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IMPORTANCE OF LUMINAL AND MUCOSAL ZINC IN THE MECHANISM OF EXPERIMENTAL GASTRIC ULCER HEALING

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Zinc has been reported to exert a gastroprotective action against various experimental gastric lesions suggesting that this trace element is involved in the integrity of the gastric mucosa. Compounds containing zinc, such as polaprezinc, were developed in Japan and used as an antiulcer drugs in the treatment of human peptic ulcer disease. However, the precise mechanism of Zn^{2+} containing compounds and their effects on mucosal integrity, gastroprotection and ulcer healing remain unclear. We have determined the efficacy of zinc hydroaspartate, a compound containing Zn^{2+}, in the mechanism of gastric secretion and ulcer healing in rats with chronic gastric ulcers induced by acetic acid (initial ulcer area = 28 mm^2). Rats with gastric ulcers were randomized into two groups: A) with gastric fistulas (GF) and B) without gastric fistulas and received a daily treatment with zinc hydroaspartate (32-130 mg/kg-d i.g.) for 3, 7 and 14 days. At the termination of each treatment, the area of gastric ulcers were examined by planimetry, the gastric blood flow (GBF) at ulcer margin was assessed by laser Doppler flowmetry and H_2-gas clearance methods. The venous blood was withdrawn for a measurement of plasma gastrin levels by radioimmunoassay (RIA). The concentration of Zn^{2+} in the gastric juice and mucosa at the ulcer margin were determined by differential pulse anodic stripping voltammetry (DP ASV) and flame atomic absorption spectrometry (FAAS) methods and the gastric biopsy samples were taken for histopathological assessment of the quality of ulcer healing. The ulcers healed gradually, with the ulcer area in the vehicle control rats being diminished by 15%, 48% and 78% upon ulcer induction at 3, 7 and 14 days, respectively. Zinc hydroaspartate dose-dependently inhibited the area of gastric ulcer, the dose reducing this area by 50% (ID_{50}) being about 60 mg/kg-d. The mucosal concentration of Zn^{2+}significantly was unchanged from the baseline immediately after ulcer induction (day 0) and at day 3 but then it rose significantly at day 7 after ulcer induction. Treatment with zinc hydroaspartate (65 mg/kg-d i.g.), which significantly raised the gastric luminal and mucosal levels of Zn^{2+}, significantly accelerated ulcer healing at day 7 upon ulcer induction. The GBF, which reached a significantly higher value at the ulcer margin than the ulcer bed, was significantly increased in rats treated with zinc hydroaspartate compared with vehicle-controls. The gastric acid output was significantly inhibited in GF rats with gastric ulcer at day 3 then restored at day 14 followed by a significant rise in the plasma gastrin levels. Treatment with zinc hydroaspartate significantly inhibited gastric secretion and also significantly raised the plasma gastrin level when compared to vehicle-control rats. We conclude that 1) trace micronutrients such as Zn^{2+} could be successfully measured in the gastric juice and gastric mucosa during ulcer healing; 2) compounds chelating of Zn^{2+} can exert a beneficial influence on the ulcer healing *via* Zn^{2+} mediated increase in gastric microcirculation, antisecretory activity and gastrin release, which may enhance the cell proliferation and differentiation during ulcer healing, ultimately exerting a trophic action on the ulcerated gastric mucosa.

Key words: *zinc, ulcer healing, ulcer margin, gastric acid secretion, gastric blood flow, gastric juice, gastrin*

INTRODUCTION

Zinc belongs to a class of microelements that are considered to play an important role in many vital biochemical reactions and physiological processes, including the growth and development of the cells (1, 2). This trace element has been also shown to stimulate the gene transcription and cell proliferation and it is also responsible for activation of DNA and RNA polymerases (3, 4). Both these enzymes are important components of cell antioxidant enzyme activity. The mechanism of zinc-induced antioxidative function remains unclear but this trace element slows down the oxidation processes and this may account for its potential antioxidant properties (5, 6). Recent evidence indicates that zinc may function as an indispensable element for optimal functioning of the human immune system (7, 8). Zinc compounds have long been used in medicine as an antidepressant (9) and in various

forms of skin injury due to its efficacy in healing skin lesions and wounds (10, 11).

The role of zinc in the mechanism of gastrointestinal integrity has not been fully understood despite the fact that zinc has been reported to have protective action against various experimental gastric lesions (12, 13). Moreover, the clinical studies have shown the antiulcer action of zinc in humans (14, 15). Previous studies revealed that zinc acts as the essential element in the physiology of the digestive system, accelerated the process of wound healing of various types of tissues including gastric ulcer in experimental animals and in humans (16-18).

Furthermore, *Helicobacter pylori* (*H. pylori*)-infected patients receiving standard anti-*H. pylori* therapy with amoxicillin, metronidazole, omeprazole and bismuth supported with capsules containing 220 mg of zinc sulphate (VI) every second day, exhibited a reduction in the ulcers size, which may reflect an acceleration of ulcer healing, that can be confirmed by endoscopy (19). It is known that the development of the gastro-duodenal ulcers may be influenced by many factors such as: *H. pylori* infection, longstanding stress and nonsteroidal anti-inflammatory drugs (NSAID) treatment, excessive gastric secretion, alcohol, cigarette smoking or irritating constituents of the diet that lead to gastric or duodenal mucosal damage, always accompanied by inflammation (20). Zinc may be helpful both in casual treatment due to its versatile activity and in palliative treatment facilitating damaged tissue regeneration. Zinc is a component of about 300 enzymes that ensure correct cell metabolism (21). It has been shown that nutrients including zinc have a positive impact on treatment of diseases such as celiac disease, HIV/AIDS and inflammatory bowel disease (21).

