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Intragastric administration of heparin enhances gastric ulcer healing through a nitric oxide-dependent mechanism in rats

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Abstract

Our preliminary finding indicated that intravenous (i.v.) injection of heparin increased gastric ulcer healing in rats. However, the anticoagulant action of i.v. heparin could produce complications in ulcer patients if the drug was used as an anti-ulcer agent. The present study aimed to investigate whether intragastric (i.g.) administration of heparin, known to have no anticoagulant activity, would have the similar ulcer healing effect and the relationship of this effect, if any, with nitric oxide (NO), a substance suggested to be important for ulcer healing. Heparin (100, 500, 1000 U/kg, i.g.) administered once daily for 4 days accelerated the healing of gastric ulcer induced by acetic acid in Sprague–Dawley rats, which was accompanied by an increase in mucosal proliferation and regeneration at the ulcer margin, microvessel number both at the ulcer margin and base, and the thickness of mucus layer. Both activity and content, but not the mRNA of constitutive nitric oxide synthase (cNOS) in the gastric mucosa were enhanced. L-N^G-nitroarginine methyl ester (L-NAME), an inhibitor of NOS activity blocked the cNOS activity activated by heparin and reversed the beneficial effects of heparin on ulcer healing. The bleeding time was not altered by i.g. heparin. These findings demonstrate that i.g. heparin promotes the healing processes of gastric ulcer. Such effect is suggested to act through the stimulation of mucosal cNOS activity. In addition, i.g. heparin is better than i.v. heparin without the potential anticoagulation effect.

Keywords: Heparin; Intragastric administration; Gastric ulcer healing; Nitric oxide (NO) synthase, Constitutive; Proliferation; Regeneration; Angiogenesis

1. Introduction

Our preliminary study found that intravenous (i.v.) injection of heparin produced an ulcer healing effect in rat stomachs (Li et al., 1998, 1999). Nitric oxide (NO) was indicated to be involved in this property of heparin (Li et al., 1999). However, the bleeding time was significantly prolonged by i.v. heparin due to its anticoagulant action (Li et al., 1999). This could produce complications in the animal. It also raises a problem that it would be extremely risky and inconvenient if i.v. heparin were applied in clinical practice for any existing peptic ulcer in humans.

An alternative route of administration of heparin differing from the general parenteral route, i.e. oral or intragastric (i.g.) administration, has been examined by different investigators. Larsen et al. (1986) reported that oral heparin resulted in the appearance of heparin fragments in the plasma of rats. Jaques et al. (1991) found that oral heparin could be absorbed into endothelium. Sakamoto et al. (1987) observed an inhibitory effect of oral administration of heparin plus cortisone acetate on endothelial cell growth in tumor masses. Gorski et al. (1991) studied the immunomodulating activity of heparin, and found that oral administration of heparin could cause immunosuppression, although the effects were weaker than after subcutaneous administration. Furthermore, orally administered heparin was demonstrated to prevent deep venous thrombosis (Gonze et al, 1998). All these findings strongly indicate that heparin can be absorbed and exert biological effects after oral administration.

The present study aimed to examine whether i.g. heparin could have a similar effect as i.v. heparin on the healing of gastric ulcer. We further investigated whether such action is mediated through nitric oxide synthase

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(NOS) in the gastric mucosa, because NO derived from this enzyme plays a pivotal role in gastric ulcer healing. It not only dilates blood vessels and increases gastric blood flow, but also stimulates angiogenesis during the ulcer healing processes (Konturek et al., 1993; Brzozowski et al., 1997). All these actions are involved in cell proliferation and regeneration for re-epithelialization of the gastric mucosa, and formation of granulation tissue at ulcer base (Szabo et al., 1995). L-N^G-nitroarginine methyl ester (L-NAME), an inhibitor of NOS, was applied in the present study to affirm the role of NO in the ulcer healing action of oral heparin.

2. Materials and methods

2.1. Drugs

Heparin (Sigma, St. Louis, USA; sodium salt, produced from porcine intestinal mucosa, 183 USP units/mg) and L-NAME (Sigma) were prepared in distilled water for i.g. administration.

2.2. Animal experiments

The use of animals in the present study had been approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Male Sprague–Dawley rats (180–200 g) were reared on a standard laboratory diet (Ralston Purina, USA), and given tap water. They were kept in a room where temperature (22 ± 1°C), humidity (65–70%), and day/night cycle (12:12 light:dark) were controlled. Rats were fasted for 24 h but with free access to water before being subjected to acetic acid to produce gastric ulcer.

2.3. Induction of gastric ulcer

Twenty-four hours after starvation, gastric ulcers were produced by luminal application of an acetic acid (E. Merck, Darmstadt, Germany) solution to rats as previously described (Tsukimi and Okabe, 1994a) with a few modifications including the ulcer size and volume of acetic acid (Li et al., 1999). One day after the injection of acetic acid solution, the ulcer size was about 125 mm^2, which was derived from the sum of ulcers on both sides of gastric walls.

