Protective Effect of Verapamil on Gastric Hemorrhagic Ulcers in Severe Atherosclerotic Rats

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Abstract

Studies concerning with pathogenesis of gastric hemorrhage and mucosal ulceration produced in atherosclerotic rats are lacking. The aim of this study is to examine the role of gastric acid back-diffusion, mast cell histamine release, lipid peroxide (LPO) generation and mucosal microvascular permeability in modulating gastric hemorrhage and ulcer in rats with atherosclerosis induced by co-administration of vitamin D_2 and cholesterol. Additionally, the protective effect of verapamil on this ulcer model was evaluated. Male Wistar rats were challenged intragastrically once daily for 9 days with 1.0 ml/kg of corn oil containing vitamin D_2 and cholesterol to induce atherosclerosis. Control rats received corn oil only. After gastric surgery, rat stomachs were irrigated for 3 h with either simulated gastric juice or normal saline. Gastric acid back-diffusion, mucosal LPO generation, histamine concentration, microvascular permeability, luminal hemoglobin content and ulcer areas were determined. Elevated atherosclerotic parameters, such as serum calcium, total cholesterol and low-density lipoprotein concentration were obtained in atherosclerotic rats. Severe gastric ulcers accompanied with increased ulcerogenic factors, including gastric acid back-diffusion, histamine release, LPO generation and luminal hemoglobin content were also observed in these rats. Moreover, a positive correlation of histamine to gastric hemorrhage and to ulcer was found in those atherosclerotic rats. This hemorrhagic ulcer and various ulcerogenic parameters were dose-dependently ameliorated by daily intragastric verapamil. Atherosclerosis could produce gastric hemorrhagic ulcer *via* aggravation of gastric acid back-diffusion, LPO generation, histamine release and microvascular permeability that could be ameliorated by verapamil in rats.

Key Words: gastric hemorrhagic ulcer, histamine, acid back-diffusion, microvascular permeability, verapamil

Introduction

Vitamin (vit) D is a prohormone that serves as a precursor to a number of biological active metabolites and as a regulator of calcium transport across the cellular membranes. Vitamin D_2 (ergocalciferol), a plant-derived form of vit. D, is present in the diet. It can increase calcium and phosphate in blood and tissues. Whereas cholesterol is a precursor to the synthesis of steroid hormones, vit. A, vit. D and bile acids (32). Vitamin D and cholesterol play important roles in the physiological homeostasis. Deficiency of vit. D may lead to hypocalcemia, osteoporosis or ricket while deficiency of cholesterol may result in type C Niemann-Pick disease (7) or Smith-Lemli-Opitz syndrome (35). These metabolism disorders can be ameliorated by supplementation of correspondent nutrient. However, co-treatment of vit. D_2 and cholesterol can cause increase in cholesterol, low-density lipoprotein (LDL) and calcium levels that are closely associated with atherosclerosis and injury of endothelial cells of blood vessels in rats (4, 24). Whether or not atherosclerosis

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induced by the mixture of vit. D_2 and cholesterol can produce gastric hemorrhage and mucosal ulceration however is totally unknown.

On the other hand, the integrity of gastric mucosa is greatly associated with offensive factors existed in the stomach. Aggravation of offensive factors, such as increased gastric acid back-diffusion (mucosal permeability to hydrogen ions), vascular permeability, histamine release and lipid peroxide (LPO) generation, may result in gastric hemorrhage and mucosal damage (19, 20). When gastric mucosal barriers are disrupted by ulcerogens, including ethanol, aspirin and bile salts, the intraluminal free acid may back diffuse through damaged mucosal barriers and consequently leads to exacerbation of gastric hemorrhagic ulcer. This back-diffusion of gastric acid is recognized as a sensitive index for mucosal integrity (6, 18) and is related to gastric oxidative stress .

Ample document indicate that the development of atherosclerosis is greatly associated with oxidative stress (36-41). Whether or not this oxidative stress can aggravate gastric mucosal damage also remains obscure.

On the other hand, histamine, one of the offensive factors, is an autacoidal neurotransmitter widely distributed in biological tissues. It is an important mediator in the process of inflammation. The release of histamine can be enhanced whenever there is urticaria or nonspecific cell-damage from any cause. Mast cells are one of major resources of histamine release. Pharmacologically, histamine is a potent gastric acid stimulator. It also produces vasodilatation and increases in permeability of blood vessel walls that may contribute to gastric hemorrhage (20). Furthermore, over-production of histamine can result in gastric ulceration (2).

Verapamil is a calcium channel blocker, which exerts potent amelioration of hypertension, angina, arrhythmia and cardiomyopathy. Verapamil also possesses antioxidant effect on various diseases, including diabetic nephropathy (23), ischemia-induced hepatic damage (34) and atherosclerotic formation (13). Whether or not verapamil can inhibit gastric hemorrhagic ulcer in atherosclerotic rats also remains obscure. The aim of the present study is to investigate aggravation of gastric acid back-diffusion, LPO generation, mast cell histamine release, gastric vascular permeability and hemorrhagic ulcer in rats with atherosclerosis induced by vit. D_2 and cholesterol. The protective effect of verapamil on various gastric ulcerogenic parameters in atherosclerotic rat stomachs also was evaluated.