The compound *N*-(3-aminopropionyl)-L-histidinato zinc (polaprezinc), a chelate of zinc and L-carnosine, is an antiulcer agent developed in Japan (22). Polaprezinc was originally designed to combine the beneficial effects of zinc with carnosine but the question remains whether zinc or L-carnosine is the active therapeutic ingredient of polaprenzinc. It is of interest that carnosine itself increased the formation of granulation tissue and accelerated gastric ulcer healing in rats. Several reports have shown the protective action of polaprezinc against experimental gastric lesions induced by various noxious agents (23-25) and that polaprezinc accelerates gastric ulcer healing in humans (26). Although the mechanism of gastroprotective and antiulcer actions of polaprezinc has not fully explained an increase in mucosal secretions, the generation of endogenous prostaglandins (PGs), the membrane-stabilizing effect, and antioxidant properties (23, 26) were proposed to contribute to its beneficial effect.

Our present study was designed to determine the role of zinc in the process of gastric ulcer healing by monitoring the alterations in the size of gastric ulcers during healing and the accompanying changes in gastric blood flow (GBF) at the ulcer margin, ulcer bed and non-ulcerated gastric mucosa. We measured the luminal as well as the mucosal concentration of zinc in gastric juice and mucosa by means of anodic stripping voltammetry (ASV) (27) and flame atomic absorption spectroscopy (F-AAS) (28) methods. Furthermore, we evaluated by histology the quality of healing of the acetic acid ulcers without and with zinc administered in the form of zinc hydroaspartate. In a separate group of conscious rats equipped with gastric fistulas, the gastric acid secretion and plasma gastrin were determined in order to assess the effect of zinc hydroaspartate on these functional parameters during ulcer healing.

MATERIAL AND METHODS

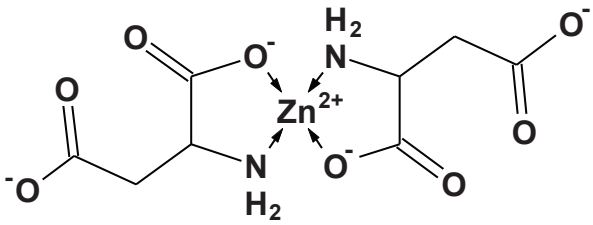
Male Wistar rats, which weighed between 200-250 g and fasted for 24 hours were used for gastric secretory studies and the induction of gastric ulcers. This study was approved by the Institutional Animal Care and Use Committee of Jagiellonian University Medical College in Cracow and run in accordance to the statements of Helsinki Declaration regarding the handling of experimental animals.

Production of gastric ulcers

Gastric ulcers were produced in rats using our modification (29) of acetic acid method originally proposed by Okabe *et al.* (30). Animals were anesthetized with pentobarbital natrium, the abdomen was opened and the stomach was exposed. The volume of 75 µl of acetic acid was poured through the plastic mold (6 mm diameter) onto the serosal surface of the anterior wall of the stomach just proximal to the antral gland area for 25 s. This produced an immediate necrosis of the entire mucosa and submucosa (but not serosa) within the area where the acetic acid was applied, *i.e.*, about 28 mm^2. The excess of acetic acid was then removed and the serosa was gently washed out with saline. Our previous studies documented that these ulcers became chronic within 2-3 days and healed completely within 2-3 weeks without perforation or penetration to the surrounding organs as described in our original technique (29, 31). After the application of acetic acid the animals were allowed to recover from anesthesia and received only water on the day of the operation (day 0). Then, they were divided into various groups and received normal chow and water *ad libitum* for the next 3, 7 and 14 days, respectively.

Effect of zinc hydroaspartate (HZnAsp) on gastric acid secretion, the rate of ulcer healing and gastric blood flow in the ulcer area

Three major series (A, B and C) of experiments were carried out in rats with chronic gastric ulcers. Series A was used to determine the effects of twice daily intragastric (i.g.) administration of vehicle (saline) or various doses of HZnAsp (Farmapol, Poland) ranging from 16.25 mg/kg-d up to 130 mg/kg-d. The chemical structure of HZnAsp bis[2-(amino-κ*N*)aspartato(2-)-κ*O* [1]]zincate(2-) is presented in *Fig. 1*. In rats of series B, the effects of HZnAsp (65 mg/kg-d i.g.) on gastric secretion was determined in separate group of 12 rats that were equipped with gastric fistula (GF) in whom gastric ulcers were induced 1 month prior to the ulcer induction (31). Series C received saline (i.g. or i.p.) and served as control. HZnAsp was dissolved in saline and given at graded doses or administered twice daily in the dose of 65 mg/kg-day i.g. that was selected based on the initial experiment with dose-dependent effect of this compound. This dose of HZnAsp was administered to rats



bis[2-(amino-κ*N*)aspartato(2-)-κ*O*[1]]zincate(2-)

Fig. 1. The chemical structure of zinc hydroaspartate (HZnAsp), a compound containing zinc (Zn^{2+}) ion.

with gastric acetic acid ulcer throughout the period of 3, 7 and 14 days.

