assessed with the ^{13}C-octanoic acid breath test after feeding the high energy meal.

Chapter 3

Association of Gastric Motility Disorders in Adverse Drug Reaction and the Clinical Efficacy of Mosapride in Dogs

In the previous chapters, I have established the ultrasonographic method of canine gastric motility, and also demonstrated the prokinetic action of mosapride in healthy dogs. However, pathogenesis of gastric motility disorders and clinical efficacy of mosapride in dogs remained unclear.

In human medicine, the cause of gastric motility disorders is mainly divided in 2 factors. One is caused secondary by underline diseases such as diabetes, cancer, or gastric ulcer (Ali et al., 2007). Another is caused as drug adverse effect. For example, some opioid drugs, anti-cholinergic agents, tricyclic anti-depressants, or calcium channel blockers may cause gastric motor abnormalities (Ali et al., 2007). These gastric motility disorders may be the cause of upper GI clinical symptoms, and therefore the therapeutic target of prokinetic agent.

In this chapter, I focused on the association of gastric motility disorders in drug adverse effect of vincristine and prednisolone, both of which have clinical trouble on its GI adverse effects not only in veterinary field but also in human medicine. Furthermore, I investigated the clinical implication of mosapride on these drugs adverse effects.

Chapter 3-1

Effect of Vincristine on Canine Gastric Motility and the Prokinetic Action of Mosapride in Dogs

Abstract

The objective of the present study was to evaluate the association of gastric motility abnormality in gastrointestinal (GI) adverse effect of vincristine, and also demonstrate the efficacy of mosapride in these adverse events in dogs. Five healthy beagles were used in this study. Dogs received vincristine *IV* at a dosage of 0.75 mg/m^2. The motility index (MI) of the antral contraction was ultrasonographically evaluated 30 minutes post-feeding before administration of vincristine and for 6 days after vincristine treatment. After a 6-week washout period, the dogs received vincristine with mosapride (2 mg/kg *PO, SID* for 6 days), and the MI was re-evaluated. Adverse GI events were evaluated in each trial according to the Veterinary Co-operative Group Common Terminology Criteria for Adverse Events (VCOG-CTCAE). After vincristine administration, a significant decrease ($P < 0.05$) in MI was observed on days 3 (6.64 ± 0.30) and 4 (8.02 ± 0.94), compared with pre-treatment levels (10.00 ± 0.62). GI adverse events were observed in 4 dogs (grade 2 decreased appetite: 3 dogs; grade 1 vomiting: 2 dogs; and grade 1 diarrhea and grade 2 hematochezia: 1 dog). When mosapride citrate was administered with vincristine and for the next 5 days, no decrease in MI was observed. Furthermore, adverse GI events occurred less frequently (grade 1 vomiting and grade 2 hematochezia in 1 dog each). In conclusion, Vincristine induces gastric hypomotility, which possibly associated with GI clinical signs in dogs. Preventive

administration of mosapride citrate (2.0 mg/kg *PO,* q24h) improves hypomotility and may decrease the adverse GI effects of vincristine.

Introduction

Vincristine is representative anti-cancer agent that is used in chemotherapy for canine lymphoma or hematopoietic tumors (Withrow et al., 2007). However, vincristine administration is known to have several adverse effects in dogs, including hematologic, GI, and neurologic effects (Withrow et al., 2007). Among them, adverse GI events are commonly observed. It was reported that 50% of dogs with lymphoma receiving vincristine experienced at least 1 adverse GI event during treatment using the University of Wisconsin-Madison chemotherapy protocol (Tomiyasu et al., 2010). Severe GI toxicity may lead to dose reduction or drug withholding and may impair ideal chemotherapy outcomes.

In humans, vincristine causes GI motility disorders that may be associated with neurotoxicity (Tomomasa et al., 1999). A previous case report suggested that metoclopramide is effective for the improvement of vincristine-induced GI motility disorders (Garewal et al., 1985). To date, there has not been reported about effect of vincristine on canine GI motility. Although use of the gastroprokinetic agent metoclopramide may also be recommended for treating chemotherapy-induced adverse upper GI events in dogs (Thamm et al., 2007), no reports have evaluated the efficacy of GI prokinetic drugs for the prevention or treatment of vincristine-induced adverse effects. The 5-hydroxytryptamine 4 receptor (5-HT_4R) agonist mosapride enhances GI

motility and is used as a prokinetic agent in humans (Curran et al., 2008). In Chapter 2, I have demonstrated that oral mosapride promotes gastric antral motility and emptying in healthy dogs.

The aim of present study was to determine the effect of vincristine administration on canine gastric motility and the prokinetic effect of mosapride in vincristine-treated dogs.

Materials and Methods

Animals

Five healthy beagles (2 males and 3 females) were used in this study. The dogs ranged in age from 3 to 4 years and their body weights ranged from 9.7 to 13.0 kg. The dogs were acclimated for at least 1 month before the experiment. No GI clinical signs were observed in any of the dogs before the experiment. During the study period, the dogs were fasted for at least 12 h before ultrasonographic evaluation of postprandial gastric motility. After assessment, the dogs were given additional commercial dry food. The experiments and animal care were carried out in compliance with the guidelines outlined by the Guide to Animal Use and Care of the University of Tokyo.

Study design

This study was composed of 2 trials. In the 1st trial, vincristine (Oncovin®, Nippon Kayaku Co., Ltd, Tokyo, Japan) alone was intravenously administered to 5 dogs at a dose of 0.75 mg/m^2. Postprandial gastric motility was assessed 1 day before the injection as baseline (day 0) and then daily for 6 days (days 1–6). In the 2nd trial, vincristine was administered on day 1 and mosapride (Pronamid®, DS Pharma Animal Health Co., Ltd, Osaka, Japan) was orally administered to the same dogs on days 1–6 at a dose of 2 mg/kg once a day. The tablet of mosapride was ground into powder, dissolved in 10 ml of

distilled water and administered *PO* to the dogs. Gastric motility was evaluated in the same manner. Adverse GI events observed during the 6-day study period were recorded in each trial. A 6-week washout period was carried out between the 2 trials. 5 dogs were carefully observed for 1 week before the 2nd trial to confirm that none had clinical signs. Blood tests (CBC, neutrophil count, plasma blood urea nitrogen, and creatinine concentrations, and alkaline phosphatase and alanine aminotransferase activity) were performed before both trials. This study was performed as an open-label study.

Gastric motility assessment

Postprandial gastric motility was ultrasonographically evaluated using the method established in Chapter 1, with a 7.5 MHz-phased array sector transducer (ProSound SSD-5000 SV; ALOKA CO., Ltd., Tokyo, Japan). The 10 g/kg of commercial canned food (SPECIFIC® CIW; Intervet Schering-Plough Animal Health, Tokyo, Japan) was given 30 min before the ultrasound examination. A cross-section of the antral area was measured by tracing the serosal side of the antrum with a built-in caliper. The antral area was measured 3 times in both the contracted and relaxed phases. The number of contractions in 3 min then was counted. The amplitude was calculated using the following formula: (mean of area relaxed – mean of area contracted)/mean of area relaxed. Frequency was defined as the number of contractions in 3 min. The motility index (MI), an indicator of postprandial gastric antral motility, was expressed as the

product of amplitude and frequency. All ultrasound examinations were performed by a single operator. On day 1 in both trials, vincristine was administered 30 min before the ultrasonographic assessment. In the 2nd trial, mosapride was administered 1 h before ultrasonography.

Assessment of GI adverse events

Adverse GI events observed during the study were estimated based on the Veterinary Co-operative Group Common Terminology Criteria for Adverse Events (VCOG-CTCAE), an objective grading system of adverse GI events in dogs and cats (Veterinary, 2004). Following the criteria, adverse GI events were classified into 5 grades on the basis of the clinical signs recorded (Table 2). Dogs were observed carefully at least 3 times a day during the 6-day study period in each trial. I also monitored the dogs during the 6-week washout period to confirm disappearance of these signs.

Statistical analysis

A Shapiro-Wilk test was used to confirm that the data were normally distributed. The MI differences between treatments at baseline (day 0) were tested using a paired *t* test. Repeated measures ANOVA was performed to assess the MI differences between treatments. When there was a significant difference, a *t* test using Dunnett's multiple comparison method was performed to assess the MI differences between baseline and

other time points of each treatment. The MI differences among treatments at each time point were analyzed using the paired t test with the Bonferroni multiple comparison method. The results were expressed as mean ± SD. Values of $P < 0.05$ were considered significant. Statistical analysis was performed by SAS system, version 9.2 (SAS Institute Inc, Cary, NC, USA).

Results

There were no fatal events in any of the dogs, and all of the adverse GI events disappeared within 1 week after vincristine administration in both trials. There were no blood test abnormalities before the trials. In the 1st trial (vincristine alone was administered), mean ± SD values of MI from day 0 to 6 were 10.00 ± 0.62, 9.17 ± 1.01, 8.58 ± 1.19, 6.64 ± 0.30, 8.02 ± 0.94, 8.39 ± 1.24, and 9.25 ± 0.87, respectively. In the 2nd trial (mosapride was administered with vincristine), mean ± SD values of MI from day 0 to 6 were 9.93 ± 1.17, 11.71 ± 1.57, 11.58 ± 1.09, 11.20 ± 0.70, 11.11 ± 1.84, 10.32 ± 1.18, and 11.55 ± 0.56, respectively (Figure 8). There was no significant difference in MI between the 2 trials at day 0 (10.00 ± 0.62 vs. 9.93 ± 1.17, $P > 0.05$). The results of repeated measures ANOVA showed significant differences between trials ($P < 0.01$). In the 1st trial, a significant decrease in MI was observed on day 3 and 4 compared with day 0. In the 2nd trial, no significant decrease in MI was observed at any time point compared with day 0, and significant increases in MI were observed on day 3 and 4 compared with the same MI time point in the 1st trial (Figure 9). GI clinical signs observed in the present study included decreased appetite, vomiting, diarrhea, and hematochezia. The grades and frequencies of the GI clinical signs observed in the present study are shown in Table 3. In the 1st trial, 4 of the 5 dogs displayed some clinical sign. The following signs were observed: grade 2 decreased appetite in 3 dogs,

grade 1 vomiting in 2 dogs, grade 1 diarrhea in 1 dog, and grade 2 colitis in 1 dog. In the 2nd trial, decreased appetite and diarrhea were not observed in any of the dogs, and only grade 1 vomiting and grade 2 colitis were observed in 1 dog each.

Discussion

I have demonstrated that vincristine (0.75 mg/m^2) administration causes gastric hypomotility in dogs. Chemotherapy-induced GI toxicity can be caused by direct damage to mucosal epithelial cells or by stimulation of the vomiting center or chemoreceptor trigger zone (CRTZ)(Withrow et al., 2007). Additionally, some anti-cancer drugs like vincristine are known to induce GI motility disorders and be one of the causes of GI toxicity in humans (Garewal et al., 1985; Tomomasa et al., 1999). Vincristine has a potent action that causes autonomic neuropathy (Tomomasa et al., 1999). In humans, vincristine-induced neuropathy can be associated with GI motility disorders such as constipation, abdominal pain, and paralytic ileus (Garewal et al., 1985; Tomomasa et al., 1999). Confirmation of the association between neurotoxicity and gastric motility disorders in dogs requires further studies.