Materials and Methods

Chemicals

All chemicals used were in reagent grade and purchased from Sigma, (St. Louis, MO, USA) or Wako Chemicals, (Tokyo, Japan). The purity of all drugs was over 98%. All chemical solutions were freshly prepared before use.

Animals

Male specific pathogen-free Wistar rats, weighing 200-250 g, were obtained from and housed in The Laboratory Animal Center, National Cheng Kung University, Tainan, Taiwan. Rats were housed individually in a room with 12-h dark-light cycle and with central air conditioning (25°C temperature, 70% humidity). They were allowed free access to water and pellet diets (the Richmond standard, PMI Feeds, Inc. St. Louis, MO, USA). The animal care and experimental protocols were in accordance with the guidelines of The National Science Council of Taiwan (NSC 2005). Rats were daily challenged intragastrically at 8:00 a.m. with 1.0 ml/kg of a corn oil mixture containing 4.5 mg/l vit. D_2 and 22.5 mg/l cholesterol for 9 days. Age-matched control rats received the same volume of corn oil in a same period of time. Rat blood samples were harvested from aorta at the end of experiment. Blood samples were centrifuged at 1000 × g for 10 min at 4°C. Serum cholesterol and LDL levels were assayed by using cholesterol and LDL kits (SERA-PAK, Cholestero LDL, Bayer Corporation, Tarrytown, NY, USA), respectively, on an ELISA (Serial RS-232C, Labsystems Multiskan Ex, Finland). Serum calcium concentration was measured on a flame photometer (Eppendorf, FCM 6341, Hamburg, Germany).

Surgical Procedures

Prior to the test, rats were deprived of food for 24 h and anesthetized with diethylether. Rat stomachs were surgically exposed for the ligation of pylorus and lower esophagus. To prevent the spontaneous gastric secretion, a bilateral diaphragmatic vagotomy was performed in all rats. A small incision was then made in the forestomach. A polypropylene tube (3.5 mm internal diameter × 20 mm long) was inserted through the same incision and secured with a ligature. The stomach was subsequently rinsed meticulously with warm normal saline (37°C). Care was taken to avoid injury of blood vessels. The residues were gently removed.

Measurement of Gastric Acid Back-Diffusion

Gastric acid back-diffusion (the H^+ loss in the gastric lumen) was quantified by the method previously described (19). Briefly, normal saline or simulated rat gastric juice (7 ml) containing 100 mM HCl, 17.4 mM pepsin and 54 mM NaCl was instilled into the cleansed stomach with a 10 ml-disposable syringe.

Luminal contents were mixed with the same syringe by three repeated aspirations and injection. Three ml of fluid were taken as an initial sample. The forestomach was tightly closed. The abdominal wound was sutured. Rats were sacrificed with an overdose of diethylether 3 h later. Gastric contents (the final sample) were collected. Both initial and final samples were centrifuged at 1000 × g for 20 min at 4°C. The volumes of initial and final samples were measured. The acidity of these samples was assessed by titrating 1.0 ml of gastric contents sample with 100 mM NaOH to pH 7.0 on an autoburette titrator (Radiometer, Copenhagen, Denmark). The net flux of ions through the gastric mucosa was calculated as follows:

$$\text{Net flux} = Fv \times Fc - (7\text{-}Iv) \times Ic.$$

Where, Fv and Iv are the volume (ml) of final sample and initial sample, respectively, and Fc and Ic are the ionic concentrations (mM) of the final sample and initial sample, respectively. A negative value for net flux indicates luminal electrolyte loss.

Morphological and Histological Studies of Gastric Mucosa

As soon as the final sample was collected, the stomach was filled with 1.0% formalin for 10 min. The length (mm) and the width (mm) of ulcer on the gastric mucosa were measured with a planimeter (1 × 1 mm) under a dissecting microscope (× 0.7 to × 3.0; American Optical Scientific Instrument 569, Buffalo, NY, USA). The ulcer area was determined as follows, as previously described (17):

$$\text{ulcer area} = \text{length} \times \text{width} \times \pi/4$$

The total ulcer area (mm^2) of each stomach was recorded.

Histological studies of the stomach were performed by methods as previously described (20). Briefly, after gross examining, the specimens taken from corpus were blocked and immersed into 10% neutral formalin for two days. Blocks were then dehydrated in series of alcohol, cleared in xylene and embedded in paraffin. Sections (7 μm thickness) were cut and stained with hematoxylene and eosin as routine histological procedures. Each section was examined under a microscope (Nikon HF, X-IIA, Tokyo, Japan), and the tissue damage was quantified. Sections were scored as 0-5, in which 0 indicated a normal appearance, 1 indicated mild injury in the epithelial cells, 2 indicated mild injury in the upper part of mucosal cells, 3 indicated hemorrhage or edema in the mid or lower part of mucosal cells, 4 indicated degranulation or necrosis of the epithelial cells, and 5 indicated serious cell disruption of lower part of the mucosa. The index score of each section was evaluated on a cumulated basis to give a maximal score of 15.