To evaluate the effects of HZnAsp administered exogenously on the gastric blood flow (GBF), the animals were anesthetized with ether and the abdomen was opened with the stomach exposed to assess the GBF at the ulcer margin, ulcer crater and in the contra-lateral intact mucosa using H_2-gas clearance technique or laser flowmetry (Model BPM 403A, blood perfusion monitor, Vasamedics Inc. St. Paul Minn., USA) (32, 33). The gastric blood perfusion was measured by laser Doppler technique and expressed in ml/min/100 g of gastric tissue. The GBF was examined in three places: at the bottom of an ulcer, at the ulcer margin and in the area not affected by ulcer. The stomach was then removed and pinned open for the determination of the area of gastric ulcers by planimetry (Morphomat, Carl Zeiss, Berlin, German) by two investigators under blinded conditions. Half of the stomach with gastric ulcer in rats with or without administration of vehicle (control) or HZnAsp was taken during autopsy and immediately fixed in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin and alcian blue/periodic acid Schiff (AB-PAS) methods for the histological assessment of the quality of the ulcer healing. Coded specimens of mucosa stained with hematoxylin and eosin were evaluated at 260x magnification under blinded conditions.

Determination of plasma gastrin levels

At the termination of some experiments with exogenously administered vehicle or HZnAsp, the blood samples (about 3 ml) were taken from the *vena cava* (into tubes containing 2500 U Trasylol, Bayer, FRG and 0.5 mg/ml of EDTA). For comparison, intact rats fasted overnight and given only vehicle saline i.g., were also anaesthetized with ether and the blood samples were collected for the determination of control values of gastrin in the plasma. The blood samples were stored at -20°C until radioimmunoassay (RIA) of gastrin using gastrin antiserum 4562 (kindly donated by Professor J.E. Rehfeld of Aarhus, Denmark) described in detail previously (34, 35). The anti-gastrin antibody recognized gastrin-17 and gastrin-34 equally well. The RIA system for gastrin was sufficiently sensitive to detect approximately 2.5 pM/L plasma equivalent to human gastrin-17 as described before (36).

Determination of gastric acid secretion and the luminal and mucosal concentration of Zn^{2+} in rats treated with vehicle or HZnAsp

The alterations of gastric secretion during ulcer healing in rats treated with vehicle (saline) and HZnAsp were tested in a separate group of 30 starved rats with acetic acid ulcers, surgically equipped with chronic gastric fistulas (GF). Control sham-operated rats with GF were also included, but instead of acetic acid, 70 µl of saline was applied to the serosal surface of the stomach for 20 s. Vehicle or HZnAsp (65 mg/kg) was applied daily in a dose of 65 mg/kg i.g., to GF animals in a manner similar to that described above. After recovery from anesthesia (day 0) or at day 1 to 3, and at day 7 and 14 after ulcer induction, GF rats with and those without gastric ulcers were placed in individual Bollman cages to prevent coprophagy and to maintain the necessary restraint. Each GF was then opened, and the stomach rinsed gently with 5-8 ml of tap water at 37°C. Basal gastric secretion was collected for 120 min, during which time the rats received saline at a rate of 4 ml/h subcutaneously (s.c.). The gastric juice was collected every 30 min, the volume was measured, and then the acid concentrations and output were determined and expressed as the output per 30 min as described previously (34-36).

After the respective days from the onset of ulcer induction, the control animals without ulcers and those with gastric ulcer were placed in special Bollman cages and the gastric fistulae were opened in order to collect gastric juice. The gastric secretion was collected into calibrated tubes during six, thirty-minute periods. In each thirty-minute fraction the volume of gastric juice in the vehicle-control animals and in those treated with HZnAsp were noted and the concentration of H^+ in each sample was measured by titration of the gastric juice with 0.1 N NaOH to calculate gastric acid output. In addition, the Zn^{2+} ions have been determined by means of F-AAS method. The samples of the juice were thoroughly mixed before analysis due to their inhomogenity. The concentration of Zn^{2+} was measured using Perkin Elmer spectrometer Model 3110 (USA) in an air-acetylene flame under standard conditions (wavelength 213.9 nm; slit 0.7 mm). In some experiments the gastric biopsy was excised, nitrogen shock frozen in Eppendorf tubes and kept in -80°C until the measurement of Zn^{2+} concentration in the gastric mucosa.

Measurement of Zn^{2+} concentrations by the differential pulse anodic stripping voltammetry (DP-ASV) procedure

An electrochemical analyzer M161 (MTM-ANKO, Poland) was used in this study as described before (27, 37). The classical three-electrode quartz cell, volume 10 mL, consisting of a control growth mercury drop electrode (CGMDE) type M164 (MTM-ANKO, Poland) as a working electrode, used in the hanging mercury drop electrode (HMDE) mode, Pt wire as the auxiliary electrode, and a Ag/AgCl/3M KCl as the reference electrode. The pH was measurements were performed with laboratory pH-meter. All solutions used for analyses were purged with argon. Experiments were carried out at room temperature. The MTM-ANKO *EAGRAPH* software enabled electrochemical measurements, data acquisition and advanced processing of the results (38-40).