In the present study, mosapride prevented vincristine-induced gastric motility disorders in dogs. Previous studies have shown that the prokinetic agent metoclopramide may be one of the treatment options for vincristine-induced adverse GI events in humans and also is empirically used to treat chemotherapy-induced GI toxicity in dogs (Garewal et al., 1985; Thamm et al., 2007). Metoclopramide has 2 potent actions: a prokinetic action and an anti-emetic effect because of its antagonistic effect on the dopamine-2 receptor in the chemoreceptor trigger zone (CRTZ) and vomiting center.

Because metoclopramide has these 2 functional mechanisms that improve clinical signs, the association between its prokinetic action and alleviation of adverse events is uncertain. In contrast, mosapride acts only on the upper GI tract and does not have a central anti-emetic action (Curran et al., 2008). The present study demonstrated that mosapride improved vincristine-induced gastric hypomotility and tended to attenuate upper GI clinical signs, suggesting that gastric hypomotility is associated with vincristine-induced adverse upper GI events and that use of gastroprokinetic agents such as mosapride may be a treatment option for these adverse events. In future study, comparison in the prophylactic action of metoclopramide and mosapride on vincristine-induced adverse reaction may be warranted.

A limitation of our study is that the experiment was not carried out as a randomized blinded trial. Because the order of the 1st and 2nd trial was not randomized, I should consider whether order effect influenced the result of this study. Therefore, I set a longer washout period (6 weeks) to exclude the possibility that vincristine administration in the 1st trial affected the result of the 2nd trial. I confirmed that there was no significant difference between the baseline MIs of the 2 trials. Adverse GI events disappeared within a week in all dogs after vincristine administration, and blood tests did not show any abnormalities before the 2nd trial. Based on these results, the order effect in this study was negligible.

In humans, mosapride is used to treat GI disorders, including chronic gastritis, functional dyspepsia, and gastroesophageal reflux disease (Curran et al., 2008). However, mosapride has not clinically applied to the patient with vincristine-induced GI adverse event yet. Present study may be served as preclinical experiment towards the novel clinical application of mosapride to the patients received vincristine.

In summary, vincristine induces gastric hypomotility in dogs, which may be associated with pathogenesis of vincristine-induced GI clinical signs. Preventive administration of mosapride improves gastric motility and attenuates vincristine-induced adverse upper GI events.

Table 2. The grading of GI adverse events (anorexia, vomiting, diarrhea, and colitis) based on VCOG-CTCAE

	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Anorexia	Coaxing or dietary change required to maintain appetite	Oral intake altered(<3days) without significant weight loss; Oral nutritional supplements indicated	Of 3-5 days duration. Associated with significant weight loss or mulnutrition; IV fruids, tube feeding or TPN	Life-threatning consequences; >5 days duration	Death
Vomiting	<3 episode in 24hrs	3-5 episodes in 24hrs; < 3/d for >2 days but >5 days; Parenteral indicated < 24hrs (IV or SC)	>5 episodes in 24hr; vomiting >4days IV fruids or PPN/TPN indicated >24hrs	Life-threatening (e.g., hemodynamic collapse)	Death
Diarrhea	Increase of > 2 stools per day over baseline	Increase of 2-6 stools per day over baseline; Parenteral (IV or SC) fluids indicated <24hrs; not interfering with ADL	Increase of >6 stools per day over baseline; IV fluids >24hrs; hospitalization; interfering with ADL	Life-threatening (e.g., hemodynamic collapse)	Death
Colitis	Asymptomatic, pathologic or radiograpic findings only	Abdominal cramping/pain; mucus or blood in stool	Abdominal pain, fever, change in bowel habits, leus, peritoneal signs	Life-threatening consequences eg, perforation, bleeding	Death

ADL, activities of daily living; PPN, peripheral parenteral nutrition; TPN, total parenteral nutrition

Table 3. The grades and numbers of GI adverse events observed in 5 dogs in each treatment

	Vincristine	Vincristine + Mosapride
Total	7	2
Anorexia	3 (Grade 2)	0
Vomiting	2 (Grade 1)	1 (Grade 1)
Diarrhea	1 (Grade 1)	0
Colitis	1 (Grade 2)	1 (Grade 2)

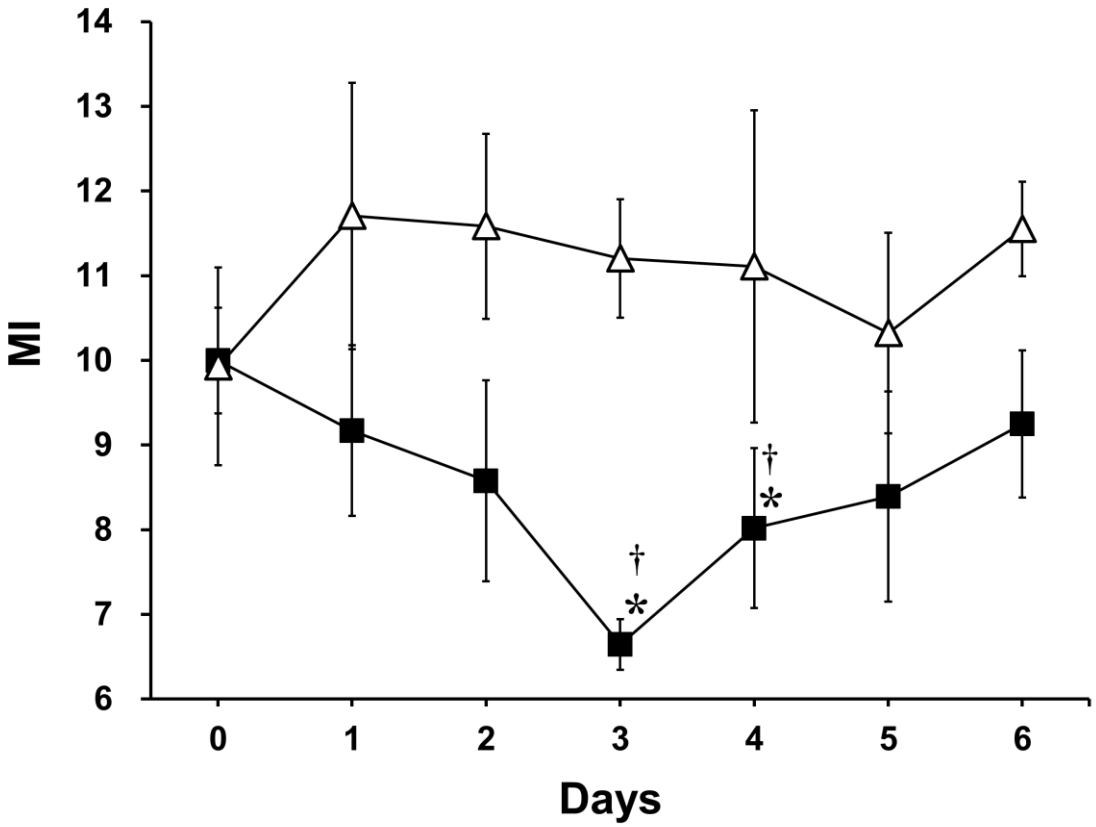

Figure 8. Change of motility index (MI) in each treatment in 5 dogs. Close squares (■) indicate MI in 1st trial (vincristine alone was administered) in each day. Open triangles (△) indicate MI in 2nd trial (mosapride was administered with vincristine) in respective days. In either trial, vincristine (0.75 mg/m^2) was administered on Day 1. *Significant difference (P< 0.05) from baseline (day 0). †Significant difference (P< 0.05) between the 2 trials on the same day.

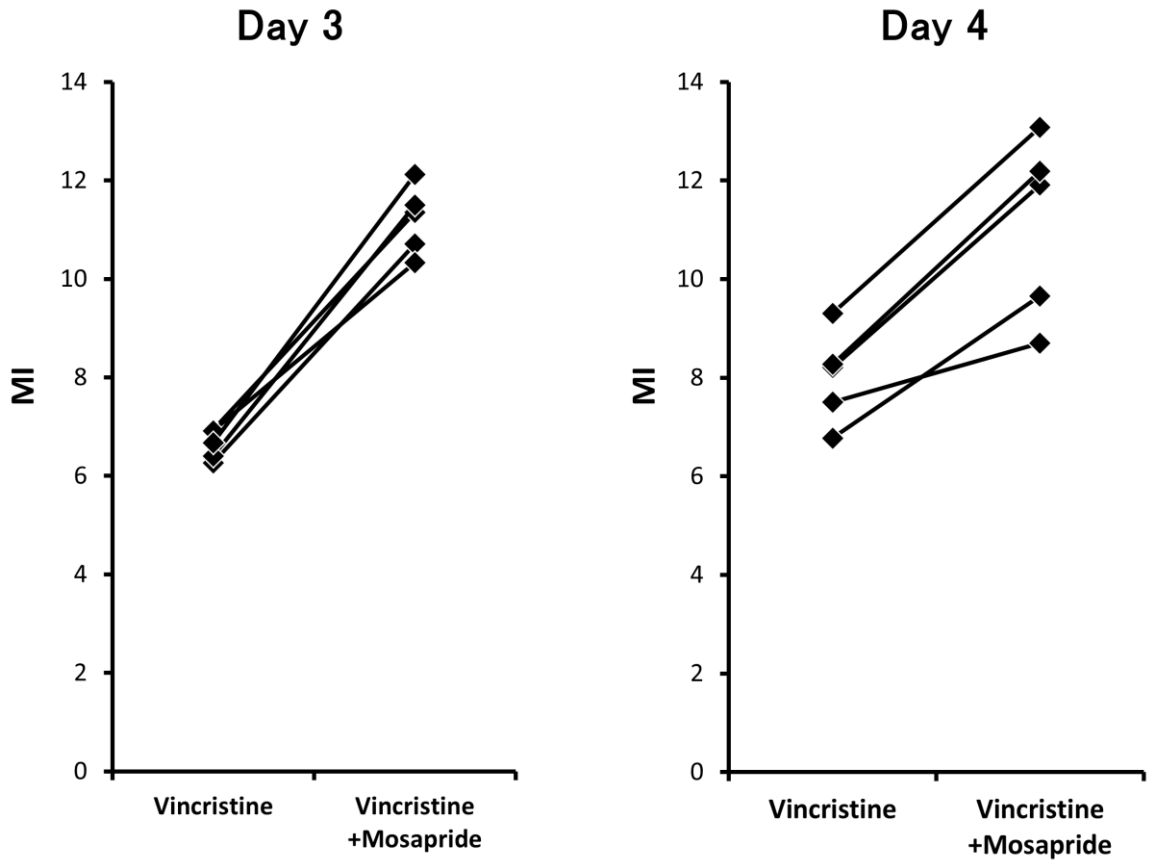

Figure 9. Results of motility index (MI) in Day 3 and 4 in 5 dogs in each treatment.

