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Induction of Heme Oxygenase-1 by Traditional Herb Mix Extract Improves MKN-74 Cell Survival and Reduces Stomach Bleeding in Rats by Ethanol and Aspirin *in vivo*

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Chinese herb medicines have traditionally been used to treat or alleviate the symptom of various diseases. The rationale for use of certain herbs to certain disorder is now getting unveiled by modern technology. In the present study, we investigated whether herb mix extract (HMX), which is alleged to be useful for gastric ulcer, protects stomach from oxidative stress. Rats were allowed to normal diet with and without HMX (1, 5, 10 mg/kg) for 30 days. To induce gastric ulcer, ethanol (75%, 1.5 ml) or acidified aspirin (100 mg/kg in 0.2 N HCl) was administered by oral route in 24 h-fasted rats and examined the gastric ulceration (bleeding) by measuring the size 1 h after the treatment. Results indicated the area of gastric bleeding was significantly less in HMX fed rats than in normal diet fed ones, and it was dependent on the duration and amount of HMX. To investigate the underlying mechanism by which HMX protects stomach from oxidative stress, expression of enzymes like heme oxygenase (HO), cyclooxygenase (COX), and inducible nitric oxide (iNOS) were investigated in MKN-74 cells, where aspirin or *H. pylori* was introduced. The results were compared with RAW 264.7 cells to check if there's cell specificities exist. The expression of HO-1 but not COX-2, iNOS was significantly increased by HMX. Furthermore, HO-1 inhibitor, SnPP IX reduced the HO-1 activity and reversed the survival rate in HMX-treated MKN-74 cells. There's no difference between RAW 264.7 cells and MKN-74 cells. We, thus, concluded that HMX is beneficial for protection from oxidative injury, and induction of HO-1 by HMX in gastric cells is, at least, responsible for protection from oxidative stress such as ethanol, aspirin and possibly *H. pylori* infection.

Key Words: Reactive oxygen species, Heme oxygenase, Alcohol, Aspirin, Stomach

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin are widely used, but major limitations to their clinical administration are serious side effects associated with oxidative stress such as acute hemorrhagic erosions, aggravating effect on stress ulceration, and interference with healing of preexisting gastric ulceration (Lanza 1984; Wang et al, 1989; Konturek et al, 1990; Levi et al, 1990; Wallace et al, 1994). *Helicobacter pylori* (*H. pylori*) infection and alcohol consumption are also increase oxidative stress in the body, where stomach is most vulnerable for these stress. Studies in humans have shown an accumulation of reactive oxygen species (ROS) in the gastric mucosa in taking aspirin and *H. pylori*-infected subjects, which was associated with increased levels of oxidative DNA damage (Kawanishi & Hiraku, 2006). Recent studies demonstrate that expression of heme oxygenase (HO)-1 is increased in oxidative stress as defense mechanism. Thus, induction of HO-1 is believed to have cytoprotective effects in models of oxidant-induced cellular and tissue injury (Otterbein & Choi, 2000).

Traditionally, many prescriptions are available to treat gastric disorders with Chinese herb medicine. In Korea, people reside near Jinju area have long been traditionally used a special herb mix extract (HMX; see the composition of specific herbs) to treat gastric ulcer or stomach ache. Furthermore, there is a belief that the plant growing in the filed of Ji-ri mountain contains much more therapeutic action than other areas. Although traditional herbs have plenty of polyphenols which have anticipated play an antioxidant role, the mechanism by which HMX relieves gastric ulcer is not clear. Therefore, the aim of the present study is to confirm 1) HMX really reduces gastric bleeding induced by ethanol or aspirin *in vivo*, and 2) to investigate the possible mechanism of action for which HO-1 activity is involved. We found that HMX significantly reduced gastric bleeding in rat stomach administered with acidified aspirin and alcohol and HMX augmented HO-1 expression in cells treated with alcohol or *H. pylori*.