All solutions and the sample preparation were realized with quadrupled distilled water (last two stages from quartz). HNO_3 65%, H_2O_2 30% and KNO_3 (Merck, Suprapur®) were used for the preparation of samples and supporting electrolyte. Also Zn(II) standard stock solution (1000 $mg \cdot l^{-1}$, Merck) was applied. About 50 mg of dried sample of the gastric mucosa was weighed and transferred into a high pressure PTFE container and treated with 4 ml of HNO_3 and 2 ml of H_2O_2 (30%). The container was then placed into a microwave oven (Microwave digestion system Anton Paar Multiwave 3000). Digestion of the samples was carried out with the following program: 20 min under microwave irradiation, a 45 min cooling time, and a 5 min waiting time. Digested samples were placed on a heated plate in order to evaporate the excess of reagents. The sample solutions were cooled to room temperature and transferred quantitatively into volumetric flasks (5 ml) and filled up to the mark with four times distilled water. Glassware was first immersed in 6 M nitric acid, and then rinsed repeatedly with distilled water.

The stripping was performed in the differential pulse (*DP*) mode. The analysis was performed in a 5 mL aliquot containing 0.01 M KNO_3 and 20-200 µL of a sample solution. The ASV procedure was performed with the following steps in an uninterrupted sequence: (a) a new drop generation for HMDE electrode; (b) the pre-concentration step: E_{acc}=-1.15 V; 15 s „t_{acc}" 60 s; (c) after a rest period of 5 s, a DP voltammogram was recorded. Conditions for the DP mode were as follows: pulse amplitude, 30 mV; potential step, 2 mV and 20 ms; potential range, from -1.15 V to -0.75 V.

Quantitative determination was performed using the standard addition method (27, 36) The voltammogram for the blank solution demonstrated the electrochemical cell and supported the electrolyte purity.

Statistical analysis

All results are presented as mean ±SEM. Statistical analysis was conducted with the following methods; comparison between two groups were done with t-Student's test for unequal variances, comparison between more than two group were done with the ANOVA, if the results shows statistical significance the post-hoc Dunnett's test. If the distribution of the data significantly differed from the normal distribution, ANOVA was replaced by nonparametric Kruscal Wallis test. Statistical analyses were carried out by using a computer program (SPSS for Windows). All statistical analysis have been proceed with SAS JMP 7.02 package (license nr GSE8SNJ0JT). Some results are presented by the box plots including mean lines, standard error boxes and standard deviation wickers.

RESULTS

Effect of vehicle and HZnAsp on gastric ulcer healing and the GBF at ulcer margin

Fig. 2 shows the macroscopic appearance of the acetic acid gastric ulcer at day 7 upon ulcer induction in an animal treated with vehicle (saline). The deep ulcer crater with clearly defined ulcer margin and the ulcer bed is visible. The results of the 7 days administration of vehicle or HZnAsp applied i.g. in graded doses ranging from 16.25 mg/kg-d up to 130 mg/kg-d on the area of gastric ulcers and the accompanying changes in GBF at the ulcer margin were presented in *Fig. 3*. In rats treated with vehicle throughout the period of 7 days, a significant reduction in the area of these ulcers was observed from initial size 28 mm^2 to 18.6±2.1 mm^2. HZnAsp given in a dose 16.25 mg/kg-d i.g. failed to affect significantly the area of gastric ulcers as compared to that obtained in vehicle-control animals. With increasing the dose of HZnAsp up to 32.5, 65 mg/kg and 130 mg/kg-d, a significant decrease in the area of gastric ulcers was observed, by about 14%, 34%, 59% and 82%, respectively, as compared with that recorded in the vehicle-treated animals (*Fig. 3*).

The GBF in the non-ulcerated mucosa of rats treated with vehicle averaged 46±6 ml/min-100 g (taken as 100%). HZnAsp applied i.g. in a dose of 16.25 mg/kg failed to affect the GBF at the ulcer margin but a significant increase in the GBF at the ulcer margin was recorded in the gastric mucosa of rats treated with HZnAsp applied i.g. at higher doses ranging from 32.5 up to 130 mg/kg-d (*Fig. 3*). HZnAsp administered at a dose of 130 mg/kg-d, produced a significant rise in the GBF and this increase was not significantly different from that obtained with HZnAsp given at the dose of 65 mg/kg-d (*Fig. 3*).

The time-course of gastric ulcer healing in rats treated with vehicle or the standard dose of HZnAsp (65 mg/kg-d i.g.) starting from the initial size recorded at day 0 to day 3, day 7 and day 14 is shown in *Fig. 4*. The acetic acid gastric ulcers healed progressively in vehicle-control rats and the area of gastric ulcer was significantly reduced within 14 days after ulcer induction but still about 65% of rats had the ulcer healing not completed. At day 7 and day 14 upon the ulcer induction, the area of gastric ulcers gradually decreased in vehicle-treated control rats by about 58% and 80%, respectively. In contrast, the area of ulcers in rats treated with HZnAsp remained significantly smaller when compared to the respective values in the vehicle-treated control rats (*Fig. 4*). As shown in *Fig. 5*, the area of gastric ulceration was significantly decreased in animals treated with HZnAsp at day 7 upon ulcer induction when compared to that measured in the vehicle-control rats.