2.4. Drug treatments and measurement of ulcer size

Heparin in doses of 100, 500, and 1000 U/kg or its vehicle (distilled water) was administered intragastrically once daily starting 1 day after ulceration for 4 days. In a separate experiment, L-NAME at the dose of 20 mg/kg was given intragastrically 30 min prior to i.g. 1000 U/kg of heparin for 4 days. After drug treatment, rats were killed and stomachs were removed, opened along the greater curvature and spread on a glass board. The ulcer size (mm^2) in the anterior and posterior walls were determined and summed blindly in each stomach.

After measuring the ulcer size, gastric tissues were excised for histological and immunohistochemical analysis. Gastric glandular mucosa was then removed by scraping with a glass slide and immediately frozen in liquid nitrogen and stored at -70°C until determinations for different parameters.

2.5. Histological studies

Gastric tissues were processed and finally stained with hematoxylin-eosin for the histological studies. The length of the regenerated gastric mucosa (mm) at the ulcer margin, and the length of the ruptured muscularis mucosae (mm) were determined under a light microscope (Nikon, Japan) according to the method described by Ogihara and Okabe (1993).

2.6. Assessment of epithelial cell proliferation at the ulcer margin

To determine cell proliferation, a single intraperitoneal injection of 100 mg/kg 5-bromo-2′-deoxyuridine (BrdU, Sigma) was administrated 1 h before the stomach was removed. Then cell proliferation was assessed by immunohistochemical staining with anti-BrdU antibody (Sigma) as described previously (Kitajima et al., 1993; Lacy et al. 1991). The percentage of cells labeled with BrdU relative to the total number of mucosal cells was counted in both margins of the gastric ulcer, using a Leica image processing and analysis system (Q500IW, Leica Imaging Systems, Cambridge, UK) for each rat, and finally expressed as the labeling index by the mean of both margins.

2.7. Determination of angiogenesis at the ulcer margin and base

The microvessels at the ulcer margin and base in the granulation tissue of submucosa were identified by immunohistochemical staining with von Willebrand factor antibody (DAKO, Denmark; Augustin et al., 1995). The number of microvessels was quantitated at the two sides of the ulcer margin and the ulcer base with the Leica image processing and analysis system. The number of microvessel at the ulcer margin was expressed by taking the average of both sides of ulcer margins.

2.8. Determination of NOS activity in the gastric mucosa

Activity of NOS in the gastric mucosa was measured by a method described by Tepperman et al. (1993) from the

conversion of [^{3}H]L-arginine to the NO co-product, [^{3}H]citrulline (Knowles et al., 1990). Briefly, the mucosal samples, 100–150 mg, were homogenized (Ultra-Turrasx, USA) for 20 s at 0°C in a homogenizing buffer (pH 7.2) followed by centrifugation (Beckman, J2-21 centrifuge, USA) at 20,000 × *g* for 30 min at 4°C. Supernatant (100 μl) was added to a 150 μl of buffered solution (pH 7.2) containing 1 μCi [^{3}H]L-arginine (1 mCi/ml; specific activity 36.1 Ci/mmol, New England Nuclear) and incubated at 37°C for 30 min. For determining the activity of inducible NOS (iNOS), EGTA (1 mM) was used to inhibit the activity of calcium-dependent cNOS. After the reaction was stopped, the resulting mixture was applied to a column containing Dowex AG50WX-8 (Na-form, Bio-Rad, Hercules, CA, USA) resins. Scintillation fluid (BCS, Amersham, UK) was mixed with the eluent, which was counted with a scintillation counter (LS-6500, Beckman Instrument). Protein assay was performed using the method developed by Lowry et al. (1951). The final result was expressed as picomoles of [^{3}H]citrulline formed per minute per gram of protein.

2.9. Assessment of gastric mucus content

Sections were stained with the Periodic Acid-Shiff (PAS) technique. They were first immersed in 1% (v/v) periodic acid for 5 min, followed by successive washing with distilled water. They were transferred to a Shiff's reagent for 3 min and then flushed with running tap water for 3 min. Finally, they were counterstained by Mayer's hematoxylin. The amount of mucus within the mucosa was assessed by a single blind method by measuring the relative thickness of the adherent mucus layer (Boon and Drjiver, 1986) with Leica image analyzer in five consecutive fields of each side of the ulcer crater. The results were averaged from two sides and expressed as the ratio of the thickness of mucus layer to the thickness of total mucosa.

2.10. Immunostaining and quantification of endothelial constitutive NOS (eNOS) expression in the gastric mucosa

The content of eNOS in gastric mucosa was immunohistochemically stained with monoclonal mouse anti-human eNOS antibody (Transduction Lab, USA) according to method described by Ma et al. (1999). Quantification of eNOS expression was performed at a 100 times magnification field consecutively starting from the edge of ulcer margin by Leica image analyzer. The areas of cells demonstrating positive staining for eNOS (dark brown) were measured. They were expressed as $\mu m^2/mm^2$ for each field. The determination for the positive staining cells was controlled by a computer throughout the measuring procedure.

2.11. Determination of mRNA expression of eNOS in the gastric mucosa

2.11.1. Isolation of RNA

Frozen gastric mucosal samples (100 mg) were homogenized in 1 ml TRIZOL reagent (GIBCO-BRL, Life Technologies, USA), and total RNA was isolated using the guanidinium isothiocyanate–phenol–chloroform method described by Chomczynski and Sacchi (1987). The total RNA amount in each sample was calculated from optical density measurements at 260 nm with spectrophotometer (DU 650, Beckman, USA).