Chapter 3-2

Anti-ulcerogenic and Prokinetic Action of Mosapride on Prednisolone-induced Gastrointestinal (GI) Adverse Effect

Abstract

Prednisolone is a representative anti-inflammatory agent in both veterinary and human medicine. However, this drug may cause gastrointestinal (GI) adverse reaction when administered higher dose. The aim of the present study was to investigate the pathogenesis of prednisolone's GI adverse reaction and present the clinical implication of mosapride in dogs. Crossover study design was employed. Prednisolone alone (2 mg/kg, *BID, SC*) and prednisolone with mosapride (1 mg/kg, *BID, PO*) was administered to 6 healthy beagles for 3 days, followed by an interval of at least 6 weeks. In each treatment, I endoscopically scored gastric mucosal injury according to the modified Lanza scale. Gastric emptying was assessed by using the ^{13}C-octanoic acid breath test. The incidence of GI clinical signs was also recorded in each medication. As the result, administration of prednisolone caused prominent gastric legions such as multiple erosions and ulcers in the dogs. Coadministration of mosapride with prednisolone significantly ($P < 0.05$) reduced the gastric mucosal injury score (median: 18, range: 14-20), compared with that of prednisolone treatment alone (median: 29.5, range: 13-36). Prednisolone treatment delayed the half-emptying time (median: 187, range: 137-262) compared with that of controls (median: 139, range: 101-159), and coadministration of mosapride improved this gastric-emptying delay (median: 143, range: 146-193). Furthermore, the incidence of the GI adverse event vomiting became less frequent upon

coadministration with mosapride. In summary, prednisolone induces gastric mucosal injury and gastric emptying delay, which can be the cause of gastorointestinal clinical signs in dogs. The use of mosapride will be effective for attenuating prednisolone-induced GI adverse reaction by its prokinetic and anti-ulcerogenic action.

Introduction

In veterinary field, the corticosteroid prednisolone has been widely used for treating immune-mediated diseases such as immune-mediated hemolytic anemia, idiopathic polyarthritis, and atopic dermatitis (Ohno et al., 2006; Balch et al., 2007; Olivry et al.). This drug also used in human medicine for immunosuppressive therapy (Tarantino et al., 1995; Ozkaya et al., 2003). However, prednisolone administration in high dose may cause gastric mucosal injury (Messer et al., 1983; Rohrer et al., 1999). Moreover, a previous study indicated that prednisolone can alter GI motility (Phillips et al., 1991)

Mosapride, a selective 5-hydroxytryptamine-4 receptor (5-HT_4R) agonist, promotes the release of acetylcholine in enteric nerves by activating 5-HT_4R and thereby enhancing GI motility. Since cisapride was withdrawn from the market for its cardiac arrhythmia side effect, mosapride has been used as a prokinetic agent for the treatment of dyspeptic symptoms of GI disorders such as chronic gastritis, functional dyspepsia, irritable bowel syndrome, and gastric esophageal reflux disease (Odaka et al., 2006; Curran et al., 2008). In the previous chapters, I have demonstrated that oral mosapride enhance the canine gastric motility and is effective for the vincristine-induced gastric motility disorder.

In addition to its prokinetic action, recent findings suggest that mosapride mediates novel actions through 5-HT_4R. Fujisawa et al. (Fujisawa et al., 2010) demonstrated that

mosapride attenuated gastric mucosal damage in a rat indomethacin gastric mucosal injury model. They demonstrated that this anti-ulcerogenic action of mosapride was mediated through cholinergic anti-inflammatory pathway; mosapride activates acetylcholine release from the enteric nervous system by 5-HT_4R activation, which finally acts on alpha7 nicotinic acetylcholine receptor (α7nAChR) expressed on immune cells. Their report suggests that mosapride can be used as an anti-ulcerogenic agent for the prevention of ulcers caused by drugs such as nonsteroidal anti-inflammatory drugs or corticosteroids. However, this anti-ulcerogenic action of mosapride has not yet been investigated in larger species, and its clinical significance has not been clarified. In addition, the prophylactic effect of mosapride on GI clinical symptoms, as well as on gastric mucosal injury, has not been investigated yet.

In this study, I evaluated the prophylactic action of mosapride on prednisolone-induced gastric mucosal injury and motility disorder. I also investigated whether coadministration with mosapride can modulate prednisolone-induced GI adverse events.

Materials and Methods

Animals

Six healthy beagles were used in this study (1 male, 5 females). The age of the dogs ranged from 4 to 5 years (median, 4.8 years), and their body weights ranged from 8.7 to 13.7 kg (median, 12.0 kg). The dogs were acclimatized for at least 1 month before the experiment. We confirmed the absence of GI clinical signs, and physical examinations showed no abnormalities. Absence of any abnormality in the blood test results (complete blood count, neutrophil count, blood urea nitrogen, creatinine, alkaline phosphatase, and alanine aminotransferase) was also confirmed. Experiments and animal care procedures were approved by the Animal Use and Care Committee of the University of Tokyo.

Study procedure

A crossover study design was employed. Six dogs were administered prednisolone alone (2 mg/kg *BID, SC*, PREDNISOLONE INJECTION SOLUTION KS®; Kyoritsu Seiyaku Corporation, Tokyo, Japan) and prednisolone with mosapride (1 mg/kg, *BID*, *PO,* GASMOTIN® powder 1%; Dainippon Sumitomo Pharmaceutical Inc., Osaka, Japan) for 3 days, followed by an interval of at least 6 weeks. The 6 dogs were divided into 2 groups. In 1 group, prednisolone alone was administered first, and after the interval,

prednisolone and mosapride were coadministered. Another group was treated with prednisolone and mosapride first. Mosapride powder was diluted in 15 ml of distilled water, and the solution was administered *PO*. After 3 days of medication, gastric emptying, gastric mucosal injury, and GI adverse events were assessed in both treatments. Gastric mucosal injury was assessed by gastroscopy, and gastric emptying was evaluated by the ^{13}C-octanoic acid breath test. Before the medication study, the baseline gastric-emptying time was determined. Gastroscopy was performed at pretreatment and 2 weeks before the second medication to confirm the absence of gastric lesions.

Evaluation of gastric mucosal injury

Gastric mucosal injury was evaluated by gastroscopy according to the modified Lanza scale a systematic scoring system for the severity of gastric lesions (Lanza et al., 1980; Ward et al., 2003; Heather Graham et al., 2009). After an overnight fast, an intravenous catheter was placed, and anesthesia was induced with propofol and maintained with isoflurane. Dogs were restrained in the left recumbent position and were subjected to gastroscopy. The severity of gastric mucosal injury was assessed in 4 regions of the stomach (cardia, gastric body, angularis incisura, and pyloric antrum). Each region was scored separately on a scale of 1–11 as previously described (Table 4), and the total gastric mucosal injury score was calculated by adding the scores of all 4

regions. Mucosal hemorrhage was defined as petechial hemorrhage without any observable defect in the mucosa. Erosion was defined as any defect in the mucosal epithelia. Ulcer was defined as a mucosal defect with observable depth and a raised margin.

I also histopathologically confirmed the presence of gastric lesions. Endoscopic biopsy specimens of the gastric mucosa were obtained from each dog in each treatment. Four biopsy samples were taken from gastric lesion sites by using biopsy forceps. The samples were fixed with neutral-buffered formalin, processed routinely, and embedded in paraffin. Sections were then cut and stained with hematoxylin and eosin.

Assessment of gastric emptying

Gastric emptying was assessed by the ^{13}C-octanoic acid breath test, as described in previous reports (Wyse et al., 2001). The dogs were fasted at least 12 hr before performing the breath test. A breath bag (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) was connected to a plastic mask, and the expired air from dogs was collected through the mask. Breath samples were collected before giving the test meal (baseline), every 15 min after feeding for 4 hr and every 30 min for an additional 2 hr. The $^{13}CO_2$ rate was analyzed using a $^{13}CO_2$-infrared spectrophotometry analyzer (POC One, Otsuka Pharmaceutical). The amount of ^{13}C in the samples was expressed as the change ($\triangle^{13}CO_2$, ‰) in the $^{13}CO_2/^{12}CO_2$ ratio before and after feeding of the test meal. Resting

$^{13}CO_2$ production was assumed stable at 0.194 l/(m_2·min) (Wyse et al., 2001). The body surface area (m^2) was computed using the following formula: 10.1 × body weight $(g)^{2/3}$/10,000 (Wyse et al., 2001). The ^{13}C excretion rate (^{13}C %dose/hr) was calculated according to a previous report (Ghoos et al., 1993) by fitting the following formula: $y = at^b e^{-ct}$ (y, %dose/hr; t, time; a, b, and c, constants; e, exponential). The cumulative ^{13}C recovery in the breath (C%D, %dose) was determined using the following formula: $C\%D = m(1 - e^{-kt})^{ß}$ (t, time; k, m, and ß, constants). The lag phase (t_{lag}) and half-emptying time ($t_{1/2}$) were used as gastric-emptying parameters in the present study and were calculated using the following formulae: $t_{lag} = b/c$ and $t_{1/2} = (-1/k) \times \ln(1 - 2^{-1/ß})$. The parameter t_{lag} reflects the time to maximum emptying speed of the substrate, and $t_{1/2}$ indicates the estimated time during which half of the total $^{13}CO_2$ is excreted. These gastric-emptying parameters were calculated using the Excel software program.

Assessment of GI clinical symptoms

The incidence of GI adverse events was assessed in the 6 dogs during each 3-day medication period. The GI clinical symptoms investigated in the present study included anorexia, vomiting, diarrhea, and hematochezia. A veterinarian carefully observed the dogs during the medication period.

Expression of α7nAChR mRNA

The gastric mucosal samples were endoscopically obtained from the gastric ulcer lesions in 3 dogs treated with prednisolone alone. Total RNA was extracted with a commercially available kit (RNAspin Mini RNA Isolation Kit; GE Healthcare UK Ltd., Buckingham shire, England) according to the manufacturer's manual. Reverse transcription was performed using a Prime Script RT Reagent Kit (Takara Bio Inc). the PCR amplification was carried out with following reactions: Pre-denaturing (95℃ for 10 sec), 40 cycling of denaturation (95℃ for 5 sec) and annealing (60℃ for 30 sec), and extension (95℃ for 15 sec). The primer pair used was as follows; α7nAChR (forward primer: 5'-ATATCCAGGCGTGAAGACGTT-3', reverse primer: 3'-CTAACACGTTGGTGGAAGT-5'), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer: 5'-TGTCCCCACCCCCAATGTATC-3', reverse primer: 3'-CTCCGATGCCTGCTTCACTACCTT-5'). The PCR products were electrophoresed in a 7.5% acrylamide, stained ethidium bromide, and visualized by UV transiluminator. The expression of α7nAChR in canine brain was also confirmed as positive control.

Statistical analysis

A Shapiro-Wilk test was performed to confirm normal distribution. A repeated-measures ANOVA was performed to compare the gastric mucosal injury score and gastric-emptying parameters in each treatment. When results were significant, Fisher's least significant difference method was performed as a post hoc test. The

incidence of each GI clinical sign in each treatment was analyzed by Fisher's exact test. $P < 0.05$ was considered significant. The all analyses were performed with commercially available software (Stat Mate IV; ATMS Co., Ltd. Tokyo, Japan).