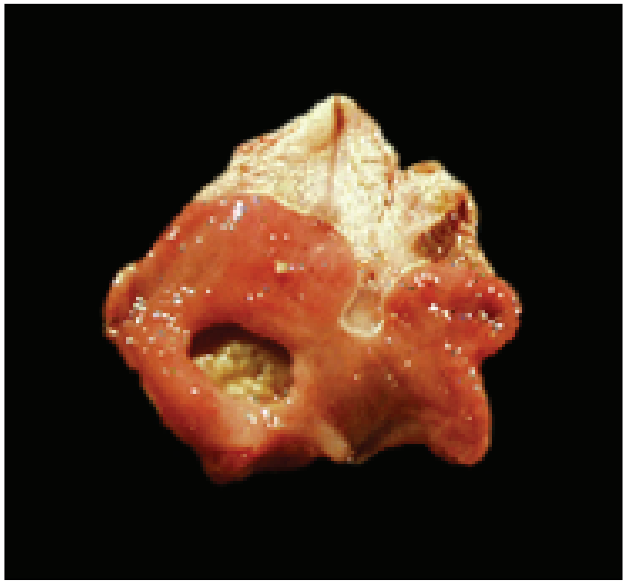


Fig. 2. Gross appearance of the gastric ulcer induced in the rat stomach by the serosal application of acetic acid (for details, see Material and Methods).

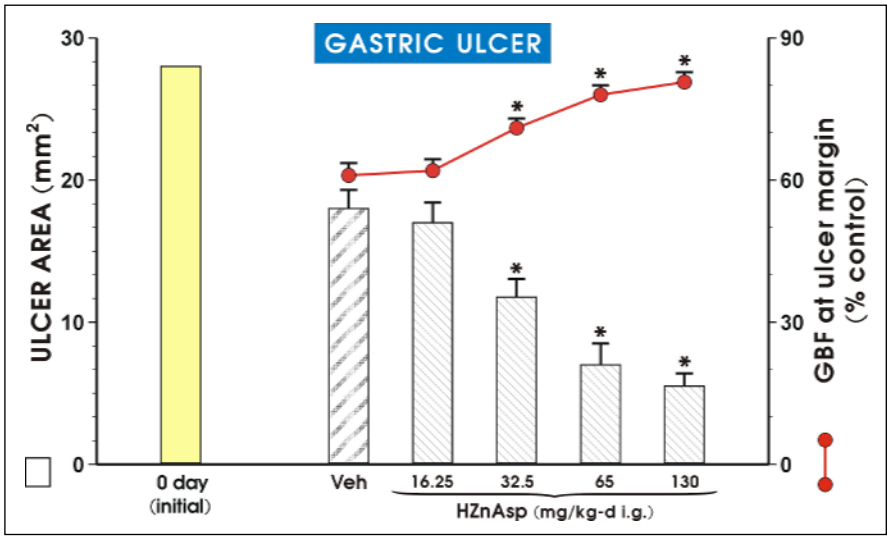


Fig. 3. The area of gastric ulcers and gastric blood flow (GBF) at ulcer margin at day 7 in rats treated with vehicle (Veh) and zinc hydroaspartate applied i.g. in graded doses ranging from 16.25-130 mg/kg-d i.g. Mean ±SEM of 6-8 rats. Asterisk indicates a significant change as compared to the value obtained in vehicle-treated gastric mucosa at day 7 upon ulcer induction.

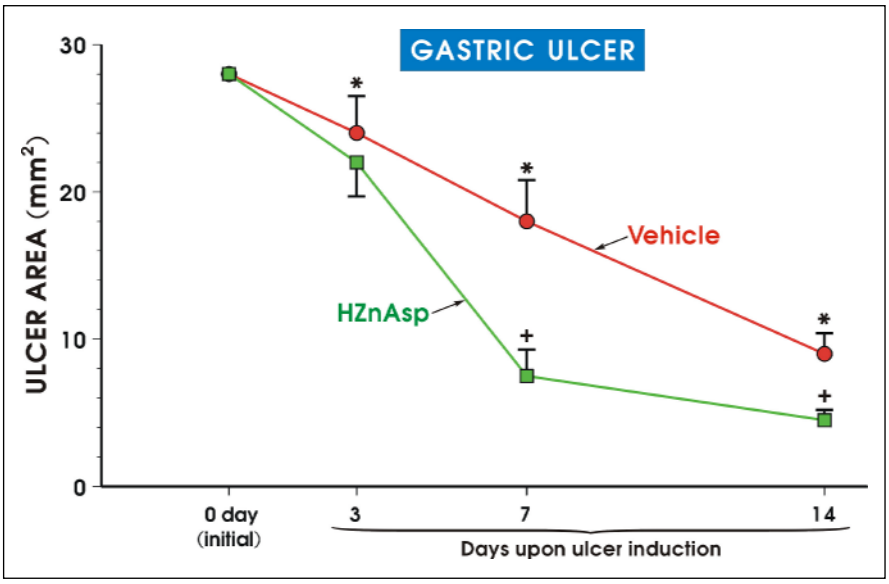


Fig. 4. Mean area of gastric ulcers in rats treated with vehicle (Veh, saline) and HZnAsp (65 mg/kg-d i.g.) and determined at day 0, 3, 7 and 14 upon ulcer induction. Mean ±SEM of 6-8 rats. Asterisk indicates a significant change as compared to the value obtained at day 0 and day 3. Cross indicates significant decrease below the values obtained in vehicle-treated animals.