2.11.2. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR was performed using a GIBCO-BRL PCR core kit (Thermoscript™ RT-PCR system, Life Technologies) and a DNA thermal cycler (GeneAmp PCR system 9700, PE Applied Biosystmes, USA) according to the manufacturer's recommended procedures. The amplification was initiated by 2 min at 94°C for denaturing followed by 35 cycles at 94°C for 45 s, 60°C for 45 s, and 72°C for 1.25 min. After the last cycle of amplification, the samples were incubated at 72°C for 7 min for extension. The specific primer set used for rat eNOS was 5′-TACGGAG-CAGCAAATCCAC-3′ (forward) and 5′-CAGGCTG-CAGTCCTTTGATC-3′ (reverse). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control and internal standard for PCR. The amplified PCR products were analyzed by electrophoresis on a 1% agarose gel containing 0.1 μg/ml ethidium bromide. The gel was then visualized by ultraviolet illumination (Gel Doc 1000,

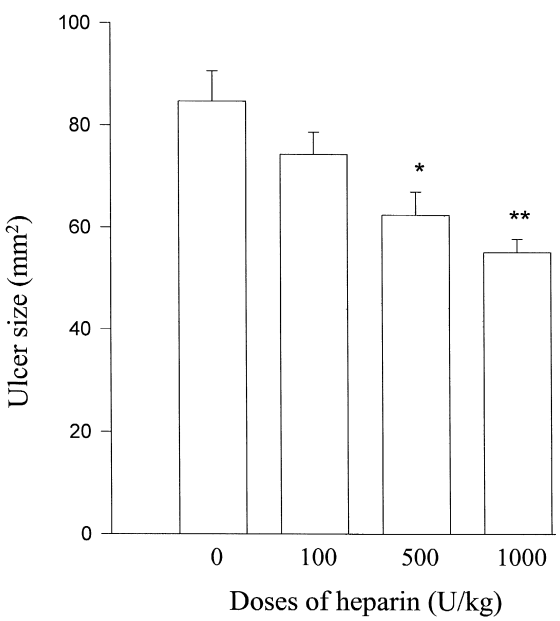

Fig. 1. Effect of the three doses of i.g. heparin given for 4 days on the healing of gastric ulcer. Data are expressed as mean ± S.E.M. of eight animals. * $P < 0.05$, * * $P < 0.01$ compared with 0 U/kg of heparin.

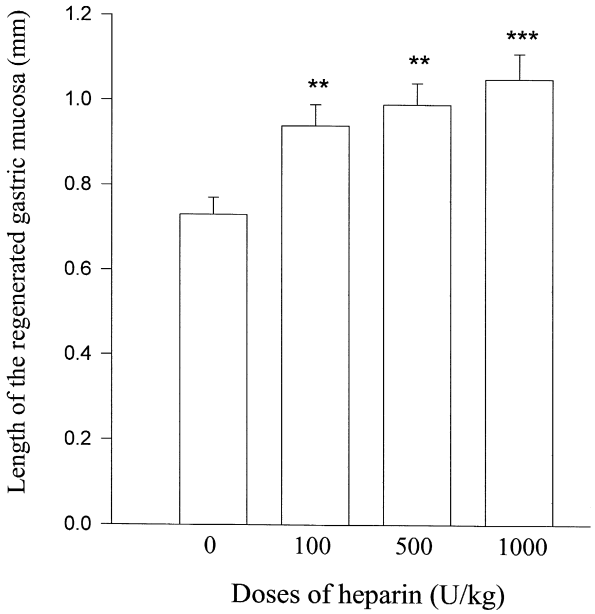

Fig. 2. Effect of the three doses of i.g. heparin given for 4 days on the regeneration of gastric mucosa at the ulcer margin. Data are expressed as mean ± S.E.M. of eight animals. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with 0 U/kg of heparin.

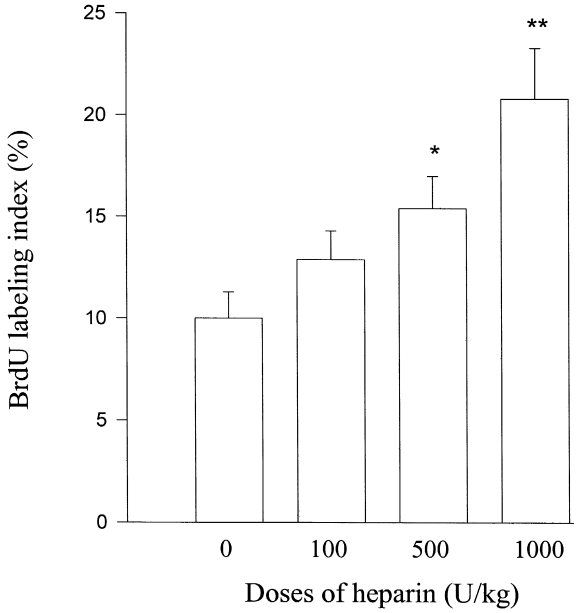

Fig. 4. Effect of three doses of i.g. heparin given for 4 days on the proliferation of gastric mucosal cells at the ulcer margin. Data are expressed as mean ± S.E.M. of eight animals. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with 0 U/kg of heparin.