Results

During the study period, none of the dogs displayed fatal events, and any gastric mucosal lesions induced by the first treatment were healed before the second treatment. The typical endoscopic findings of the gastric mucosa after prednisolone treatment in the dogs are shown in Figure 10A. When prednisolone alone was administered, multiple erosions were observed in 5 of the 6 dogs, and 3 had more than 1 ulcer lesion. The remaining dog had no erosion or ulcer lesions, but showed multiple hemorrhages in the gastric body, angularis incisura, and pyloric antrum. Histopathological findings of biopsy samples from the erosion and ulcer sites revealed defects in mucosal epithelia with mucosal hemorrhage and infiltration of neutrophils and mononuclear cells (Figure 10B). In the ulcer tissue, mRNA expression of α7nAChR was detected (Figure 11).

The gastric mucosal injury scores in each treatment and in each region are shown in Figure 12. The median of the total gastric lesion score in 6 dogs after prednisolone treatment was 29.5 (range, 13–36). No significant difference was found in gastric mucosal lesion scores among the cardia (median: 7, range: 1-10), gastric body (median: 9, range: 1-10), angularis incisura (median: 6.5, range: 4.-8), and pyloric antrum (median: 7, range: 4-8) regions after prednisolone treatment. When mosapride was coadministered with prednisolone, the median value of total gastric lesion score was 18 (range, 14–20). Treatment with mosapride significantly decreased the total gastric

lesion score compared with that of prednisolone treatment alone. The attenuation of gastric lesions by mosapride administration was especially marked in the gastric body (Figure 13) and angularis incisura.

The gastric-emptying curves determined by ^{13}C-octanoic acid breath test was shown in Figure 14. The mean ± SD values of t_{lag} (min) and $t_{1/2}$ (min) in the 6 dogs were 98 ± 20 and 137 ± 19 in the control, 132 ± 28 and 184 ± 45 in prednisolone treatment, and 100 ± 18 and 143 ± 29 in prednisolone with mosapride treatment, respectively (Figure 14). When prednisolone alone was administered, there was a significant increase in t_{lag} and $t_{1/2}$, compared with that of the control. Mosapride treatment significantly decreased t_{lag} and $t_{1/2}$ compared with prednisolone treatment, and no significant difference was observed with that of the control (Figure 15).

The incidence of each GI clinical symptoms in each treatment is shown in Table 5. When prednisolone alone was administered, vomiting was observed in 5 of the 6 dogs at least once during the medication period. In contrast, only 1 dog had vomiting when mosapride was coadministered with prednisolone. Anorexia, diarrhea, and hematochezia were not observed in either treatment. The difference in the occurrence of vomiting between the treatments was statistically significant.

Discussion

In this chapter, I have demonstrated that prednisolone (2 mg/kg *BID, SC*) induces prominent gastric mucosal injury in dogs. A deficiency of endogenous prostaglandins has been reported to be associated with corticosteroid-induced gastric mucosal injuries (Nobuhara et al., 1985). Prostaglandin inhibition causes mucosal ischemia, gastric acid and mucus imbalance, and HCO_3^- diffusion, leading to local gastric mucosal necrosis (Main et al., 1973; Bickel et al., 1981; Konturek et al., 1981; Nobuhara et al., 1985; Takeuchi et al., 1985). In necrotic tissues, endothelial and infiltrating mononuclear cells produce pro-inflammatory cytokines and activate local fibroblasts or neutrophils. This inflammatory process has a role in ulcer formation and healing (Arakawa et al., 1998; Gudis et al., 2005; Tanigawa et al., 2005). A previous study demonstrated that the expression of inflammatory cytokines is elevated in aspirin-induced gastric mucosal injury in humans (Hamlet et al., 1998).

According to previous reports, the attenuation of gastric mucosal damage by mosapride may be associated with cholinergic receptor stimulation following 5-HT_4R activation. The cholinergic anti-inflammatory pathway has been recently recognized; this pathway modulates inflammation through the cholinergic neuron stimulation by acting on the α7 nicotinic acetylcholine receptor (α7nAChR) expressed in immune cells such as macrophages or neutrophils (Borovikova et al., 2000; Pavlov et al., 2005;

Rosas-Ballina et al., 2009). A previous study on rodents demonstrated that mosapride attenuated gastric mucosal damage through the acceleration of acetylcholine release, followed by activation of 5-HT_4R, which acts on α7nAChR located in macrophages (Fujisawa et al., 2010). Another rodent study reported that activation of α7nAChR ameliorates indomethacin-induced intestinal ulceration by modulating neutrophil activity in mice (Kawahara et al., 2011). In the present study, the mRNA expression of α7nAChR was observed in gastric mucosal lesions in dogs treated with prednisolone. To definitively confirm the association of α7nAChR with the anti-ulcerogenic action of mosapride in dogs, a further large-group experiment with an α7nAChR agonist and antagonist is needed.

In this study, gastric emptying was delayed by prednisolone administration. The pathogenesis of the prednisolone-induced gastric-emptying disorder may be associated with prostaglandin deficiency and gastric mucosal injury. Prostaglandin E_2 inhibition promotes contractile force generation in circular muscles, while the force in longitudinal muscles is suppressed in the stomach (Sanders, 1984). This action may cause gastric motility abnormalities. Gastric mucosal injury may also alter gastric motility. A previous report described gastric hypomotility in human patients with gastric ulcers (Fujimura et al., 1994). Although the pathogenesis of gastric-emptying disorders was not definitively determined, our study indicates that mosapride is effective for the improvement of prednisolone-induced gastric-emptying abnormalities.

The present study demonstrated that the incidence of prednisolone-induced vomiting was attenuated by mosapride coadministration. The pathogenesis of this vomiting may be associated with gastric mucosal injury and the gastric-emptying disorder. The decreased incidence of vomiting in dogs treated with mosapride could be a result of mosapride's anti-ulcerogenic and prokinetic actions. From the present study, it is indicated that mosapride is effective for the prevention of GI adverse effect of prednisolone in dogs. As anatomical and physiological properties of canine stomach is close to humans, this study may also be served as preclinical study towards the novel clinical application of mosapride as anti-ulcerogenic agent in humans.

In summary, mosapride ameliorates prednisolone-induced gastric mucosal injury and gastric-emptying disorder in dogs. The preventive administration of mosapride reduces the incidence of prednisolone-induced GI clinical signs by prokinetic and anti-ulcerogenic action.

Table 4. Criteria of the gastric mucosal injury scoring according to Modified Lanza Scale.

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

Table 5. Incidence of GI clinical symptoms in 6 dogs in each treatment.

	Prednisolone	Prednisolone + Mosapride
Anorexia	0	0
Vomiting*	5	1
Diarrhea	0	0
Hematochezia	0	0

* Significantly different between the 2 treatments at $P < 0.05$

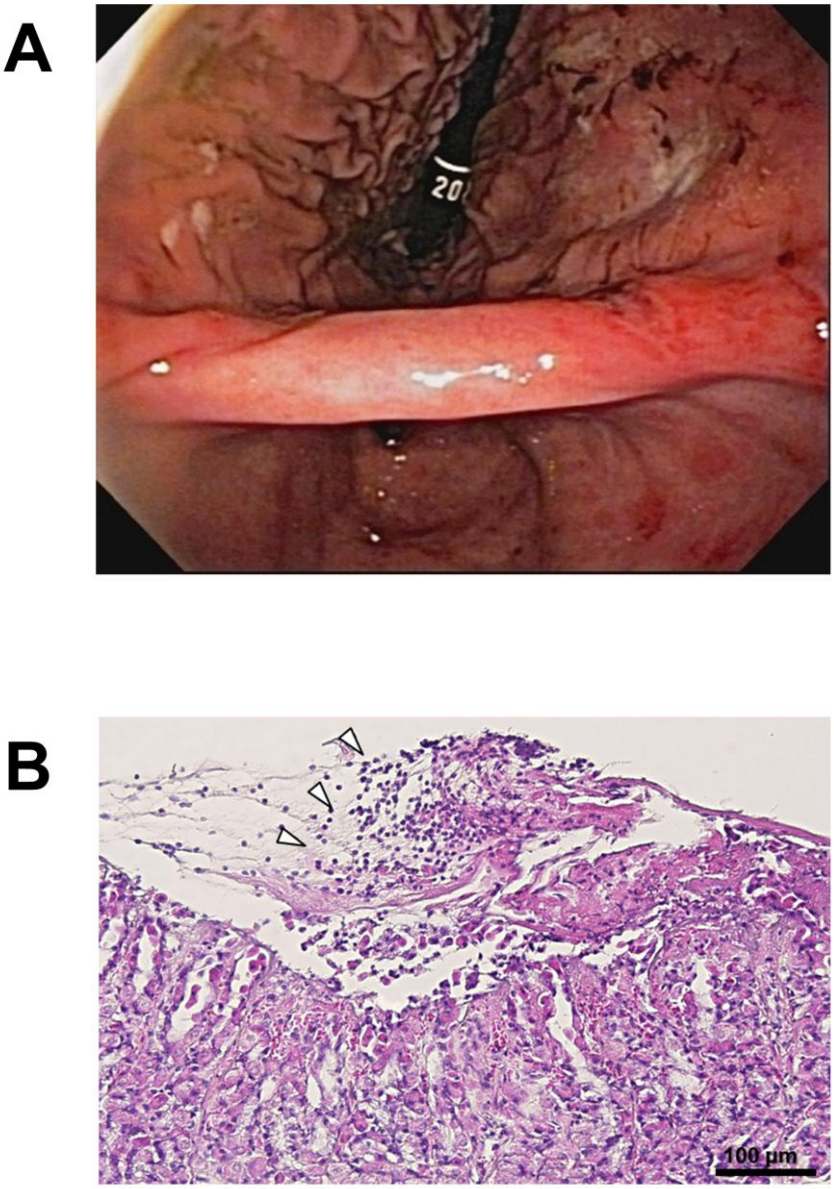

Figure 10. Macroscopic and microscopic findings of gastric mucosa after prednisolone treatment. (A). Endoscopic view of stomach (cardia, angularis incisura and pyloric antrum) after prednisolone treatment in a dog. In cardia, and pyloric antrum, multiple erosions with bleeding were observed. An ulcer was found in the angularis incisura. (B). Microscopic observations of a gastric mucosal erosion site in a dog. A defect of the mucosal epithelia with infiltration of neutrophils and mononuclear cells (shown by arrowheads) was observed.

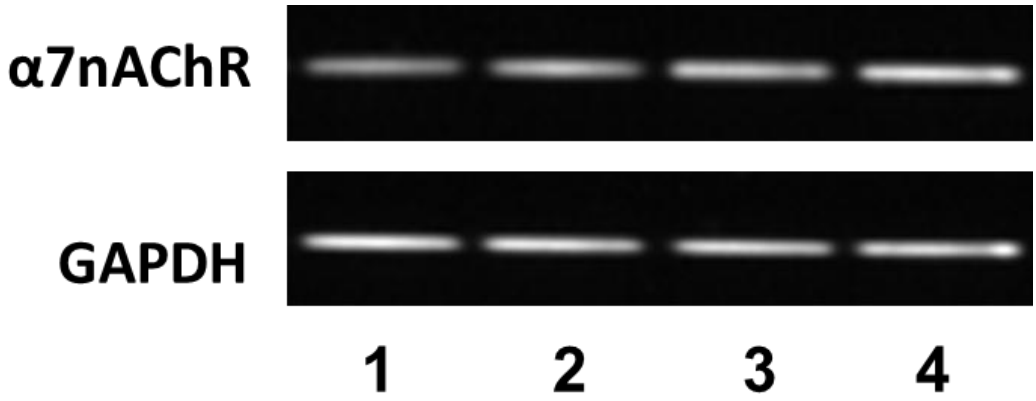

Figure 11. Expression of α7nAChR and GAPDH mRNA. (1) Brain. (2-4) Gastric ulcer lesions in 3 dogs.