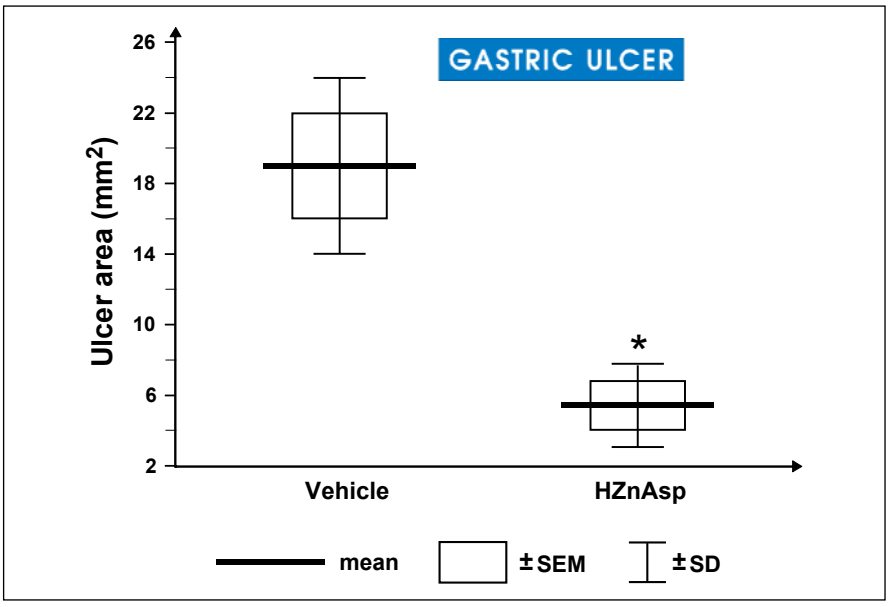


Fig. 5. The changes in the area of gastric ulcers at day 7 upon ulcer induction in rats treated daily with vehicle (saline) and HZnAsp (65 mg/kg-d i.g.). Mean ±SEM of 8-10 rats. Asterisk indicates a significant change as compared to the value obtained in vehicle-treated gastric mucosa.

By histology, the non-ulcerated gastric mucosa showed the normal gland architecture and an intact continuity of the surface epithelium and this histological appearance of gastric mucosa was similar in animals without ulcer induction who received i.g. treatment with HZnAsp (*Figs. 6A* and *6B*). At day 7, only 15% of control vehicle-treated rats showed almost complete healing of gastric ulcer as reflected by the remnant ulcer crater, but in those with macroscopically healed ulcer, microscopically the ulcer scar exhibited marked gland dilation and an incomplete reconstruction of mucosal cells at ulcer margin (*Fig. 6C*). In contrast, in the majority of rats treated with HZnAsp, the gastric ulcers were not observed both macroscopically or microscopically and the healing zone at the ulcer margin showed well developed restoration of surface epithelium, however the "healed" gastric mucosa confirmed abnormal glandular appearance (*Fig. 6D*).

The GBF at the ulcer margin was significantly decreased in the vehicle-control rats when compared to that in the intact non-ulcerated gastric mucosa (*Fig. 7*). Administration of HZnAsp significantly increased the GBF at the ulcer margin when compared to the respective values of GBF at the ulcer margin recorded in the vehicle-control animals (*Fig. 7*).

Fig. 8 shows the values in GBF in the intact gastric mucosa and for comparison those recorded at the ulcer margin and the ulcer bed in rats treated for 7 days with vehicle or HZnAsp. The GBF in the non-ulcerated gastric mucosa was not significantly affected by the treatment with HZnAsp. In contrast, the GBF at the ulcer margin and ulcer bed reached significantly lower values when compared to that in the non-ulcerated gastric mucosa, though the GBF was significantly increased at the ulcer margin than that in the ulcer bed. The GBF at the ulcer margin and the ulcer bed was significantly higher in rats treated with HZnAsp when compared to the respective values of GBF at the ulcer margin and the ulcer bed recorded in vehicle-controls (*Fig. 8*).

Effect of HZnAsp on gastric acid secretion and plasma gastrin during ulcer healing

The results of the gastric secretory studies in conscious rats equipped with gastric fistula with or without induction of gastric ulcers are presented in *Table 1* and *Fig. 9*. In control rats without gastric ulcers, the basal acid output averaged 137±12