Determination of Hemoglobin (Hb)

After initial and final samples were collected, the corpus mucosa was dissected. The blood attached to it was carefully scrapped and added to the final sample. Subsequently, both initial and final samples were adjusted to pH 1.5 with 100 mM HCl. The Hb content of the samples was measured spectrophotometrically (14). The absorption maximum of Hb was measured at 376 nm. Appropriate irrigating solutions, adjusted to pH 1.5, were used as blank. Absorbances of the samples were measured against a standard curve ($r^2 > 0.98$) constructed with freshly prepared rat Hb (0.05 -1.00 mg/ml) treated in the same manner as gastric samples. The luminal Hb content was calculated as:

$$\text{Luminal Hb content} = Fv \times F_{Hb} - (7\text{-}Iv) \times I_{Hb}$$

Where Fv and Iv are the volume (ml) of the final sample and the initial sample, respectively, while F_{Hb} and I_{Hb} are the luminal Hb concentration (mg/ml) in the final sample and the initial sample, respectively. The results obtained from gastric samples were expressed in mg Hb per stomach.

Determination of LPO

The concentration of gastric mucosal LPO was determined by estimating malonedialdehyde using the thiobarbituric acid test (30). Namely, rat stomachs were promptly excised and rinsed with cold normal saline. To minimize the possibility of interference of Hb with free radicals, any blood adhering to the mucosa was carefully removed. The corpus mucosa was scraped, weighed and homogenized in 10 ml of 100 g/l KCl. The homogenate (0.5 ml) was added with a solution containing 0.2 ml of 80 g/l sodium laurylsulfate, 1.5 ml of 200 g/l acetic acid, 1.5 ml of 8 g/l 2-thiobarbiturate and 0.3 ml distilled water. The mixture was incubated at 98°C for 1 h. Upon cooling, 5 ml of n-butanol: pyridine (15:1) was added. The mixture was vortexed for 1 min and centrifuged for 10 min at 1300 × g at 4°C. The supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane. The recovery was over 90%. The results were expressed as nmole malonedialdehyde/g wet tissue.

Measurement of Mucosal Histamine

The concentration of gastric mucosal histamine was determined by the methods as described previously (31). The corpus of gastric mucosa was scrapped and

homogenized with trichloroacetic acid (90 mM) in a final concentration of 100 mg/ml tissue. The homogenate was centrifuged at 4°C for 10 min at 6000 × g. The o-phthaldialdehyde (OPT)-NaOH solution was prepared by dissolving 10 mg OPT in 1.0 ml methanol (1% w/v) and 4 ml of NaOH (0.06 mol/l). This mixed solution was then gassed with nitrogen for 10 min. Then, 2.2 ml of a 2% OPT-NaOH solution was added to 100 µl of a 1/10- fold diluted sample of supernatant or histamine test solution. The mixture was then placed at -20°C for 10 h. Two hundred µl of 0.35 mol H_2SO_4/ (final pH 1.6-2.4) was added to this frozen mixture. After thawing, the sample was vortexed for 1 min. The fluorescence of the sample was read at room temperature (25°C) at 350 and 450 nm on a fluorescent spectrophotometer (Model 251-0030, Tokyo, Japan) using 1 cm^2 quartz cells. All samples were measured in duplicate. The fluorescence of the sample was calculated against a standard curve constructed with freshly prepared histamine solutions (0.78-25 µg/ml) that were treated in the same manner as tissue samples. The results obtained from tissue samples were expressed as µg histamine/g wet tissue.

Measurement of Gastric Microvascular Permeability

After collection of final samples, the rat stomach was rinsed with warm normal saline (37°C). Rats were injected with 1 ml of 1% Evans blue (EB) intravenously. Thirty minutes after EB was injected, rats were sacrificed under deep diethylether anesthesia. The stomachs were removed and the amount of dye trapped in the corpus mucosa and in the gastric contents was determined. After collecting gastric contents carefully by lavaging with 5 ml of cold distilled water, the stomach was opened along the greater curveture and the corpus mucosa was scrapped off using two glass slides, weighed and put into a tube containing 5 ml of distilled water. The extraction of dye was performed according to the modified method described by Takeuchi *et al*. (38). Namely, 2 ml of gastric samples were soaked overnight in stoppered glass tubes containing 2 ml of 3.5 M KOH at 37°C. Then, 18 ml of a mix solution of 4N H_3PO_4 and acetone (1.75: 16.25) was added to each tube to make up a total volume of 25 ml. The tube was shaken vigorously for a few seconds and centrifuged at 4°C for 15 min at 1000 × g. Absorbance of the supernatant was measured at 620 nm on a Hitachi spectrophotometer (model U-3210, Tokyo, Japan). A previous study shows that the recovery rate of dye was about 95% under the present conditions (20). The total amount of dye was calculated by adding the amount of dye recovered from the gastric content and to that recovered from extract of corpus mucosa. The result was expressed as microgram of EB per stomach.

Drug Administration

Daily intragastric verapamil (0-20 mg/kg) was challenged to rats 30 min before co-administration of vit. D_2 and cholesterol.

Statistical Analysis

The data obtained from the experiments were expressed as mean ± SEM. Significant differences in the data of experiments for single measurement traits were analyzed statistically by using ANOVA or the Turkey honestly significant difference test for pairwise comparison after ANOVA (28). Statistical significance was set at $P < 0.05$. A simple regression analysis was used to determine the correlation between two different variances.