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ABBREVIATIONS: CO, carbon monoxide; *H. pylori, Helicobacter pylori;* HMX, herb mix extract; HO, heme oxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

METHODS

Source and reparation of HMX

Dried leaves, stems, fruit or root of *Atractylodes japonica koidzumi, Platycodon grandiflorum, Leonurus joponicus Houll, Cornus officinalis Sieb.et Zucc, Acanthopanax sessiliflorus, Adenophora triphylla var. japonica Hara, Chrysanthemum sibiricum, poncirus trifoliate, and Glycyrrhiza uralensis Fisch* were purchased from local practitioner. These materials (total 300 g) were put together in a 200 L jar and boiled at 100°C for 24 h. Then filtered the filtrate with gauze and the obtaining filtered fluids (herb mix extract, HMX) were dried under liquid freezing dryer. The ratio of each component that makes HMX was shown in Table 1.

Gastric protective effects of HMX against ethanol and aspirin injury

Spague-dawley rats were divided into 2 experimental groups in which each group was consisted 5 animals. Control groups were administered distilled water, while experimental groups were administered HMX (10 mg/ml) for 10, 20, and 30 days. In separate experiments, rats were administered HMX (1 mg/ml, 5 mg/ml, 10 mg/ml) for 30 days. All animals were fasted 24 h prior to oral administration of acidified aspirin (100 mg/kg in 0.2 N HCl) or alcohol (1.5 ml of 75% ethanol). One hour later, rats were killed under anesthesia (ketamine and rumpun) and excised the stomach and measured the bleeding area after fixed with 10% formalin.

Effect of HMX in the presence of ethanol on MKN-74 cell viability

MKN-74, human gastric cancer cell line, was obtained from Korea cell bank. The cells were cultured in RPMI with 10% FBS and 1% antibiotics under CO_2 incubator at 37°C until confluent. After cells were grown, cells were divided into 24 well-plate as concentration of 1×10^5 per well which was incubated for 2 days. On the day of experiment, media was replaced with new one and treated with extract as well as 10% alcohol as indicated. For MTT, cells were incubated for 3 h and media were discarded. MTT reagent (1 mg/ml) was diluted 10 : 1 with RPMI and further incubated for 3.5 h. The cells are mixed with DMSO and absorbance was measured at 570 nm.

Table 1. The composition of HMX

Medicinal herbs	Dry weight (g)	Remarks
Atractylodes japonica koidzumi	157	root
Platycodon grandiflorum	40	root
Leonurus joponicus Houll	14	stem, root, leaf
Cornus officinalis Sieb.et Zucc	6	fruit
Acanthopanax sessiliflorus	12	root
Adenophora triphylla var. japonica Hara	30	root
Chrysanthemum sibiricum	12	flower, stem
Poncirus trifoliate	8	fruit
Glycyrrhiza uralensis Fisch	36	root

These medicinal plants are naturally growing in the fields of mountain "Ji-ri", and all the materials were purchased from local practitioner. These materials (300 g) were put together in a 200 l jar and boiled for 24 h. After filtering the filtrate with gauze, only the solution was used. The obtaining fluids (herb mix extract, HMX) were dried under liquid freezing dryer. HMX was weighed and used as mg/kg (for *in vivo*) or mg/ml (*in vitro*).

Western blotting analysis

Cells were treated with aspirin for 6 h. After this, media were washed 3 times with cold PBS solution. Then cells were harvest and extract with protein extract solution provided by Intron. The protein concentrations were measured by Bradford method and SDA-PAGE was applied with 30 μg proteins. After electrophoresis, the proteins were transferred to nylon membrane which was subjected to Western blotting with HO-1, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) polyclonal antibody.