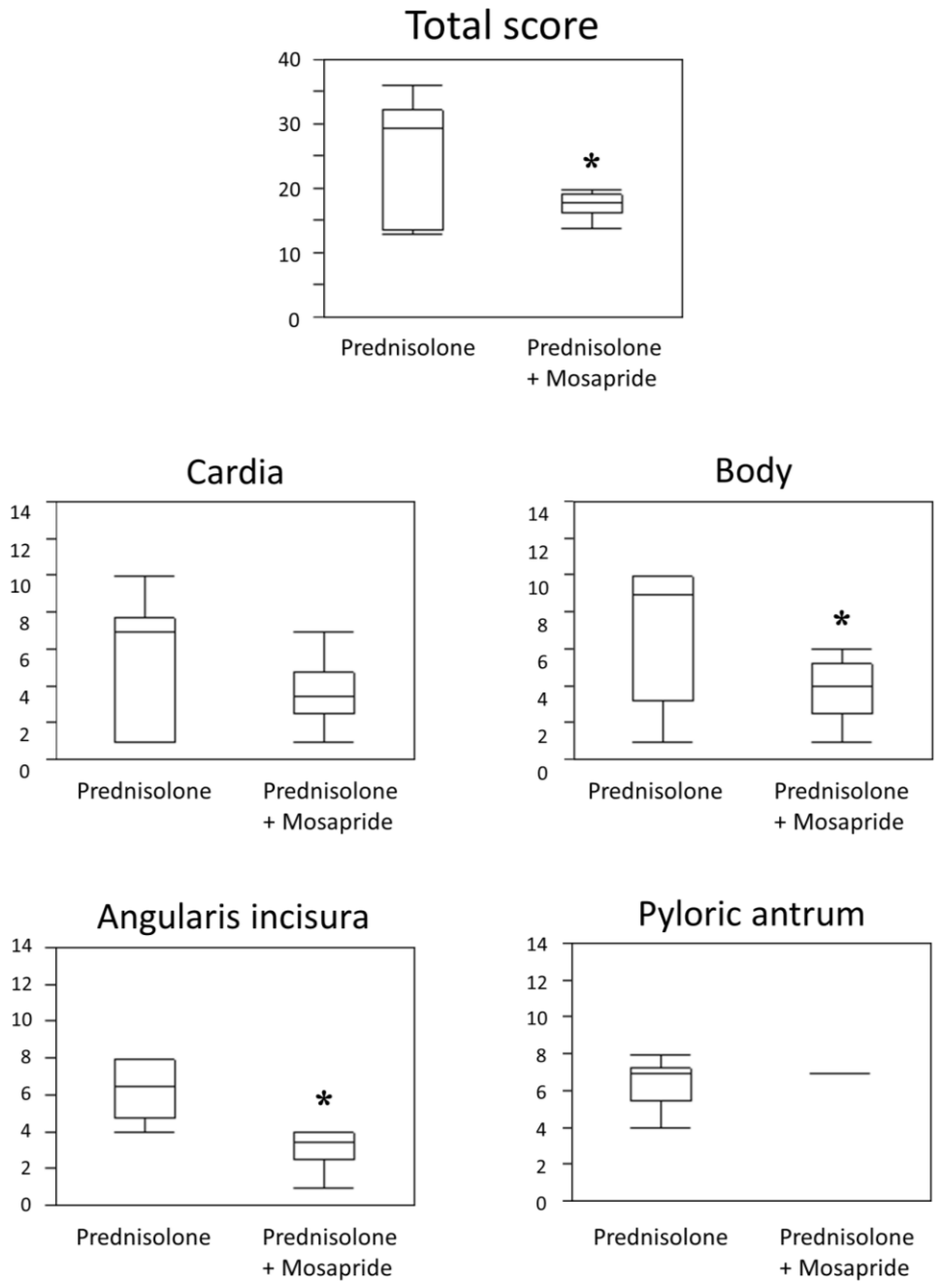

Figure 12. Gastric mucosal injury scores in each treatment. *Significant difference ($P < 0.05$) with prednisolone treatment alone.

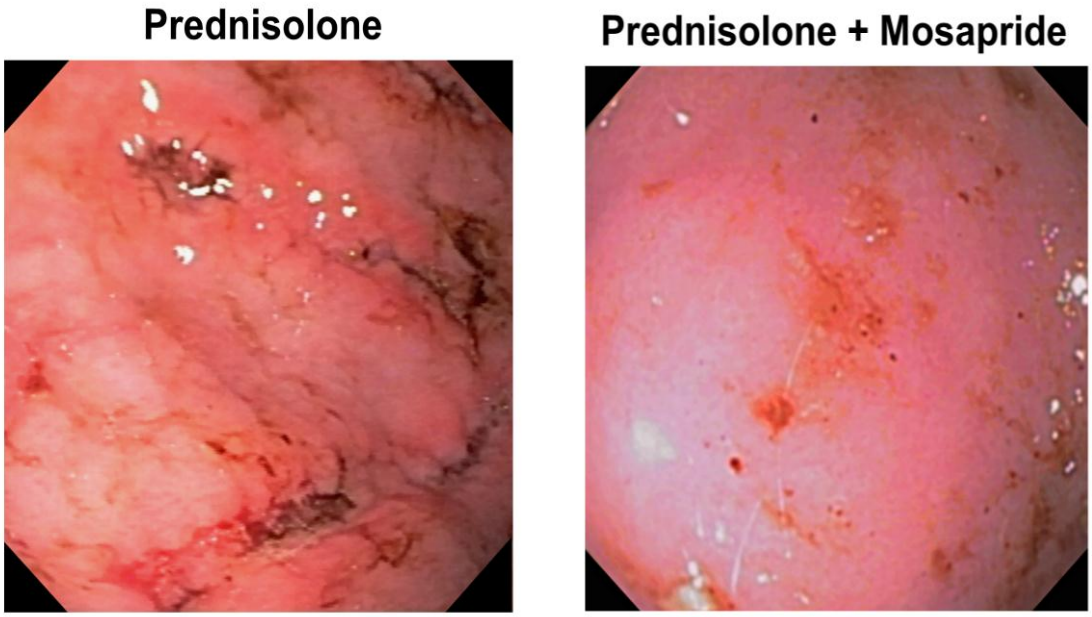

Figure 13. Endoscopic findings in the gastric body after each medication in the same dog. In this dog, multiple ulcers were observed in the gastric body after prednisolone administration. When mosapride citrate was co-administered, petechial hemorrhage was observed widely in the gastric body, but no erosion or ulcers were detected. The gastric mucosal injury scores of the gastric body in each treatment were 10 and 4, respectively.

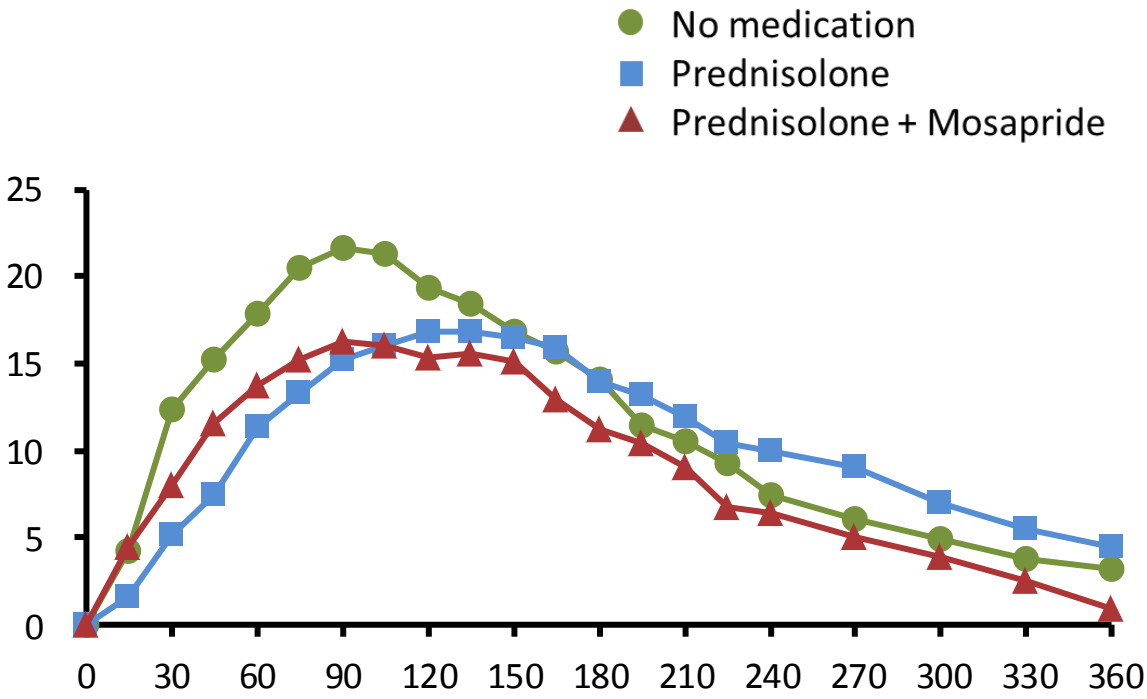

Figure 14. Gastric emptying curves determined by ^{13}C-octanoic acid breath test. Data are expressed as mean.

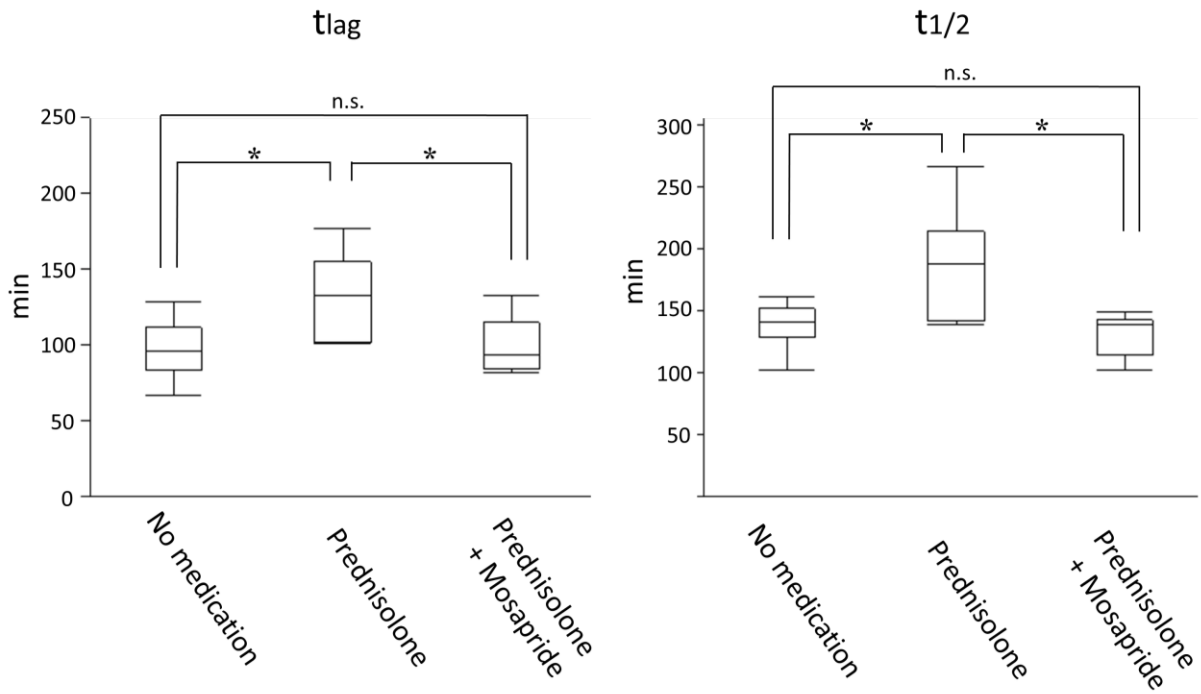

Figure 15. Results of gastric emptying parameters (t_{lag} and $t_{1/2}$) in each treatment. *$P < 0.05$. n.s. :no significance.