Results

Changes in Eerum Lipoproteins and Serum Calcium in Atherosclerotic Rats

After receiving daily intragastric corn oil for 3 days, serum total cholesterol, LDL, HDL and calcium levels in control rat were 55.4 ± 3.0, 4.9 ± 0.14, 50.2 ± 4.3 and 10.1 ± 0.6 mg/dl, respectively. The atherosclerosis index (AI) as calculated by (cholesterol-HDL)/HDL, was 0.1 ± 0.03 (n = 5). However, rats received the same volume of a corn oil containing vit. D_2/cholesterol in a same period as control, the serum total cholesterol, LDL, HDL and calcium levels were increased to 120.6 ± 6.5, 14.6 ± 2.4, 38.3 ± 3.3 and 20.8 ± 1.2 mg/dl, respectively, whereas HDL was decreased to 38.3 ± 3.3. The AI was increased to 2.13 ± 0.45 ($P < 0.05$, n = 5). After daily administration of corn oil for 9 days, the serum total cholesterol, LDL, HDL and calcium levels were 73.2 ± 5.0, 5.8 ± 0.7, 48.6 ± 3.3 and 12.1 ± 0.5 mg/dl, respectively. The AI was 0.51 ± 0.03. When rats received daily administration of vit. D_2 and cholesterol for 9 days, the serum total cholesterol, LDL and calcium levels were increased to 448.0 ± 6.5, 25.5 ± 3.4 and 28.8 ± 2.2 mg/dl, respectively. While HDL decreased to 20.6 ± 3.1 (n = 5), the AI increased to 9.7 ($P < 0.05$, n = 5). Calcification and ischemia of the heart, aorta intimae and gastric smooth muscle layers were visible in these animals. Apparently, co-administration of vit. D_2 and cholesterol could result in atherosclerosis and calcification of soft tissues in rats.

Time-Dependent Increase in Gastric Acid Back-Diffusion, Histamine Levels and Hemorrhagic Ulcer in Normal and Atherosclerotic Rats

As shown in Fig. 1, gastric acid back-diffusion

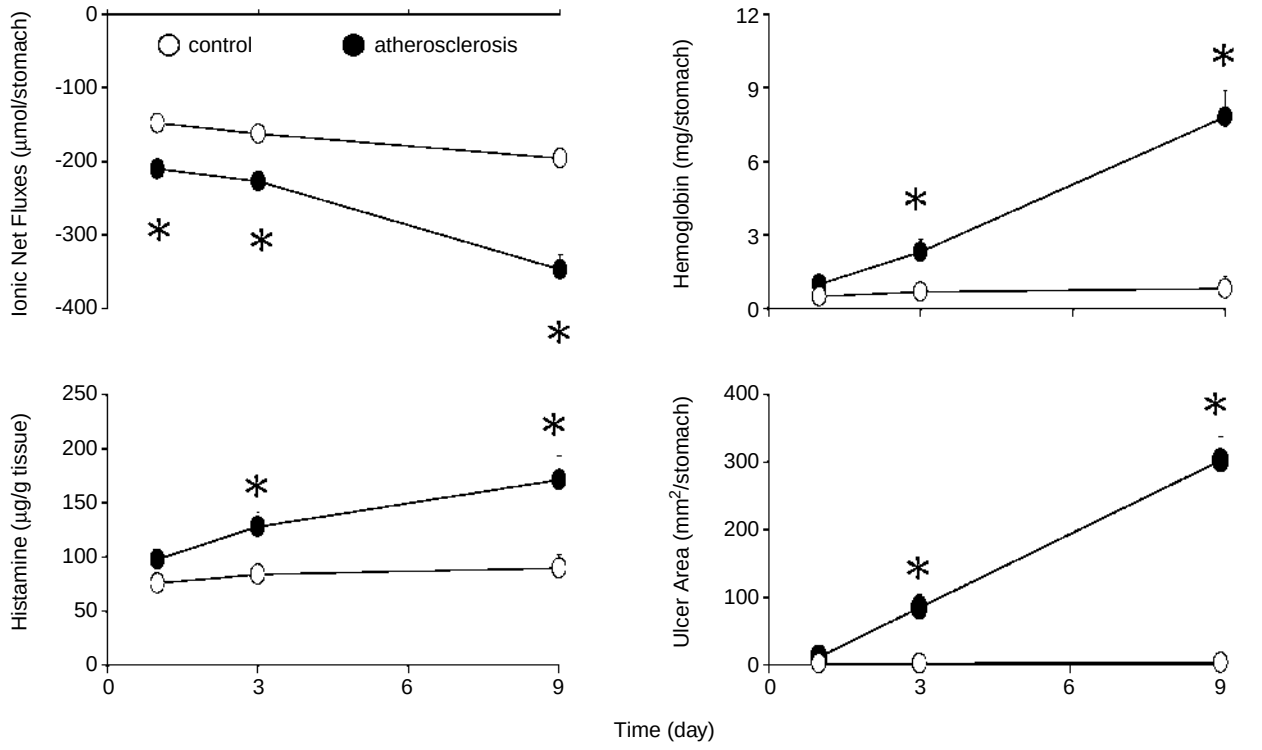

Fig. 1. Time-course of increased acid back-diffusion, histamine level, Hb content and ulceration in stomachs of atherosclerotic rats. Rat stomachs were irrigated for 3 h with gastric juice in control or atherosclerotic rats. Data are mean ± SEM, n = 8 for each point. Significant differences were analyzed by ANOVA. *$P < 0.05$ *vs.* correspondent control (non-atherosclerotic) rats.