Heme oxygenase activity

To determine HO enzyme activity, confluent cells were incubated in 100 mm culture dishes for 9 h with or without HMX. Some experiments were done in the presence of a HO enzyme inhibitor, SnPP IX. After incubation, the cells were washed twice with 1×phosphate-buffered saline (PBS, pH 7.4) and centrifuged (100,000 g, 5 min, 4°C). The cell pellet was suspended in 2 mM $MgCl_2$ in 100 mM phosphate buffer (pH 7.4), frozen at −70°C, thawed three times and finally sonicated on ice before centrifugation at 100,000 g for 15 min at 4°C. The supernatant (400 μl) was added to an NADPH-generating system containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate-1-dehydrogenase, 2 mg protein of rat liver cytosol (prepared from the 105,000 g supernatant fraction), 100 mM potassium phosphate buffer (pH7.4), and hemin (10 μM) in a final volume of 200 μl. The reaction was incubated for 1 h at 37°C in the dark and terminated by addition of 600 μl chloroform. The extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm using a quartz cuvette (ε.= 40 μM/cm). HO-1 activity was represented as picomoles of bilirubin formed per milligram of protein per hour.

Statistical evaluations

Values are expressed as mean±S.E.M. The significance of the difference from the respective controls for each experimental test condition was assayed by using student's t test for each pair experiments. $p<0.05$ was regarded as a significant difference.

RESULTS

Protective effects of HMX on gastric bleeding injury by aspirin and ethanol

As shown in Fig. 1A, the size of gastric bleeding was 81.4 mm^2 and 79.2 mm^2 by aspirin and ethanol, respectively. However, depending on the administrative duration and amount of HMX, the size of was decreased. For example,

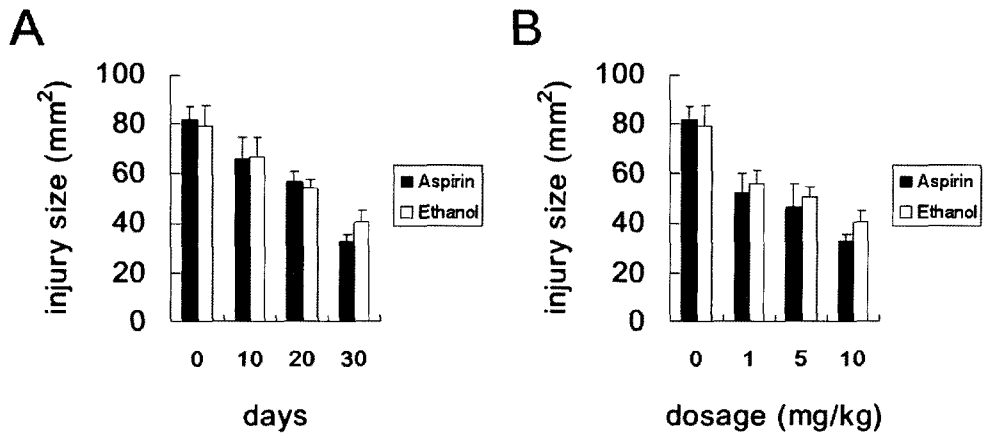

Fig. 1. Effects of HMX against ethanol and aspirin-induced injury in rat stomach. Rats were administered HMX (10 mg/ml) for 10, 20, and 30 days (A) and 1, 5, and 10 mg/ml for 30 days (B). All animals were fasted during 24 h before administration of acidified aspirin (100 mg/kg in 0.2 N HCl) or 1.5 ml of 75% ethanol. One hour later, rats were killed under anesthesia (ketamine and rumpun) and measured the area of injury (bleeding) in the stomach, which was fixed with 10% formalin.