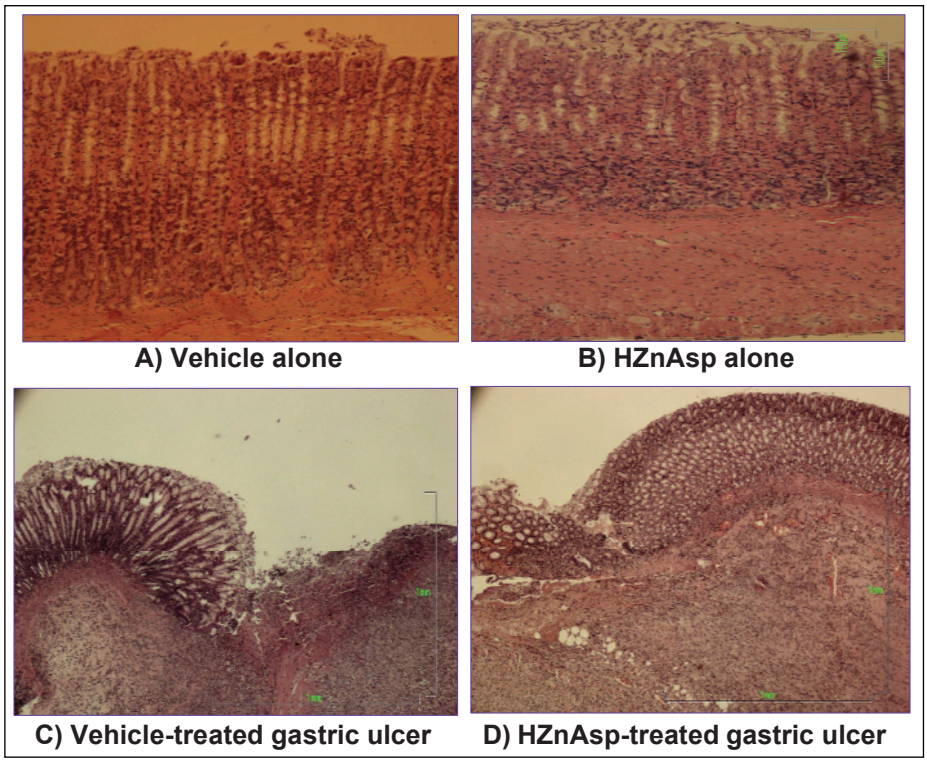


Fig. 6A-D. Histological appearance of the intact gastric mucosa without gastric ulcer. A: in rat treated with vehicle for 7 days, the surface epithelium is continued and the gastric glands show normal architecture. B: the non-ulcerated gastric mucosa of rat treated with HZnAsp (65mg/kg-d i.g.) shows the normal appearance not significantly different from that treated with vehicle. C: gastric ulcer in rat treated with vehicle (control) at day 7 after ulcer induction. Note, that ulcer crater is clearly visible, the healing zone is poorly developed and non-healed ulcer consists of inflammatory cell reach exudate. D: gastric ulcer in rat treated with HZnAsp at day 7 after ulcer induction. Comparing to vehicle-control rat, the gastric ulcer is almost completely healed but the ulcer scar is filled with poorly differentiated epithelium forming dilated irregular glands. So called "healed mucosa" reflecting advanced healing process still shows abnormal appearance. H&E, magnification x260.

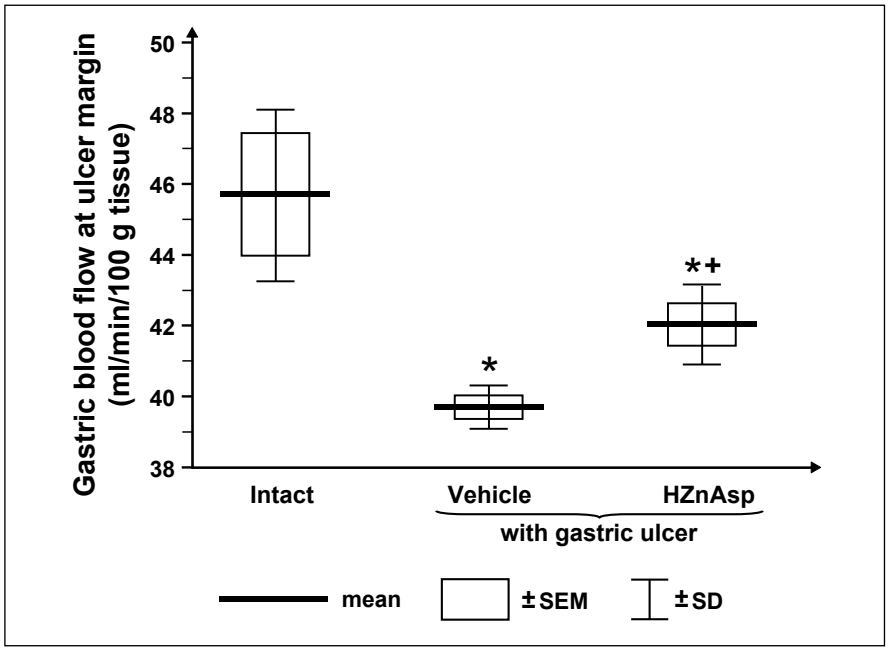


Fig. 7. The gastric blood flow (GBF) in gastric mucosa of intact rats and those with acetic acid induced gastric ulcer treated throughout the period of 7 days with vehicle (saline) or HZnAsp (65 mg/kg-d i.g.). Mean ±SEM of 6-8 rats. Asterisk indicates a significant change as compared to the value obtained in intact animals. Asterisk and cross indicate a significant change as compared to the value obtained in vehicle-control rats.

µmol/30 min and plasma gastrin level reached the value of 38±3 pM/L. Immediately after induction of gastric ulcers, the gastric acid output was significantly reduced by about 55% and the plasma gastrin level was significantly increased by about 15% as compared with the respective values in control rats without ulcer induction. Treatment with HZnAsp (65 mg/kg-d i.g.) resulted in a further significant decrease in gastric acid outputs and the significant rise in plasma gastrin as compared to those treated with vehicle (saline) (*Table 1*). On day 3, the gastric secretion tended to increase but still significant inhibition of gastric acid output was observed in vehicle-treated animals when compared to those in rats without gastric ulcers. HZnAsp significantly reduced gastric acid output and also significantly increased the plasma gastrin concentration comparing to the values obtained in animals treated with vehicle. After 7 days, the values of gastric acid output in vehicle-control rats were still significantly lower when compared to those obtained in intact rats without ulcer (*Fig. 9*). In rats treated with HZnAsp, a significant decrease in the gastric acid output was still observed on day 7 when compared to respective vehicle-controls (*Table 1, Fig. 9*). On day 14, the gastric acid outputs in the vehicle-treated animals reached the value similar to those attained in rats without ulcer induction but, in contrast, the decrease in the gastric acid output was still observed in HZnAsp-treated rats when compared to those treated with vehicle (*Table 1*).