and mucosal histamine levels were in a normal range in corn oil-treated rats. Gastric luminal hemoglobin content and mucosal ulceration also were in normal values. When the mixture of vit. D_2 and cholesterol was challenged to rats for 1-9 day(s), a time-dependent greater gastric acid back-diffusion and mucosal histamine levels as well as luminal hemoglobin and mucosal ulceration were achieved. The maximal response was obtained at day 9.

Morphological and Histological Examination of Gastric Mucosa of Atherosclerotic Rats

Gastric mucosa in control rat stomachs irrigated with gastric juice looked intact (Fig. 2A), however, numerous severe necrotic ulcers with remarkable hemorrhage were observed in corpus portion of gastric mucosa in 9-day atherosclerotic rat stomachs irrigated with gastric juice (Fig. 2B). The microscopy of mucosal damage in gastric juice-irrigated stomachs of control or atherosclerotic rats was shown in Fig 3. In control rats, gastric juice did not produce appreciable injury or atrophy of gastric mucosal cells (Fig. 3a). However, in gastric juice-irrigated stomachs of atherosclerotic rats, not only the epithelial layers but also the lamina propria of the mucosa was greatly damaged. In most cases, gastric edema was also observed (Fig. 3b). The degrees of histological tissue damage produced by normal saline or gastric juice in control or atherosclerotic rats were illustrated in Table 1. When stomachs of control rats were irrigated with either normal saline or gastric juice, no appreciable damage of gastric mucosal cells was observed. In atherosclerotic rats, normal saline produced more mucosal cell damage than did irrigation with gastric juice or normal saline in control rats. Furthermore, a remarkable deterioration of mucosal cell damage was observed in gastric juice-irrigated stomach of atherosclerotic rats.

Aggravation of Various Ulcerogenic Parameters in Gastric Juice-Irrigated Stomachs of Atherosclerotic Rats

Table 1 also shows that gastric acid back-diffusion, mucosal LPO generation, histamine levels, luminal Hb content and mucosal ulceration in normal saline-irrigated stomachs of control rats were within normal levels. A slight but significant ($P < 0.05$) increase in acid back-diffusion, mucosal histamine levels, LPO generation and mucosal damage was observed in gastric juice-irrigated stomachs of these rats. In atherosclerotic rats, normal saline-irrigated stomachs produced significant increase ($P < 0.05$) in these ulcerogenic parameters compared to those found in control rat stomachs irrigated with normal saline. When gastric juice was used instead

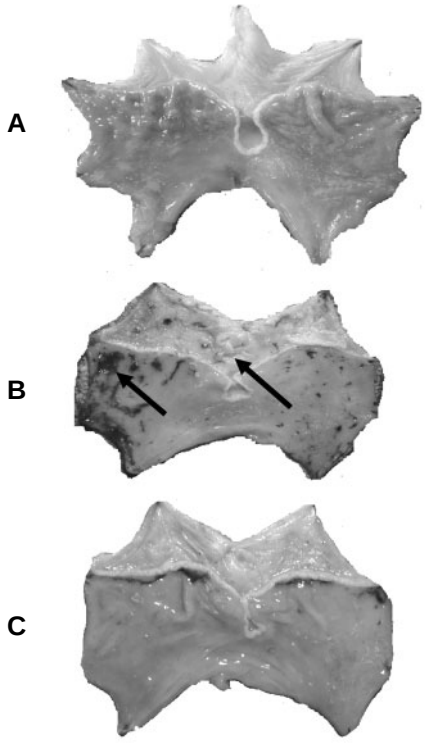

Fig. 2. Morphological study of gastric mucosa of control and atherosclerotic rat stomachs irrigated with gastric juice Gastric mucosa was exposed for 3 h with gastric juice in control (A) and atherosclerotic (B) rats. Note that gastric mucosal cells look intact in gastric juice-irrigated control rat mucosa (A). However, in atherosclerotic rat stomachs (B), a complete disruption of the upper mucosal cells and lamina propria is observed. Daily verapamil (20 mg/kg, p.o.) shows potent protective effect on gastric hemorrhagic ulceration in atherosclerotic rats (C).