Bio-Rad) and quantified by optical density using the GAPDH cDNA as control.

2.12. Assessment of anticoagulant activity

The bleeding time was used as a determinant of coagulation function (Ogle et al., 1977). The rats were anesthetized with an i.p. injection of pentobarbitone sodium and the abdomen opened with a crucial incision to expose the liver. A piece of liver was excised from the edge of a lobe, then pieces of filter paper were dipped at 5-s intervals into the blood oozing from the cut surface until the

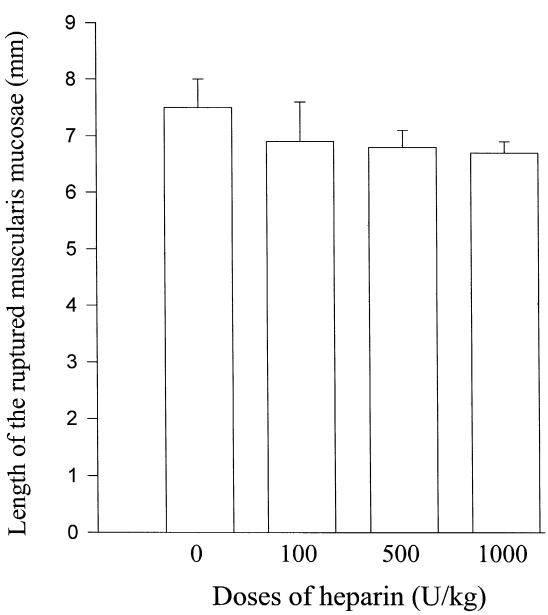

Fig. 3. Effect of three doses of i.g. heparin given for 4 days on the contraction of the ulcer base. Data are expressed as mean ± S.E.M. of eight animals.

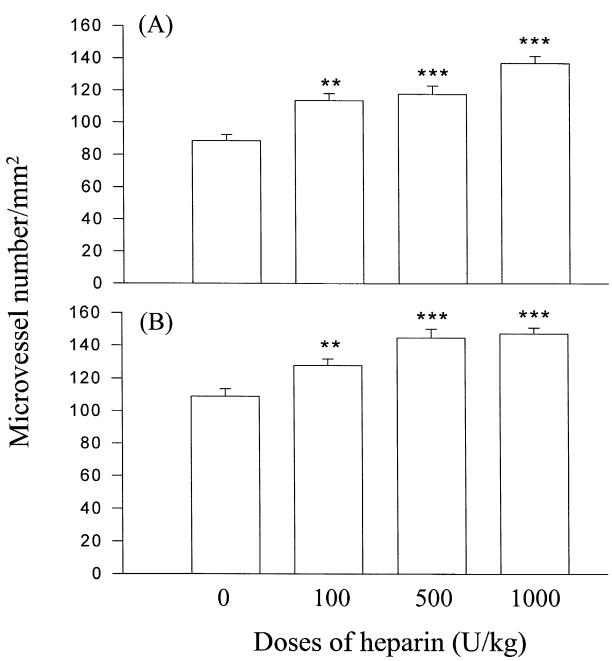

Fig. 5. Effects of three doses of i.g. heparin given for 4 days on the angiogenesis in granulation tissue of submucosa at (A) ulcer margin and (B) ulcer base. Data are expressed as mean ± S.E.M. of eight animals. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with corresponding 0 U/kg of heparin.