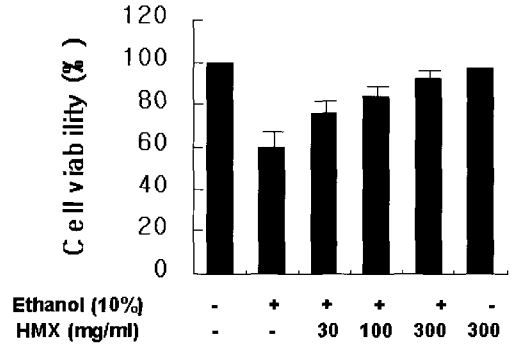

Fig. 2. Effects of HMX on alcohol-induced cell viability. MKN-74 cells were cultured in RPMI with 10% FBS and 1% antibiotics under CO_2 incubator at 37°C until confluent. After cells were grown, they were divided into 24 well-plate as concentration of 1×10^5 per well and incubated for 2 days. On the day of experiment, media was replaced with new one and treated with HMX as well as 10% alcohol as indicated. For MTT, cells were incubated for 3 h and discarded media. MTT reagent (1 mg/ml) was diluted 10 : 1 with RPMI and further incubated for 3.5 h. The cells are mixed with DMSO and measured the absorbance at 570 nm.

it reduced to 32.4 mm^2 (aspirin) and 40.2 mm^2 (ethanol) by HMX (10 mg/kg) for 30 days. Likewise, dosage also influenced the protective effect against ethanol or aspirin. In aspirin-induced gastric injury, the protective effect as represented by percentage of the dosages 1, 5, and 10 mg/kg was gradually increased to 36, 43 and 61%, respectively. In ethanol-induced injuries, 1 mg/kg HMX treatment resulted in 28.8% reduction in bleeding area compared to the positive control, which was increased to 35.9% (5 mg/kg) and 49.3% (10 mg/kg)(Fig. 1B).

Cell viability

Fig. 2 shows that HMX do not influence the cell survival. However, increased toxicity induced by alcohol was significantly reduced by the presence of HMX and it was concentration dependent.

Induction of HO-1 by HMX in cells treated with aspirin and H. pylori

Aspirin induced HO-1 protein in MKN-74 cells, which was significantly augmented by treatment with HMX (Fig. 3A). There was no cell specificity in the induction of HO-1 by HMX as shown in RAW 264.67 cells (Fig. 3A). Fig 4B shows that *H. pylori* infection also induced HO-1 expression in both RAW 264.7 cells and MKN-74 cell. However, in the presence of HMX, HO-1 protein expression increased as the HMX concentration increases. HO-2 protein, internal control, was not increased even though HMX concentration goes up.

Effect of HMX on COX-2 expression in cells infected with H. pylori

COX-2 protein was induced by *H. pylori* infection in both RAW 264.7 cells and MKN-74 cells. This indicates that *H. pylori* infection can increase inflammation-related genes. As shown in Fig. 4A, COX-2 protein expression was significantly and concentration-dependently decreased by the presence of HMX, indicating that administration of extract reduces inflammation. Another inflammation-related gene, iNOS was induced by *H. pylori* infection in both RAW 264.7 cells and MKN-74 cells. As shown in Fig. 4B, iNOS protein expression was also significantly and concentration-dependently decreased by the presence of HMX.

Effects of SnPP IX on the HO-1 activity and cell survival in HMX-treated MKN-74 cells.

As shown in Fig. 5A, HMX concentration-dependently increased HO-1 activity in MKN-74 cells. The increased HO-1 activity was significantly inhibited by SnPP IX, HO-1 inhibitor. The reduced cell viability due to ethanol was reversed by the HMX, which was significantly antagonized by SnPP IX (Fig. 5B).