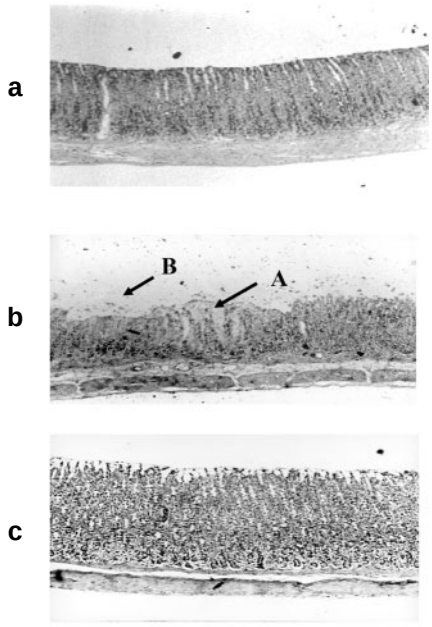

Fig. 3. Histological study of gastric mucosa of control and atherosclerotic rat stomachs irrigated with gastric juice. Gastric mucosa was exposed for 3 h with gastric juice in control (A) and atherosclerotic (B) rats. Note that gastric mucosal cells look intact in gastric juice-irrigated control rat mucosa (a). However, in atherosclerotic rat stomachs (b), a complete disruption of the upper mucosal cells and lamina propria is observed (A). The injured cells are characterized by karyorrhexis and dense homogenous acidophilic cytoplasm. In all cases, exfoliated mucosal cell plaques are also observed (B). Daily verapamil (20 mg/kg, p.o.) shows potent protective effect on gastric hemorrhagic ulcera in atherosclerotic rats (c) × 200.

of normal saline, a remarkable exacerbation of gastric hemorrhage and mucosal ulcerations accompanied with a great enhancement in acid back-diffusion, mucosal LPO generation and histamine release was found in atherosclerotic rats. Moreover, the enhanced mucosal histamine concentrations in those atherosclerotic rats were closely related to mucosal ulceration and gastric hemorrhage (Fig. 4). These results indicated that intraluminal gastric juice-exacerbated gastric mucosal hemorrhagic ulcer was greatly associated with gastric oxidative stress and histamine release in stomachs of atherosclerotic rats.

Effect of Verapamil on Serum Cholesterol, LDL and Calcium Concentration in Atherosclerotic Rats

As shown in Fig. 5, greater serum cholesterol, LDL and calcium levels were observed in 3 day-atherosclerotic rats. Daily intragastric verapamil (20 mg/kg) produced a significant inhibition ($P < 0.05$) in serum cholesterol, LDL and calcium levels in those rats.

Effects of Verapamil on the Aggravation of Various Gastric Parameters in Atherosclerotic Rats

Table 2 demonstrated that greater acid back-diffusion, LPO generation, mucosal histamine release and microvascular permeability, as well as luminal Hb contents and mucosal ulcerations were produced in gastric juice-irrigated stomachs of atherosclerotic rats. Intragastric verapamil (0-20 mg/kg) dose-dependently attenuated these gastric parameters. Gastric mucosal morphological and histological damage produced in atherosclerotic rats also were remarkably inhibited by verapamil (Fig.2C; Fig. 3c).

Discussion

In the present study, treatment of vit. D_2 and

Table 1. Changes in various gastric ulcerogenic parameters in normal saline- or gastric juice-irrigated stomachs of normal and atherosclerotic rats

	acid back-diffusion μmol /stomach	histamine μmol /g tissue	lipid peroxide nmol MDA /g tissue	hemoglobin mg /stomach	ulcer area mm^2 /stomach	histological score
Control						
normal saline	15.4 ±2.1 [a]	78.1 ±11.3 [c]	44.6 ±3.4 [d]	0.2 ±0.1 [d]	0.2 ±0.2 [d]	0.1 ±0.1 [d]
gastric juice	-150.8 ± 5.0[c]	100.3 ±11.8 [b]	65.5 ±3.3 [c]	0.5 ±0.1 [c]	5.7 ±0.6 [c]	0.5 ±0.3 [c]
Atherosclerosis						
normal saline	20.5 ±4.8 [a]	110.8 ±10.2 [b]	118.4 ±11.2 [b]	2.2 ±0.3 [b]	85.5 ±10.5 [b]	4.5 ±0.7 [b]
gastric juice	-358.6 ±25.0 [d]	169.3 ±22.0 [a]	181.0 ±18.9 [a]	7.7 ±1.5 [a]	282.3 ±32.2 [a]	9.8 ±1.8 [a]

Rats received daily intragastric corn oil or vitamin D_2 and cholesterol mixture dissolved in corn oil for 9 days. Data are mean ± SEM (n = 12). Significant differences are analyzed by using the Tukey honestly significant difference tests for pairwise comparison after ANOVA. The differences between those treatments with different letters are statistically significant ($P < 0.05$). Abbreviations: MDA= malonedialdehyde.

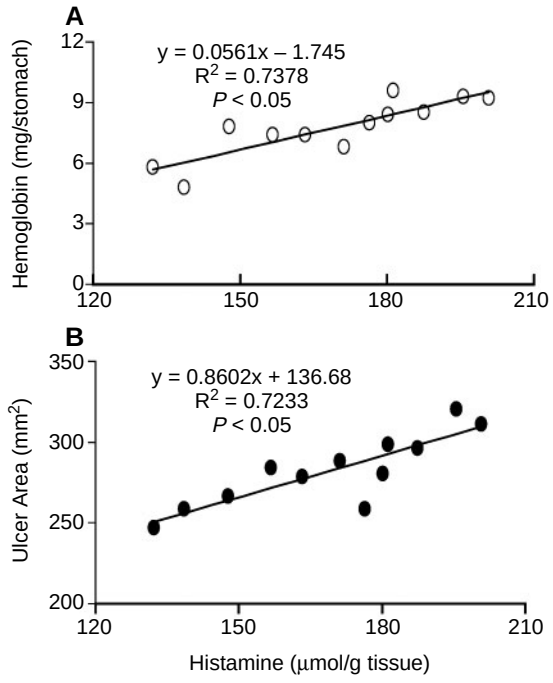

Fig. 4. Relationship between mucosal histamine levels and gastric hemorrhage (A) or between mucosal histamine levels and ulcer formation (B) in atherosclerotic rat stomachs irrigated for 3 h with gastric juice.