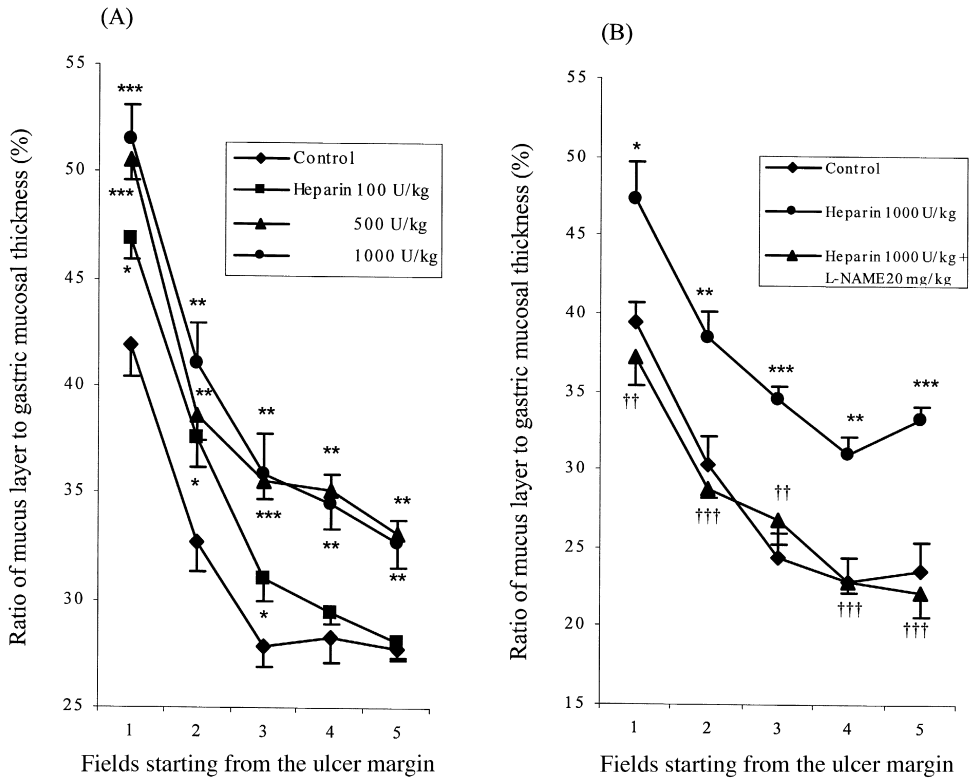

Fig. 6. Effect of three doses of i.g. heparin given for 4 days on the thickness of gastric mucus layer (A) and L-NAME on the stimulating effect of heparin on the thickness of gastric mucus layer (B). Data are expressed as mean ± S.E.M. of eight animals. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with corresponding control; $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ compared with heparin 1000 U/kg.

end point was reached, indicated by a piece of blood clot clinging to the filter paper. The bleeding time was taken as the time elapsing between cutting the liver edge and the end point of bleeding. Three separate consecutive readings

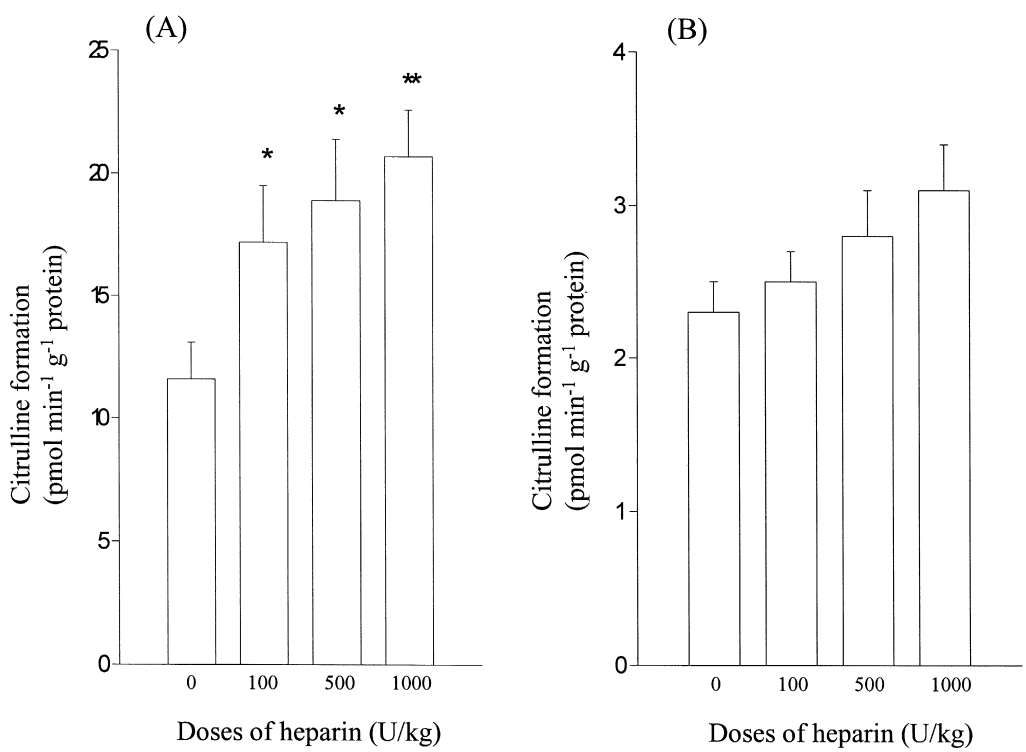

Fig. 7. Effects of the three doses of i.g. heparin given for 4 days on the gastric mucosal (A), cNOS and (B) iNOS activities. Data are expressed as mean ± S.E.M. of eight animals. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with corresponding 0 U/kg of heparin.

Table 1
Effect of heparin on the expression of eNOS content in the gastric mucosa 4 days after i.g. administration

	eNOS level ($\mu m^2/mm^2$)				
	Field 1	Field 2	Field 3	Field 4	Field 5
Control	692.9 ± 57.4	1277.1 ± 138.5	1322.6 ± 144.9	1758.6 ± 77.9	1467.9 ± 464.1
Heparin 100 U/kg	755.5 ± 48.9	1344.5 ± 190.7	1620.3 ± 94.2	1841.4 ± 175.5	1572.5 ± 231.7
Heparin 500 U/kg	956.9 ± 138.5	1921.6 ± 221.3[a]	1923.4 ± 147.8[a]	2011.4 ± 301.5	1792.8 ± 211.4
Heparin 1000 U/kg	1620.9 ± 187.2[b]	4140.4 ± 630.9[b]	3436.2 ± 620.8[a]	3316.9 ± 321.5[b]	3011.5 ± 383.1[a]

Data are expressed as mean ± S.E.M. of eight animals. Fields 1 to 5 represent consecutive fields starting from the ulcer margin.
[a] $P < 0.05$ compared with the control.
[b] $P < 0.01$ compared with the control.

were taken by a person unaware of the type of treatment from three lobes in each rat and the values were averaged.