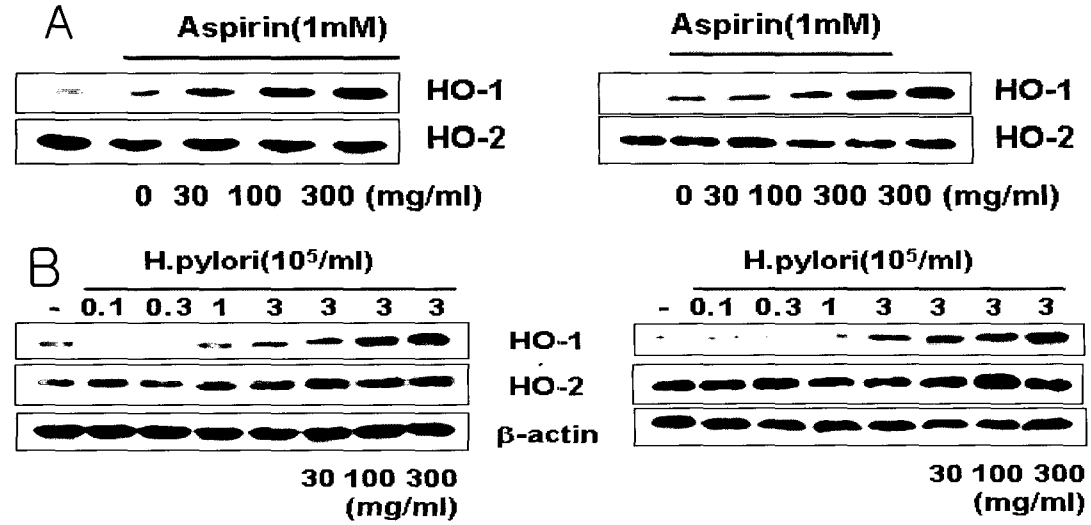

Fig. 3. HMX increases HO-1 and HO-2 expression in aspirin (A), and *H. Pylori* (B)-treated RAW 264.7 cell (left) and MKN-74 cell (right). Cells were treated with aspirin for 6 h. After this, media were washed 3 times with cold PBS solution. Then cells were harvest and extracted the protein. The protein concentrations were measured by Bradford method and SDS-PAGE was applied with 30 μg proteins. After electrophoresis, the proteins were transferred to nylon membrane which was subjected to Western blotting with HO-1 polyclonal antibody.