Chapter 4

Effect of Mosapride on Pro-inflammatory Cytokines Expression in Canine Primary Cultured Macrophage

Abstract

Recent *in vitro* studies have suggested that the 5-hydroxytryptamine-4 receptor (5-HT_4R) was expressed in monocytes and associated with the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α). The aim of the present study was to investigate the direct action of mosapride on the expression of pro-inflammatory cytokines in canine macrophage. Primary cultured macrophages were isolated from canine peripheral blood mononuclear cells (PBMCs) and stimulated with lipopolysaccharide (LPS) with or without mosapride (1-30μM). The mRNA expression of 5-HT_4R, TNF-α, interleukin-1ß (IL-1ß), interleukin-6 (IL-6) was quantified by real-time PCR. As the result, mosapride dose-dependently decreased the expression level of TNF-α (3-30μM). Mosapride at a concentration of 30 μM also significantly inhibited the IL-1ß and IL-6 mRNA level. Although the mRNA expression of 5-HT_4R mRNA was confirmed in the canine primary macrophage, the inhibitory effects of mosapride on cytokines were not antagonized by 5-HT_4R antagonist, GR113808. In conclusion, mosapride inhibits the expression of pro-inflammatory cytokines, especially TNF-α, in PBMC-derived canine macrophage without activation of 5-HT_4R.

Introduction

Previous rodent studies have demonstrated that mosapride, a 5-hydroxytryptamine-4 receptor (5-HT_4R) agonist shows anti-inflammatory action in gastric mucosal injury and post-operative ileus models (Fujisawa et al., 2010; Fujisawa et al., 2011; Tsuchida et al., 2011). This anti-inflammatory action was mediated through the cholinergic stimulation followed by 5-HT_4R stimulation. Recent studies have indicated that activation of cholinergic neurons modulates inflammation through the α7-nicotinic acetylcholine receptor (α7nAChR) expressed in immune cells such as macrophages (cholinergic inflammatory pathway). Borovikova et al. reported that acetylcholine attenuated the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1ß (IL-1ß) through α7nAChR (Borovikova et al., 2000). In Chapter 3, I have demonstrated that mosapride attenuates the prednisolone-induced gastric mucosal injury. Although the definitive mechanism of the action has not been determined, it has been suggested that the anti-ulcerogenic effect of mosapride may be associated with its anti-inflammatory action, when considering the previous studies in rat (Fujisawa et al., 2010; Tsuchida et al., 2011).

Recently, it was indicated that 5-HT_4R was expressed in immune cells such as monocyte and associate with the release of pro-inflammatory cytokines. The activation of 5-HT_4R was found to decreased the TNF-α production, whereas the release of IL-1ß

and IL-6 was promoted (Durk et al., 2005). Thus, it is possible that mosapride can regulate the pro-inflammatory cytokines not only via the cholinergic stimulation, but also with direct activation of 5-HT_4R.

In the present chapter, I assessed the direct action of mosapride on the expressions of pro-inflammatory cytokines in canine primary cultured macrophage.

Materials and Methods

Reagents

Phorbol-12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), and GR113808 were purchased from Sigma Aldrich Japan Co. LLC (Tokyo, Japan). Mosapride was kindly obtained from Dainippon Sumitomo Pharma (Osaka, Japan). Dimethylsulfoxide (DMSO) was used as the vehicle of PMA, mosapride, and GR113808. LPS was diluted in sterile PBS. All reagents were stored at -20 ◦C until use.

Cell culture

Primary cultured macrophages were obtained from peripheral blood mononuclear cells (PBMCs) of healthy beagles according to previous report (Goto-Koshino et al., 2011). PBMCs were obtained using an ordinary gravity sedimentation method. A buffy coat was isolated from EDTA-treated peripheral blood and overlaid on Optiprep (AXIS-SHIELD, Oslo, Norway) adjusted to a density of 1.079, and centrifuged at 800 × g for 30 min. Isolated PBMCs were resuspended in PBS and overlaid on Optiprep adjusted to a density of 1.063 and centrifuged at 500 × g for 15 min. Obtained PBMCs were resuspended in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % FCS and penicillin, streptomycin and 10 ng/ml of PMA. PBMCs (2×10^6 cells) were plated on a 35-mm tissue culture-treated dish (Corning, Lowell, MA, USA), and

cultured for 7 days. Following the culture, isolated canine macrophages were stimulated with 200 ng/ml LPS and cultured with or without mosapride (1 -30 μM) and the 5-HT_4R antagonist GR113808 (10 μM). The pharmacological action of selective 5-HT_4R antagonist GR113808 has previously been confirmed in dogs (Prins et al., 1999). The final concentration of DMSO in all samples was adjusted to 0.1 %.

Total RNA extraction and quantification of mRNA transcription by real-time PCR

After the 24hr incubation, total RNA was extracted from the cells using a commercially available kit (RNAspin Mini RNA Isolation Kit; GE Healthcare UK Ltd., Buckingham shire, England) according to the manufacturer's manual. Genomic DNA was removed from each sample during the procedure of RNA extraction using the kit. Extracted RNA samples were stored at -80 ◦C until use. Reverse transcription was performed using a Prime Script RT Reagent Kit (Takara Bio Inc, Tokyo, Japan). the PCR amplification was carried out with the following reactions: pre-denaturation (95 ◦C for 10 sec), 40 cycles of denaturation (95 ◦C for 5 sec) and annealing (60 ◦C for 30 sec), and extension (95 ◦C for 15 sec). The primer pairs used in the present study are shown in Table 6. The mRNA transcription was quantified using the comparative cycle threshold (Ct) method, by which the relative transcription of the target gene is reported as the n-fold difference relative to that of the calibrator gene (HPRT1). For this purpose, Ct values of the calibrator gene were subtracted from Ct values of the targets (DCt). The

transcription levels of TNF-α, IL-1β, and IL-6 in the samples of cells treated under each condition were compared. The transcription level of 5-HT_4R in canine primary macrophage, which was not treated with LPS, was compared with that in brain. The PCR products were electrophoresed in a 7.5% acrylamide, stained ethidium bromide, and visualized using an ultraviolet transiluminator.

Statistical analysis

All experiments were performed in triplicates. One way ANOVA was performed to compare the differences in cytokine mRNA levels between treatment groups. When significant, Dunnett's *t* test was performed to adjust for multiple comparisons. Data are expressed as mean ± SEM. $P < 0.05$ was considered significant.

Results

Firstly, the mRNA expression of 5-HT_4R in canine PBMCs-derived primary cultured macrophage was investigated. The RT-PCR experiments revealed the mRNA expression of 5-HT_4R in the primary cultured macrophage (Figure 16). The mean relative mRNA level of 5-HT_4R in the primary cultured macrophage was compared with that in the positive control tissue brain, which showed an approximately 1.2 fold expression of brain (Figure 17).

Next, the effect of mosapride on the mRNA level of TNF-α, IL-1β, and IL-6 level was assessed by real-time PCR. Mosapride decreased the relative mRNA level of TNF-α in a dose dependent manner (Figure 18A). Significant decreases in the TNF-α level were observed when mosapride was applied at concentrations of 3-30 μM. Mosapride also significantly decreased of the mRNA levels of IL-1β (Figure 18B) and IL-6 (Figure 18C) at a concentration of 30 μM.

Finally, I assessed whether the inhibitory action of mosapride was mediated through 5-HT_4R, by using the selective 5-HT_4R antagonist GR113808. As the result, no significant antagonistic action was observed for any of the cytokines (Figure 18A, B, C).

Discussion

I have shown that mosapride inhibits the expression of inflammatory cytokines in dose-dependent fashion, especially in TNF-α.

TNF-α is produced in the early phase of the inflammatory process, which activates the production of other pro-inflammatory cytokines and chemokines, as well as other inflammatory mediators such as cyclooxygenase, leading to the activation of early host defense (Locksley et al., 2001). Prolonged production of TNF also causes connective tissue re-modeling and frank tissue destruction. This action was associated with local inflammatory process of wound healing or mucosal injury (Kanno et al., 2011). The attenuation of gastric mucosal damage by mosapride demonstrated in chapter 3-2 may have been mediated by the anti-inflammatory action, including TNF-α inhibition. The association with the present findings and the anti-ulcerogenic action of mosapride in dogs requires further investigation.

In the present study, a significant decrease in TNF-α mRNA level was observed in canine primary macrophage at mosapride concentrations of 3-30 μM. A previous report showed that single oral administration of mosapride (10 mg/kg *SID, PO*) in healthy dogs resulted in a concentration of 0.33 μM in the plasma (Sakashita et al., 1993). The concentration of mosapride used in the present study was higher than the plasma concentration in healthy dogs after single oral administration of mosapride. However, it

has been indicated that the concentration of mosapride in liver, kidney, stomach, and small intestine after oral administration in rats were approximately 10 times higher than that in the plasma concentration (Matsumoto et al., 1993). When considering the clinical significance of the inhibitory actions of mosapride, it is necessary to investigate the dose achievable in various tissues after oral administration of the drug, as well as other routes of administration, and to determine the highest single dose that can be administered safely to dogs.

Although mosapride mediated significant decrease of the pro-inflammatory cytokines levels in the primary cultured macrophage, these actions were not antagonized by the 5-HT_4R antagonist, GR113808. I have also confirmed that other selective 5-HT_4R agonist CJ-033466 did not inhibit the expression of these cytokines in canine primary macrophage (data not shown). Therefore, the inhibitory action of mosapride was not associated with 5-HT_4R activation. The affinity of mosapride for the various neurotransmitter receptors has previously investigated. Mosapride was found to have a high affinity for 5-HT_4R and also weak affinity on 5-hydroxitriptamine-3 receptor (5-HT_3R) and the benzodiazepine receptor in rat (Yoshida, 1993). A Previous report indicated that 5-HT_3R antagonist decreases the production of TNF-α and IL-1β in LPS-stimulated monocytes (Fiebich et al., 2004). It has also been reported that midazolam, a benzodiazepine receptor antagonist, decreases the serum concentration of TNF-α, IL-1β, and IL-6 in a rodent-burn wound injury model (Babcock et al., 2011).