2.13. Statistical analysis

All data are presented as mean ± S.E.M. Statistical analysis was performed with an analysis of variance (ANOVA) followed by Dunnett's *t* test or Student's two-tailed unpaired *t* test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of heparin on reduction of gastric ulcer size

The size of gastric ulcer in the control group after 4 days of vehicle treatment was about 84.7 ± 5.9 mm^2, which was smaller than that of 1 day after ulceration (125.0 ± 4.4 mm^2), indicating a spontaneous reduction of the ulcer size. Three doses of heparin given for 4 days elicited a dose-dependent reduction of ulcer size than that in the control group. Significant effect was found at the two higher doses of heparin when compared with the control (Fig. 1).

3.2. Effects of heparin on histological changes

Heparin treatment for 4 days provoked an increase of the length of the regenerated gastric mucosa at the ulcer margin in a dose-dependent manner. This effect was significantly different from the control (Fig. 2). However, heparin only slightly shortened the length of the ruptured muscularis mucosae (Fig. 3).

3.3. Effect of heparin on epithelial cell proliferation at the ulcer margin

The BrdU labeling index, an indication of epithelial cell proliferation, was dose-dependently increased by heparin at the ulcer margin after 4 days of treatment. Heparin at the two higher doses showed a significant stimulatory effect on the proliferation of gastric epithelial cells (Fig. 4).

3.4. Effect of heparin on angiogenesis at the ulcer margin and base

The three doses of heparin increased the microvessel number in the granulation tissue both at the ulcer margin and base in a dose-dependent manner after 4 days of treatment. They were all significantly higher than that in the control groups (Fig. 5A,B).

3.5. Effect of heparin on thickness of the gastric mucus layer

The thickness of mucus layer was the highest at the ulcer margin and decreased from there to the adjacent normal mucosa. The three doses of heparin increased the thickness of the mucus layer in a dose-dependent and significant manner (Fig. 6A).

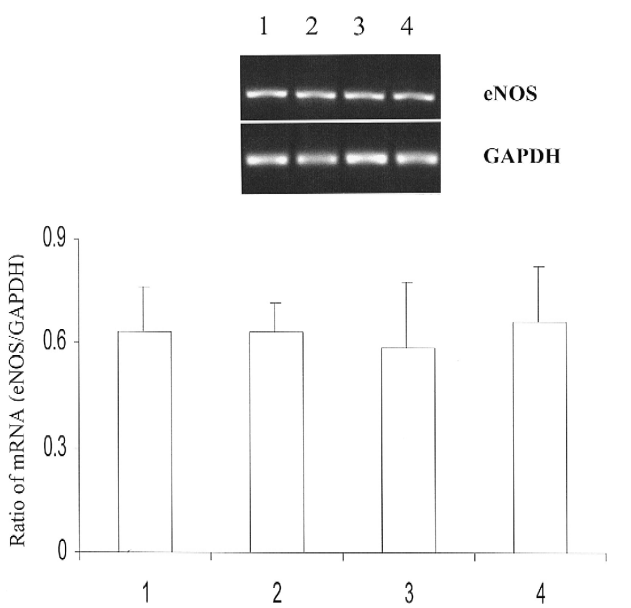

Fig. 8. Representation of RT-PCR for mRNA expression of gastric mucosal eNOS and effect of three doses of i.g. heparin given for 4 days on the mRNA expression of gastric mucosal eNOS. Data are expressed as mean ± S.E.M. of eight animals. Line 1: control (vehicle); line 2: heparin 100 U/kg; line 3: heparin 500 U/kg; line 4: heparin 1000 U/kg.

Table 2
Effects of L-NAME on the healing effect of i.g. heparin on gastric ulcer

Parameters	Control (distilled water)	Heparin (1000 U/kg)	L-NAME (20 mg/kg)	L-NAME + heparin (20 mg/kg + 1000 U/kg)
Ulcer size (mm^2)	89.7 ± 4.7	61.0 ± 7.4[b]	91.2 ± 4.3	84.7 ± 5.3[d]
Length of the regenerated gastric mucosa (mm)	0.86 ± 0.08	1.28 ± 0.1[b]	0.83 ± 0.05	1.0 ± 0.04[d]
Length of the ruptured muscularis mucosae (mm)	7.6 ± 0.6	6.8 ± 0.2	7.3 ± 0.3	7.4 ± 0.1
BrdU labeling index (%)	8.6 ± 0.9	18.5 ± 1.6[b]	10.2 ± 0.8	12.9 ± 1.2[d]
Microvessel number/mm^2 (ulcer margin)	82.7 ± 2.6	132.5 ± 6.8[c]	91.7 ± 5.6	100.4 ± 6.1[e]
Micrivessel number/mm^2 (ulcer base)	105.7 ± 6.0	153.7 ± 8.0[c]	110.5 ± 7.4	112.5 ± 7.1[e]
cNOS activity (pmol min^{-1} g^{-1} protein)	11.2 ± 1.3	17.4 ± 1.6[a]	8.9 ± 1.2	9.8 ± 1.2[e]
iNOS activity (pmol min^{-1} g^{-1} protein)	2.4 ± 0.2	3.0 ± 0.3	2.0 ± 0.2	2.7 ± 0.4