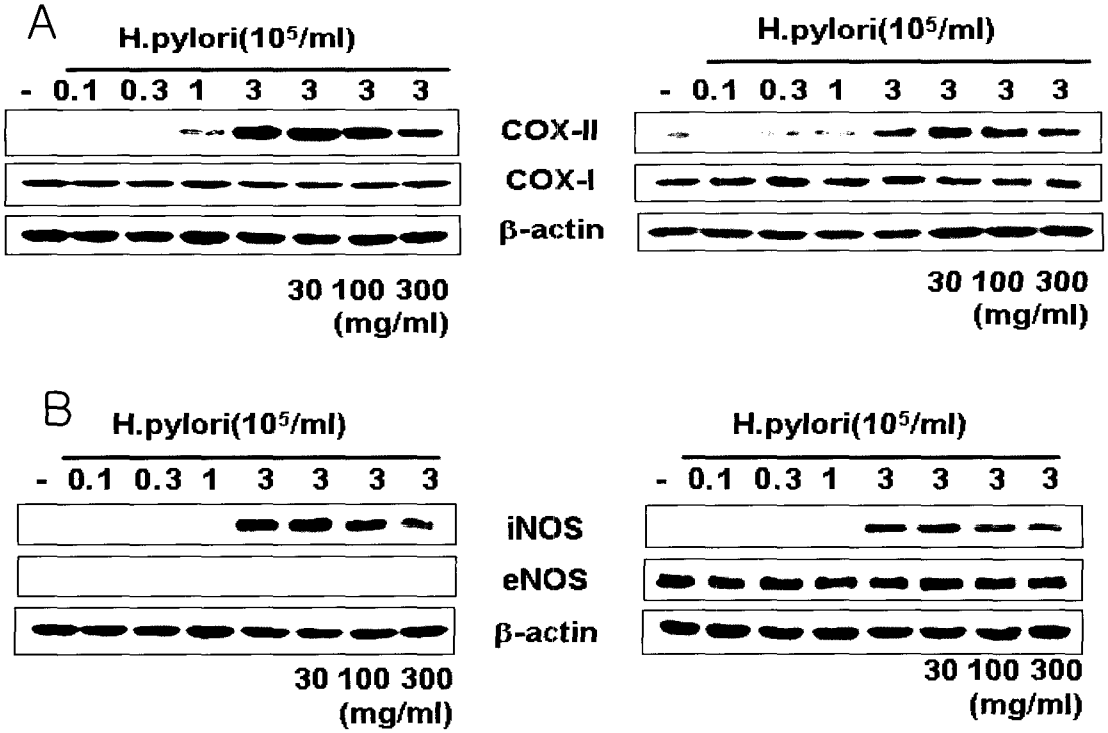

Fig. 4. Effects of HMX on COX-1 and COX-2 (A) and iNOS and e-NOS (B) expression in *H. Pylori*-treated RAW 264.7 cell (left) and MKN-74 cell (right). Cells were treated with *H. Pylori* for 6 h. After this, media were washed 3 times with cold PBS solution. Then cells were harvest and extracted the protein. The protein concentrations were measured by Bradford method and SDS-PAGE was applied with 30 μg proteins. After electrophoresis, the proteins were transferred to nylon membrane which was subjected to Western blotting with COX-1, 2, iNOS, and e-NOS polyclonal antibody.

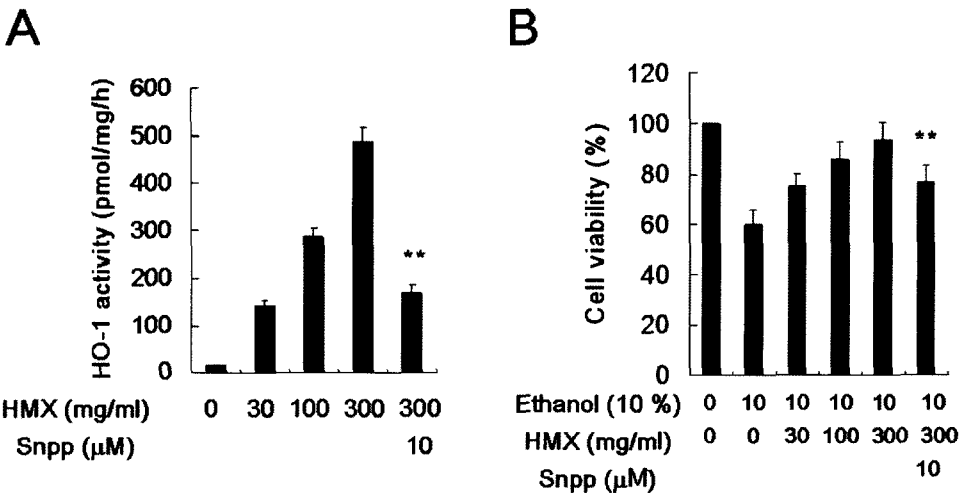

Fig. 5. HMX increases HO-1 activity (A), and cell viability in alcohol-treated cells which is reversed by SnPPIX (B). After incubation, the cells were washed twice with 1×phosphate-buffered saline (PBS, pH 7.4) and centrifuged (100,000 g, 5 min, 4°C). The cell pellet was suspended in 2 mM $MgCl_2$ in 100 mM phosphate buffer (pH 7.4), frozen at −70°C, thawed three times and finally sonicated on ice before centrifugation at 100,000 g for 15 min at 4°C. The reaction was initiated by incubating for 1 h at 37°C in the dark and terminated by addition of 600 μl chloroform. The extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm using a quartz cuvette (ε.= 40 μ M/cm). HO-1 activity was represented as picomoles of bilirubin formed per milligram of protein per hour.