Although the affinity of mosapride for 5-HT_3R and the benzodiazepine receptor found to be very low in a previous report, it is undeniable that mosapride inhibits the pro-inflammatory cytokines through these receptors in canine primary macrophage.

The present study revealed that 5-HT_4R is expressed in canine primary cultured macrophage. Serotonin, a ligand of 5-HTRs, is a well-characterized neurotransmitter and vasoactive amine involved in the regulation of a large number of physiological functions such as sleep, appetite, and behavior (Lovenberg et al., 1993). Serotonin also has immunomodulatory effects regulating a wide variety of cell responses such as migration, phagocytosis, and cytokine production (Young et al., 1993; Iken et al., 1995; Laberge et al., 1996). A previous report in human indicated that monocyte expresses 5-HT_4R and activation of these receptors alters the production of TNF-α, IL-1ß, and IL-6 (Durk et al., 2005). It has also been demonstrated that human dendritic cell expresses 5-HT_4R, which regulates the release of TNF-α, IL-1ß, IL-8, and IL-12 through the elevation of intracellular cyclic adenosine monophosphate (Idzko et al., 2004). However, it has not previously been reported in any species, that macrophage express 5-HT_4R. Along with cytokine production, macrophage provides a variety of functions in host defense, such as phagocytosis, reactive oxygen production, and antigen presentation (Taylor et al., 2005). Further studies are required to investigate the function of 5-HT_4R expressed in macrophage.

In summary, the present study shows that mosapride inhibits the expression of pro-inflammatory cytokines without the activation of 5-HT_4R in canine primary macrophage. The clinical significance of this finding and the mechanism of action require further investigation.

Table 6. Sequences of oligonucleotide primers used for RT-PCR and quantitative real-time PCR.

Primer set		Primer sequence (5'-3')	Position of primers	Genebank accession number
HPRT1	Forward	CACTGGGAAAACAATGCAGA	414 - 433	NM_001003357.1
	Reverse	ACAAAGTCAGGTTTATAGCCAACA	513 - 536	
5-HT$_4$ receptor	Forward	GACAGGCAGCTCAGGAAGA	174 - 192	XM_546316.2
	Reverse	CAGCTCAATGGCACCAAAG	260 - 278	
TNF-α	Forward	TCATCTTCTCGAACCCCAAG	235 - 254	NM_001003244
	Reverse	CTGGTTGTCTGTCAGCTCCA	350 - 369	
IL-1β	Forward	CATATGAGCTTCGGGCTCTC	407 - 426	NM_001037971.1
	Reverse	ATGCCCAAGACCACAGGTAT	505 - 524	
IL-6	Forward	AAAGAGGCACTGGCAGAAA	229 - 247	AF275796.1
	Reverse	GCAGGTCTCCTGATTGAACC	299 - 318	

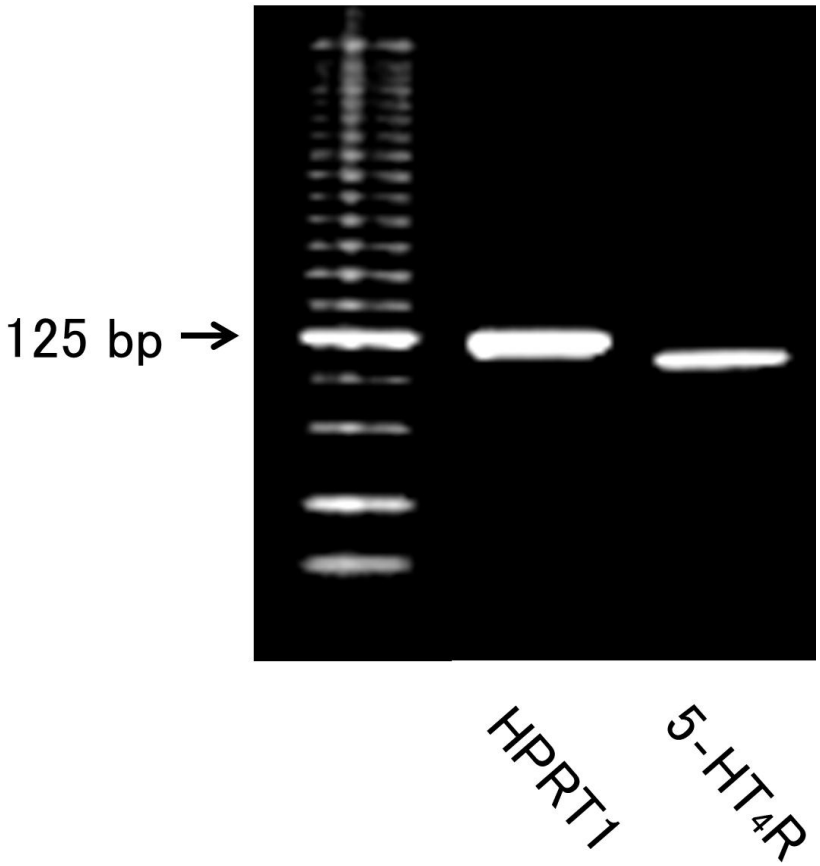

Figure 16. Expression of 5-HT_4R and HPRT1 mRNA in the primary cultured macrophage.

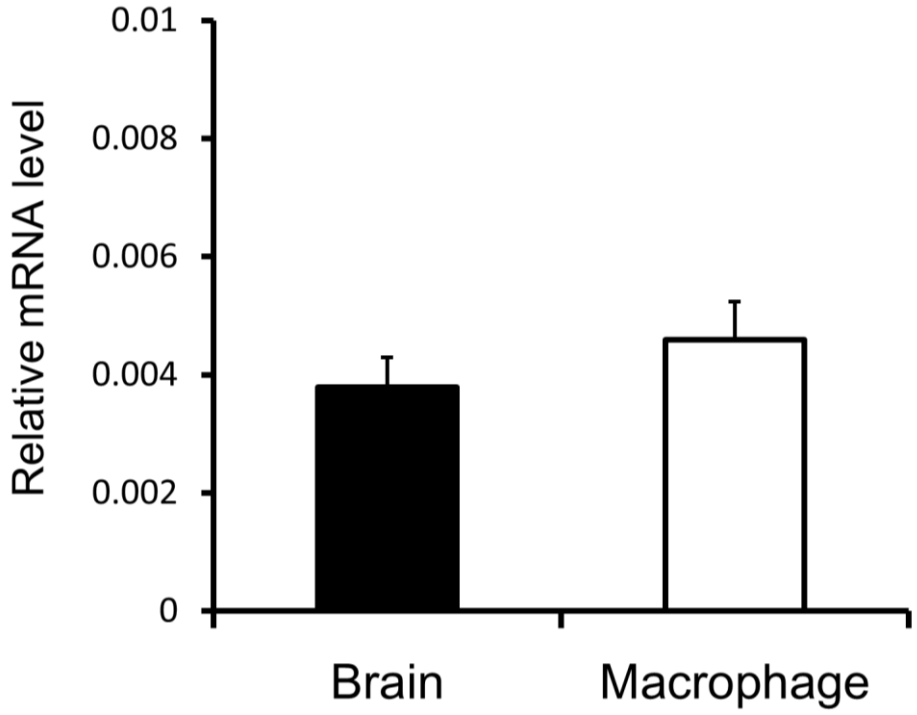

Figure 17. Relative transcription level of 5-HT_4R in primary cultured macrophage and brain.

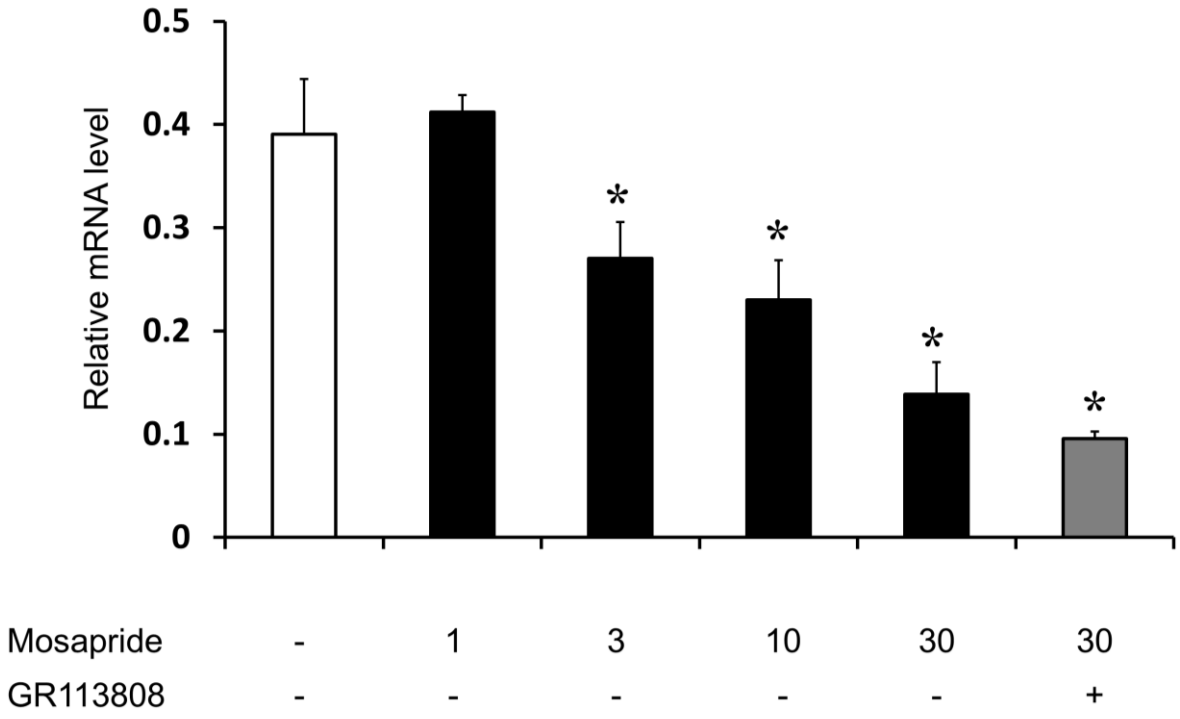

Figure 18A. Relative transcription level of TNF-α mRNA in primary cultured macrophage. LPS stimulated primary cultured macrophages were incubated in the absence or presence of mosapride and GR113808 for 24hr. *Significant difference ($P < 0.05$) with LPS treatment alone