Data are expressed as mean ± S.E.M. of eight animals. L-NAME was i.g administered 30 min prior to i.g heparin for 4 days.
[a] $P < 0.05$ compared with the heparin 1000 U/kg.
[b] $P < 0.01$ compared with the control.
[c] $P < 0.001$ compared with the control.
[d] $P < 0.05$ compared with the control.
[e] $P < 0.01$ compared with the heparin 1000 U/kg.

3.6. Effects of heparin on NOS activity in the gastric mucosa

Heparin enhanced the constitutive NOS (cNOS) activity in the gastric mucosa after 4 days of treatment. The effect showed a dose-related fashion and the enhancement of cNOS activity by the three doses of heparin was all significantly higher than that of the control (Fig. 7A). However, heparin did not significantly affect the inducible NOS (iNOS) activity after 4 days of treatment (Fig. 7B).

3.7. Effect of heparin on eNOS content in the gastric mucosa

The positive staining of eNOS was found at the parietal cells in the gastric mucosa, microvessels in the middle part of mucosa and blood vessels at the lower part of mucosa, as well as in the submucosa. The expression of eNOS content in the vehicle control group showed a field-related increase from fields 1 to 4, i.e. the eNOS content was the lowest just beside the ulcer crater and increased thereafter away from the ulcer site. Heparin increased the expression of gastric mucosal eNOS content in a dose-dependent manner in each field after 4 days of treatment (Table 1).

3.8. Effect of heparin on expression of eNOS mRNA in the gastric mucosa

Expression of eNOS mRNA was detected in the gastric mucosa of ulcerated rats. Heparin treatment for 4 days did not significantly affect the mRNA expression of the enzyme. The mRNA levels in heparin-treated groups were similar to that of the vehicle control group (Fig. 8).

3.9. Effects of L-NAME on the healing effect of heparin on gastric ulcer

L-NAME itself, at the dose of 20 mg/kg, did not significantly affect NOS activity and the ulcer healing process after being used for 4 days. However, L-NAME reversed the ulcer healing effect of heparin. The blocker attenuated the increases of regeneration and proliferation of gastric mucosa at the ulcer margin, and microvessel number both at the ulcer margin and base. L-NAME also reversed the increased cNOS activity induced by heparin. All these effects were significantly different when compared with those of heparin treatment alone. However, L-NAME did not affect the length of the ruptured muscularis mucosae (Table 2). In addition, L-NAME blocked the stimulatory effect of heparin on gastric mucus layer thickness (Fig. 6B).

3.10. Effect of intragastric heparin on the coagulation function

Intragastric administration of heparin did not prolong the bleeding time. The bleeding time (s) measured 1 h after i.g. heparin was 44.4 ± 1.9, 45.0 ± 2.7, 46.3 ± 2.4 or 45.6 ± 1.9 for the control group or three doses of heparin, respectively.

4. Discussion

The present study demonstrated for the first time that i.g. heparin produced an ulcer healing effect in the rat stomach. The effect was related to the increases of proliferation and regeneration of the gastric mucosa, as well as angiogenesis and mucus in the stomach. The increases of cNOS content and its activity in the gastric mucosa were associated with the healing property of heparin. Such ulcer healing property was as effective as that of the i.v. heparin route (Li et al., 1998, 1999).

The contributory roles of the regeneration of the mucosa and the contraction of the ulcer base in the healing process of gastric ulcer have been demonstrated (Ogihara and Okabe, 1993; Tarnawski et al., 1990; Tsukimi and

Okabe, 1994b). Intragastric administration of heparin increased the length of the regenerated gastric mucosa at the ulcer margin, but did not shorten the length of the ruptured muscularis mucosae. This result indicated that heparin accelerated gastric ulcer healing via the regeneration of gastric mucosa, but not the contraction of the ulcer base. The increased regeneration of gastric mucosa could be the result of cell proliferation. Our results also revealed an increase of BrdU incorporation in gastric mucosal cells at the ulcer margin after heparin treatment. This correlated well with the faster reduction of ulcer size. In our previous studies, i.v. heparin also provoked a similar effect on gastric mucosal proliferation and regeneration (Li et al., 1999). These findings demonstrated further that heparin has a stimulatory effect on gastric mucosal proliferation and regeneration after ulceration regardless of route of administration.

The formation of new microvessels, i.e. angiogenesis, is an important factor for ulcer healing. Heparin stimulated the angiogenesis both at the ulcer margin and base. Increased angiogenesis at the ulcer site could supply more oxygen and nutrients to support cell proliferation and regeneration for the re-epithelialization of the ulcer crater. Angiogenesis at the ulcer base is important to facilitate the formation of granulation tissue that supplies connective tissue for the restoration of lamina propria and endothelial cells in the formation of microvasculature within the mucosal scar (Szabo et al., 1995; Tarnawski et al., 1991). The angiogenic action of i.g. heparin in the ulcerated rat stomach was similar to that of i.v. heparin (Li et al., 1999). The present study demonstrated further the importance of the angiogenic action of heparin for its ulcer healing property.