DISCUSSION

To our knowledge, this is the first report that HMX augmented HO-1 expression in MKN-74 cells treated with *H. pylori* or aspirin. The present investigation clearly shows that oral administration of HMX significantly reduces gastric bleeding in rats treated with ethanol or acidified aspirin *in vivo*. The protective effect was closely related with HMX dosage and duration of administration. In fact, HMX has long traditionally been used for treatment of stomach ache, in particular, to those who reside in the rural area of Jinju city, and it is said to be good for the stomach ache if one takes it before or after heavy drink. ROS were originally implicated in the gastric injury provoked in animals using various experimental models including aspirin and NSAIDs (Son et al, 1996; Tanaka et al, 1996). Ethanol also has deleterious effects on cells in relation with ROS: ethanol is converted into acetaldehyde via intracellular oxidation, eventually generating ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydrogen radical (· OH). These ROS create oxidative conditions, resulting in alterations of DNA, lipids, and proteins that affect cell survival (Sagara et al, 1998). Thus, the deleterious effects of ethanol on cells have been associated with oxidative stress (Pirlich et al, 2002). The production of ROS in peripheral blood is increased in individuals with *H. pylori* infection (Mashimo et al, 2006) and *H. pylori* water extract-induced mouse skin carcinogenesis by ROS have been recently reported (Ishikawa et al, 2006). Thus, ROS can be one of major factors to cause gastric bleeding by aspirin or ethanol. Although administration of HMX reduced gastric bleeding, the precise mechanism to protect stomach from ethanol- or aspirin-induced damage is not known. It is reported that the root of *Atractylodes japonica,* the main and large proportion composing HMX, is digestive and stomachic (Yeung, 1985; Brown, 1995). We believe that HO-1 induction seems, at least, to be responsible for this. The reason is based on the present results that 1) HMX increased HO-1 induction in MKN-73 cells treated with aspirin and *H. Pylori in vitro,* 2) it increased survival rate of ethanol-treated cells, which was reversed by SnPP IX, and 3) SnPP IX, HO-1 inhibitor, decreased the HO-1 activity in HMX-treated cells. In fact, accumulating evidence indicates that HO-1 induction protects cells from ROS such as ischemia reperfusion injury (Lee et al, 2006), and overexpression of HO protects cells from oxidative stress (Chen et al, 2000; Satoh et al, 2003). HO is present in most mammalian tissues and catalyzes the degradation of heme to biliverdin, releasing equimolar amounts of biliverdin IXa, iron, and carbon monoxide (CO) (Mains, 1997). The expression of HO-1 is sensitive to induction by oxidants. We found that ethanol and *H. pylori* induced HO-1 in cells, thus confirming ethanol and *H. pylori* acts as an oxidant in the present study. At the present time, how HMX augmented HO-1 expression in those cells treated ethanol or *H. pylori* is not known. Then, does HMX act as an oxidant? It is unlikely because it protected the gastric cell from oxidative stress. It, however, may be possible HMX affect cyclic nucleotides contents in the cells (Ko et al, 2002; Oh et al, 2006) or redox states by acting as antioxidant, which are responsible for the induction of HO-1. Cells have multiple defense systems against ROS. As shown in the present study, upon exposure to ROS (aspirin, ethanol), both RAW 264.7 cells and MKN-74 cell induce HO-1 to protect themselves (Ishii et al, 1999). Induction of HO-1 gene expression and the related production of CO in many cells play essential roles in protecting the cells against inflammation or oxidative stress (Li et al,

2000; Chow et al, 2005; Lin et al, 2005; Sawle et al, 2005). These findings indicate that immune (RAW 264.7 cell) or non-immune cell (MKN-74 cell) alike induce HO-1 expression against oxidative stress, where HMX further increased the HO-1 induction. The beneficial effect of HO-1 is more evident from the result that SnPP IX, HO-1 inhibitor, reversed the increased cell survivability by HMX in ethanol-treated cells.

In conclusion, we found that administration of HMX palliated the gastric injuries occurred by acute ethanol ingestion or acidified aspirin *in vivo*. This confirms the fact HMX relieves gastric pain in those who took this before or after strong liquor in humans. We also found that HMX increased HO-1 expression in RAW 264.7 cells and MKN-74 cells when treated with ethanol or *H. pylori*. Therefore, HMX can be useful for the treatment of gastric ulcers in susceptible patients who take NSAIDs (rheumatoid arthritis), habitual drinkers or *H. pylori*-infection.

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