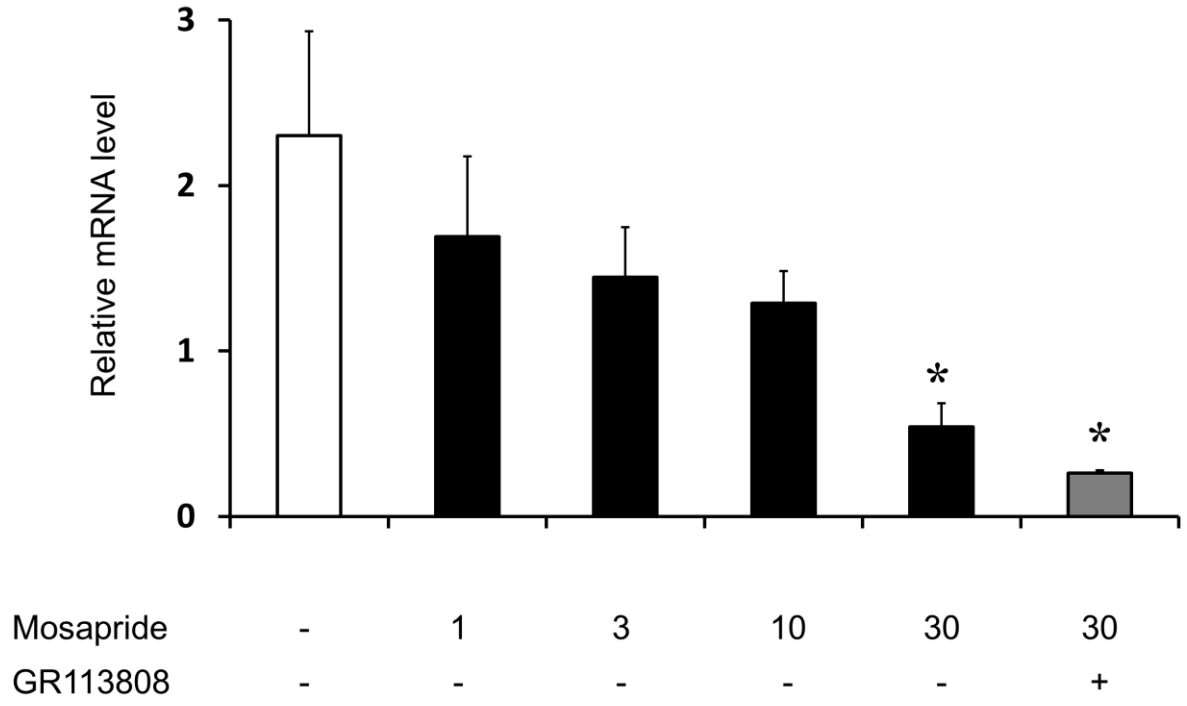

Figure 18B. Relative transcription level of IL-1β mRNA in primary cultured macrophage. LPS stimulated primary cultured macrophages were incubated in the absence or presence of mosapride and GR113808 for 24hr. *Significant difference ($P < 0.05$) with LPS treatment alone

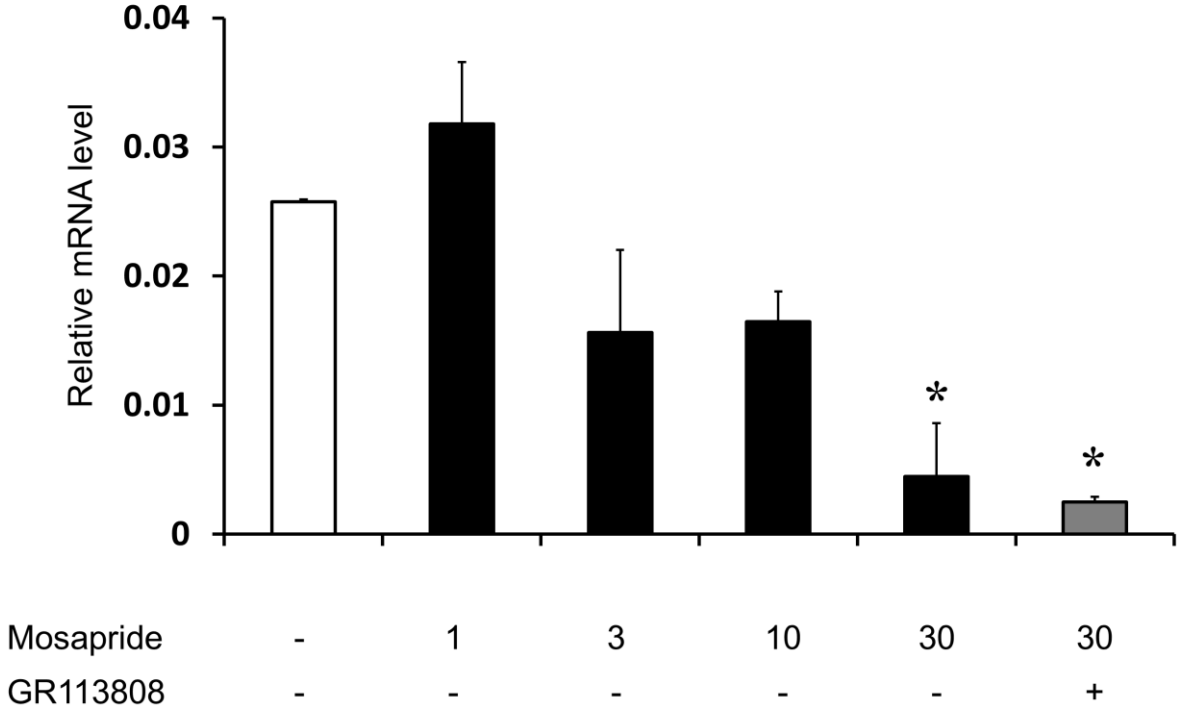

Figure 18C. Relative transcription level of IL-6 mRNA in primary cultured macrophage. LPS stimulated primary cultured macrophages were incubated in the absence or presence of mosapride and GR113808 for 24hr. *Significant difference ($P < 0.05$) with LPS treatment alone

Conclusion

A series of studies in this thesis were carried out for the investigation of canine gastric motility disorders and the efficacy of 5-HT_4R agonist prokinetic agent mosapride in dogs.

In chapter 1, a method for postprandial gastric antral motility assessment was established using ultrasonography. Although several methods for the evaluation of gastric motility have been established previously in dog (Choi et al., 2002; Wyse et al., 2003; McLellan et al., 2004), many of these methods had limitations on the requirement of specific facility or the invasion to the dogs. These limitations make it difficult to investigate the pathogenesis of canine gastric motility disorders and the clinical efficacy of prokinetic agent. Ultrasonography is non-invasive device which allows clinician to assess gastric motility at bedside. Compared with conventional methods, the novel method can assess gastric motility in a short time with minimum invasion to dogs (Choi et al., 2002; Wyse et al., 2003; McLellan et al., 2004). The novel method also enables us to assess the replicate measurement of gastric antral motility. Some of the previous methods like schintigraphy or ^{13}C-octanoic acid breath test had limitation of replicate measurement, since it requires washout period for the elimination of specific substrate. This novel ultrasonographic method supplies the real-time gastric motility information which could apply to both clinical and experimental use in dogs.

In chapter 2, the prokinetic action and safety of oral mosapride was assessed in dogs. In the study, prokinetic action of mosapride was investigated in the standpoint of

gastric antral motility and solid-phase gastric emptying. As a result of the ultrasonographic antral motility assessment, mosapride dose-dependently increased gastric antral motility in healthy dogs. This result was consistent with the study on intravenous administration of AS-4370, a drug substrate of mosapride (Yoshida et al., 1991). In the gastric emptying analysis, mosapride significantly accelerated the solid-phase gastric emptying rate, while metoclopramide administration did not. Metoclopramide was representative canine prokinetic agent that has long been used in veterinary field (Hall et al., 1999). Present study suggests that mosapride may be more recommended than metoclopramide in the cases with severe gastric motility disorder, such as gastroparesis. I also performed safety assessment to ensure the safety of mosapride in repeated administration, which resulted in no adverse events in any dog. From the studies on chapter 2, appropriate dose of mosapride that produce prokinetic action on gastric motility was determined. These studies will be served as basic study toward the clinical application of mosapride in dogs.

Based on the studies of previous chapters of this thesis, I assessed the pathogenesis of canine gastric motility disorders and clinical actions of mosapride in Chapter 3. I focused on the association of gastric motility disorders in drug-induced adverse effect of vincristine and prednisolone, and present the clinical efficacy of mosapride in dogs.

In chapter 3-1, I have demonstrated that vincristine induces gastric hypomotility, with adverse GI events of anorexia, vomiting, diarrhea and hematochezia. Mosapride

co-administration improved the gastric motility and reduced the incidence of adverse GI adverse effects, suggesting that the gastric hypomotility is associated with the pathogenesis of the vincristine-induced GI clinical signs. It is also suggested that preventive administration of mosapride is effective for the prevention of gastric motility disorder and GI adverse effects. In dogs, vincristine-induced GI adverse effects are commonly observed, which impair the QOL of dogs during chemotherapy (Tomiyasu et al., 2010). Present result will contribute to the management of vincristine-induced GI adverse events in dogs.

In chapter 3-2, I assessed the pathogenesis of GI adverse events in prednisolone. Prednisolone is a representative corticosteroid anti-inflammatory agent in veterinary field. In the present study, prednisolone induces prominent gastric mucosal damage, gastric emptying disorders, causing the GI clinical signs. The dosage of prednisolone (2 mg/kg *BID*) administered in the study was within the clinical dosage, indicating that supportive treatment is essential to prevent the GI adverse events of prednisolone when administered at this dose. Mosapride co-administration attenuated gastric mucosal injury and improves gastric emptying, which resulted in the reduction of GI clinical signs. Therefore, mosapride will be therapeutic option for the attenuation of prednisolone's GI adverse reaction by prokinetic and anti-ulcerogenic action.

Series of studies in chapter 3 revealed the association of gastric motility disorders on drug adverse reaction and demonstrated the clinical efficacy of mosapride in dogs.

These studies will contribute to the management of drug-induced adverse effect of prednisolone and vincristine in dogs. In humans, the clinical approval of mosapride is limited to the chronic gastritis or functional dyspepsia (Curran et al., 2008). As well as dogs, the GI adverse reaction of vincristine and prednisolone are common clinical problems in humans (Messer et al., 1983; Tomomasa et al., 1999). From the standpoint of the attenuation of drug adverse reaction, present study will be served as preclinical studies for the novel application of mosapride in human medicine.

Recent reports have demonstrated that mosapride had anti-inflammatory action through the promotion of accethylcholine release followed by 5-HT_4 receptor activation (Fujisawa et al., 2010; Tsuchida et al., 2011). In chapter 4, I assessed the direct action of mosapride on the pro-inflammatory cytokines expression in canine primary cultured macrophage. The mRNA expression of 5-HT_4R was observed in canine PBMC derived macrophage and mosapride dose-dependently decreased the mRNA levels of TNF-α, IL1-ß, and IL-6, especially TNF-α. However, the inhibitory action of mosapride was not mediated through 5-HT_4R. Clinical significance and the function of 5-HT_4R expressed in the macrophage requires further investigation.

In humans, gastric motility disorders are associated with GI clinical symptoms in various diseases, such as diabetes, gastroenteritis, or cancer (Ali et al., 2007). These diseases are also observed in veterinary field, and sometimes occur with upper GI signs. By using the ultrasonographic method established in chapter 1, the association of GI

clinical signs and gastric motility disorders in cases should be evaluated in future studies. The present studies revealed the various actions of mosapride, which include prokinetic, anti-ulcerogenic and possibly anti-inflammatory actions. The clinical trials of mosapride in dogs may also be an issue on future.

In conclusion, the series of studies on this thesis will contribute to the management of upper GI clinical symptoms associated with gastric motility disorders in dogs. Furthermore, these works serve as preclinical study towards novel clinical application of mosapride in dogs and also humans, leading to the better management of upper GI symptoms.

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