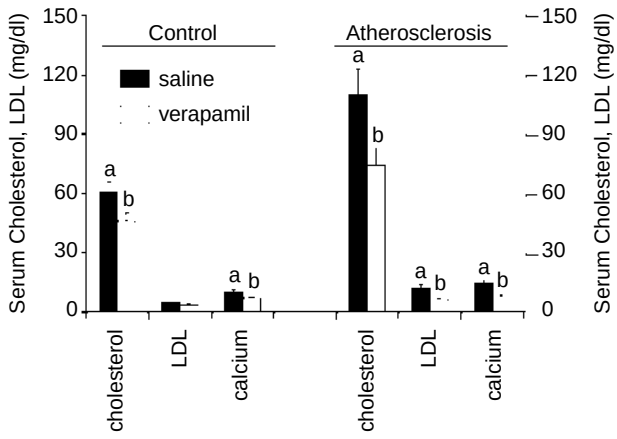

Fig. 5. Effect of verapamil on serum cholesterol, LDL and calcium levels in 3-day atherosclerotic rats. Daily intragastric saline or verapamil (0-20 mg/kg) was given to rats 30 min before corn oil or corn oil mixture. Data are mean ± SEM, n = 7 for each point. Statistic differences were analyzed by ANOVA. The differences between those treatments with different letters are statistically significant ($P < 0.05$). LDL = low-density lipoprotein.

cholesterol mixture caused severe atherosclerosis accompanied with substantial gastric hemorrhage and mucosal ulceration in rats. Mechanisms underlying inflammation and ulcerogenesis of this hemorrhagic ulcer model may be complex. The augmentation of gastric acid back-diffusion, LPO generation, histamine release and mucosal vascular permeability as well as luminal Hb content and mucosal ulceration was found in stomachs of atherosclerotic rats. These results implied that high cholesterol; LDL and calcium levels found in atherosclerotic rats could cause disruption of gastric mucosal barriers and led to the enhancement of acid back-diffusion and histamine release. The back-diffusion of gastric acid may also stimulate histamine release and thus elevate gastric mucosal and microvascular permeability that contribute to gastric hemorrhage and ulceration. In the development of atherosclerosis, high concentration of blood cholesterol and calcium can result in oxyradical generation and damage gastric mucosa cells. Oxyradicals also can increase the oxidative modification of LDL. This is one of the important mechanisms, which increases gastric damage in atherosclerotic rats. In the stomach, oxyradicals can also directly attack mast cells and cause cell degranulation (6, 22). In turn, gastric mucosal histamine concentration is elevated. Gastric inflammation produced by atherosclerosis is greatly associated with increased mucosal histamine concentrations. In the experimental animal, increased mucosal histamine has been reported to elicit gastric secretion and mucosal lesion (8). Histamine also can cause increase in gastric mucosal permeability to free acid and renders the stomach more susceptible to acid-induced damage (9, 17). High relations of mucosal histamine concentrations to gastric hemorrhage and to mucosal ulceration shown in the present study further supported the importance of histamine in gastric damage in atherosclerotic rats. The released mast cell histamine has been proposed to play a role in the development of ethanol-induced gastric ulcer (39). Degranulation of mast cells can be prevented by mast cell stabilizers, such as ketotifen and zinc sulfate (13, 19). In the gastrointestinal tract, histamine causes potent contraction of the smooth muscle cells and gastric mucosal internal leak (16) that contributes to plasma protein, electrolyte and water loss. This effect may be involved in the etiology of histamine-induced gastric mucosal hemorrhagic damage, particularly when gastric juice is present in the lumen. Ample documents indicate that diphenhydramine and / or ranitidine, the specific histamine H1 and H2 receptor antagonist, respectively, can protect histamine-provoked gastric hemorrhagic damage (16, 19, 20, 21).