Intragastric administration of heparin increased the thickness of gastric mucus layer within the mucosa. This mucus layer was generally referred to be the adherent mucus layer. The adherent mucus layer is responsible for mucosal protection and epithelial recovery in the stomach (Bell et al., 1982, 1985; Wallace and Whittle, 1986). A number of drugs or substances, which increase the synthesis and secretion of gastric mucus, have been demonstrated to accelerate gastric ulcer healing (Takahashi and Okabe, 1998; Seidler et al., 1988; Slomiany et al., 1991). Therefore, the effect of heparin to increase the thickness of gastric mucus could contribute in part to its ulcer healing activity.

In the present experiments, the ulcer healing effect of i.g. heparin was associated with an increase of cNOS activity in the gastric mucosa, indicating that more NO was produced. NO is a pivotal mediator to accelerate gastric ulcer healing. It maintains the integrity of the gastric epithelium (Whittle et al., 1990), regulates gastric mucosal blood flow (Pique et al., 1992), and stimulates gastric mucus secretion and synthesis (Brown et al., 1992; Takahashi et al., 1995). NO donor glyceryl trinitrate and NOS substrate L-arginine were demonstrated to accelerate gastric ulcer healing in the rat, and this effect was related to the increase of angiogenesis in the stomach (Konturek et al., 1993; Brzozowski et al., 1997). The stimulating effect of heparin on NO formation in the vascular system was also demonstrated (Yokokawa et al., 1993; Minami et al., 1995). All these findings suggest that NO derived from cNOS could contribute significantly to the ulcer healing effects of i.g. heparin. We further substantiate such phenomenon by using an NOS inhibitor of L-NAME which blocked the increase of cNOS activity by heparin and abolished the beneficial effects of heparin on ulcer healing. Moreover, neither the basal NOS (cNOS or iNOS) activity nor ulcer healing process was significantly affected by L-NAME alone given at the present dose, indicating that this compound was more active to inhibit the enhanced NOS activity and its related ulcer healing action. A similar result was also found in our study with protamine sulfate on ulcer healing (Li and Cho, 1999). Furthermore, in another study, it was also shown that L-NAME was more effective to abolish the ulcer healing effect of basic fibroblast growth factor (bFGF) than that in the basal ulcer healing process (Akiba et al., 1997). Other reports demonstrated that inhibition of NOS activity not only attenuated the stimulatory effects of NO on mucus synthesis and secretion (Takahashi et al., 1995), but also inhibited angiogenesis and impaired ulcer healing (Konturek et al., 1993; Ma et al., 1999). In addition, iNOS activity in the gastric mucosa was not significantly altered after i.g. heparin treatment. This result affirms further that the ulcer healing effects of i.g. heparin is indeed acting through the activation of cNOS activity.

In order to study further the mechanism of how heparin affected cNOS activity, the content and mRNA expression of eNOS in the gastric mucosa were investigated. The results revealed that i.g. heparin dose-dependently increased the content of eNOS, but not the expression of eNOS mRNA. The location of eNOS was mainly found at the parietal cells, and blood vessels of both the mucosa and submucosa. This finding indicated that eNOS was involved in the modulation of heparin on cNOS activity, and this modulation was at the translation level other than the transcription level. The increased eNOS protein level offered a support for the elevation of cNOS activity. How exactly heparin modulates the content and activity of cNOS in the gastric mucosa is unclear, and needs further investigation.

It is traditionally known that oral administration of heparin is ineffective for its anticoagulant activity because of poor absorption in the gastrointestinal tract (American Hospital Formulary Service, 1976). This was confirmed in the present study that the liver bleeding time, an index of general coagulation (Ogle et al., 1977; Li et al., 1999) was not significantly altered by i.g. heparin, which was in contrast with the results of i.v. heparin in our previous study (Li et al., 1999). However, it was reported that although heparin did not act as an anticoagulant when administered orally, it resulted in anti-angiogenic and anti-

tumor effects in the presence of cortisone. This intriguing finding suggested that when heparin is taken orally, it was degraded by the gastrointestinal tract, and the non-anticoagulant heparin fragments might enter the bloodstream and retain their biological capacity (Folkman et al., 1983). Similar conclusion was also made by other studies (Larsen et al., 1986; Jaques et al., 1991; Sakamoto et al., 1987; Gorski et al., 1991). All these findings suggested that the biological active fragments of heparin could be absorbed after oral administration. Our result not only supports this phenomenon, but also further extends the pharmacological action of i.g. heparin to that on ulcer healing in the stomach.

Taken together, it was concluded that i.g. heparin could accelerate gastric ulcer healing. This effect was acting through the elevations of cNOS content and activity in the gastric mucosa, followed by increases of gastric mucosal cell proliferation and regeneration, angiogenesis and mucus production. Oral heparin, with lack of its anticoagulant activity, could be an effective alternative for the treatment of gastric ulcer.

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