Greater acid back-diffusion as well as gastric morphological and histological observation in the present study strongly indicated that gastric mucosal barriers in atherosclerotic rats were severely disrupted. Mechanism of the gastric mucosal barrier-disruption in atherosclerotic is totally unknown. However, atherosclerosis is associated with an augmentation of serum cholesterol and LDL levels as well as increased

Table 2. Changes in varapamil on various gastric parameters in gastric juice-irrigated stomachs of atherosclerotic rats

mg/kg	acid back-diffusion µmol /stomach	histamine µmol /g tissue	lipid peroxide nmol MDA /g tissue	MVP mg EB /stomach	hemoglobin mg /stomach	histological mm^2 /stomach
Control						
vehicle	-146.2 ±3.8	100.3 ±11.8	60.4 ±5.3	74.6 ±8.2	0.6 ±0.2	3.4 ±0.6
verapamil 20	-120.8 ± 4.0*	67.8 ±6.5 *	42.3 ±3.1 *	50.5 ±4.4 *	0.1 ±0.1 *	0 ±0
Atherosclerosis						
vehicle	-362.8 ±7.4	168.2 ±17.9	172.1 ±9.3	112.5 ±8.5	7.6 ±1.2	308.5 ± 44.3
verapamil 1	-325.4 ±14.5 *	150.5 ±17.2	142.7 ±11.6	96.7 ±10.6	5.5 ±0.8	263.0 ±22.5
verapamil 5	-266.2 ±10.2	120.4 ±14.8 *	108.3 ±8.4 *	78.4 ±8.6	3.6 ±0.6 *	186.5 ±26.5 *
verapamil 20	-144.6 ±8.4 *	72.8 ±8.0 *	85.4 ±5.3 *	52.6 ±5.5 *	2.0 ±0.4 *	82.5 ± 3.4*

Nine-day atherosclerotic rat stomachs were irrigated for 3 h with gastric juice. Data are mean ± SEM (n = 6-8). Significant differences are calculated by ANOVA. *P< 0.05 *vs.* correspondent vehicle-treated group. MDA = malonedialdehyde, EB = Evans blue, MVP = microvascular permeability.

serum calcium concentration. During metabolism of cholesterol, very low-density lipoprotein (VLDL) is formed and converted to remnant VLDL by lipolysis *via* lipoprotein lipase in the vessels of liver and peripheral tissues. Some of the VLDL remnant is then converted to LDL by loss of triglyceride and apoliprotein E (26). The LDL may be oxidized and causes generation of oxyradicals in the macrophage. This cellular oxidative stress mediated by oxidized LDL and an enrichment of the cell plasma membrane with cholesterol might be responsible. Cholesterol enrichment of the smooth muscle cell membrane may occur rapidly and is associated with an increase in membrane bilayer width, calcium permeability, and cell proliferation (40). Increase in calcium permeability may elevate intracellular calcium and consequently enhances calcification and apoptosis of cells. In clinics, it is reported that calcium polysulfide ingestion produces hemorrhagic necrosis of gastric mucosa (15). Taken together, pathological alterations induced by cholesterol, oxidized LDL and calcium can greatly affect the cellular homeostasis and lead to oxidative stress of tissues.

The aggravation of various gastric ulcerogenic parameters might also be associated with atherosclerosis-induced hemodynamic changes, such as gastric ischemia, decreased mucosal blood flow and increased platelet activating factor.

On the other hand, verapamil has been reported to have gastric mucosal cytoprotection against ethanol-induced damaging effect *via* endogenous prostaglandins and sulfhydryls stimulation (12).

In the present study, verapamil exerted significant inhibition in serum cholesterol, LDL and calcium levels in atherosclerotic rats. Verapamil has been shown to possess anti-atherosclerotic actions by inhibition of cholesterol and calcium levels (40). It has also been shown to inhibit LDL oxidation (22) that may play a role in the gastric mucosal damage. The increase in mucosal microvascular permeability found in the present study may be due to increased calcium levels and histamine release. The increase in vascular permeability with histamine may be associated with increased calcium binding to vascular endothelial membranes (3).

Verapamil also produced dose-dependent attenuations of gastric acid back-diffusion, mucosal LPO generation, histamine release, microvascular permeability and hemorrhagic ulcer. Increased gastric acid back-diffusion in atherosclerotic rats may be due to increased mucosal histamine and intracellular calcium concentration that is associated with cellular contraction and increased mucosal permeability. Furthermore, the increase in vascular permeability with histamine may be associated with increased calcium binding to vascular endothelial membranes. Taken together, verapamil could inhibit intracellular calcium level and histamine release that was contributed to the attenuation of gastric mucosal and microvascular permeability as well as mucosal hemorrhage.

Ample document demonstrate that verapamil exert potent antioxidant effects on tissue lipid peroxidation (11, 22, 25, 37). Verapamil also can inhibit oxidation and toxicity of LDL (29, 33). In gastrointestinal studies, verapamil has been shown to protect gastric mucosa against stress-induced (1) gastric lesions and histamine- and other inflammatory mediator-stimulated increase in vascular permeability (10, 27). Taken together, verapamil can be used as cytoprotective agents for therapy or prevention of gastric hemorrhagic ulcer produced by atherosclerosis. The present study is clinically significant. It provides evidence that atherosclerosis induced by chronic co-treatment of vit. D_2 and cholesterol can lead to severe gastric hemorrhage and stomach ulcers, particularly when gastric acid is presented in the lumen. Vit. D_2 should be carefully treated on patients with hypercholesterolemia.

In conclusion, severe atherosclerosis could result in gastric hemorrhage and mucosal ulceration by increasing serum cholesterol, LDL and calcium concentration that can result in exacerbation of gastric acid back-diffusion, oxyradical generation, histamine release and vascular permeability; and these parameters were effectively ameliorated by daily treatment of verapamil.

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