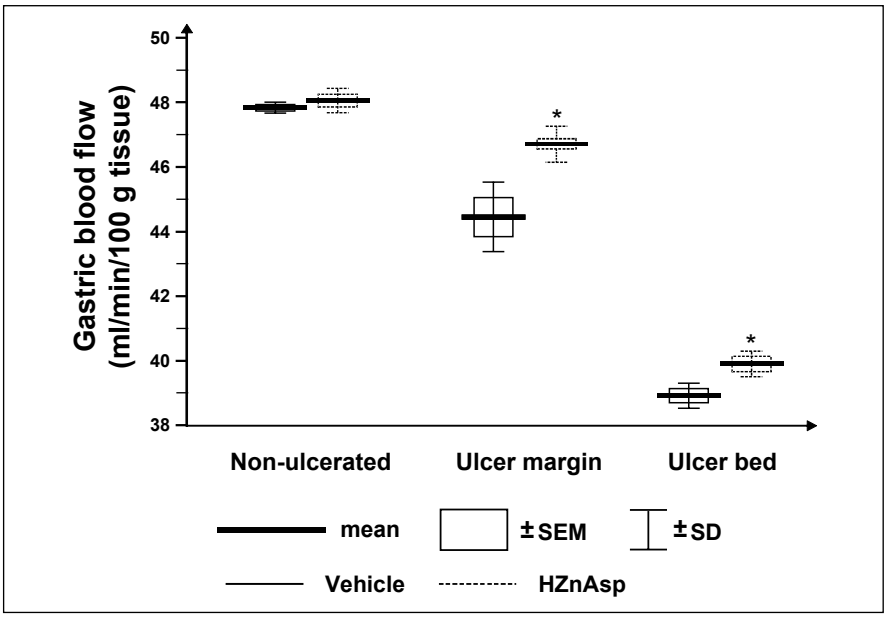


Fig. 8. The alterations in gastric blood flow (GBF) in non-ulcerated gastric mucosa, at ulcer margin and ulcer bed in rats treated throughout the period of 7 days with vehicle (saline) or HZnAsp (65 mg/kg-d i.g.). Mean ±SEM of 6-8 rats. Asterisk indicates a significant change as compared to the value obtained in vehicle-treated animals.

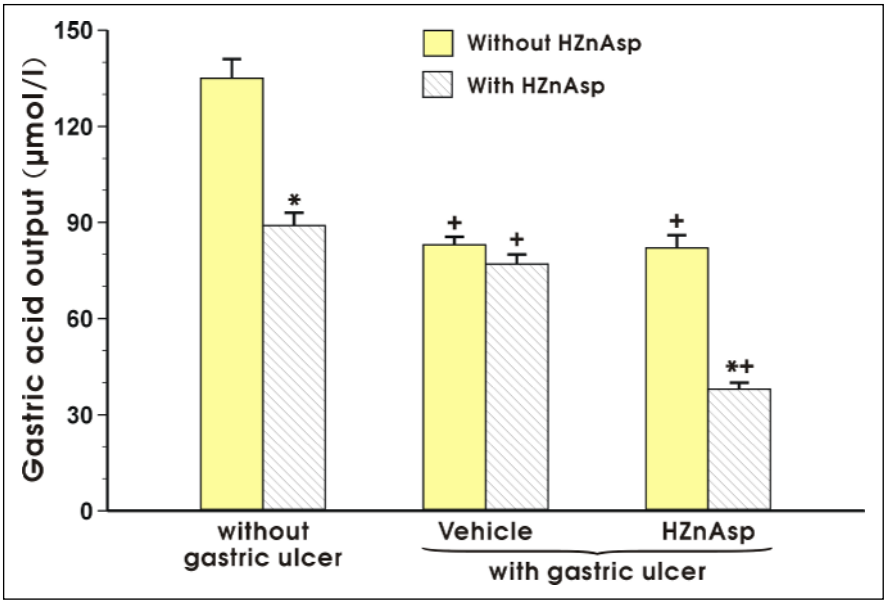


Fig. 9. The gastric acid output in rats without or with gastric ulcer equipped with gastric fistulas and treated with vehicle (saline) and HZnAsp (65 mg/kg-d i.g. for 7 days). Mean ±SEM of 6-8 rats. Asterisk indicates a significant change as compared to the value obtained in vehicle-treated animals. Cross indicates a significant change as compared to the value obtained in rats without gastric ulcer. Asterisk and cross indicate a significant change as compared to the value obtained in rats with gastric ulcer without HZnAsp treatment.

Effect of vehicle or HZnAsp administration on the concentration of Zn2+ in gastric juice and gastric mucosa in rats without or with gastric ulcer

Fig. 10 shows the time sequence of the effect of vehicle or HZnAsp administration on the luminal Zn^{2+} content in intact rats and those treated with vehicle of HZnAsp (65 mg/kg-d i.g.) as determined on day 1, day 3 and day 7 upon ulcer induction. *Fig. 11* shows the effect of 7 days administration of vehicle and HZnAsp on the concentration of Zn^{2+} in the gastric mucosa at ulcer margin. The luminal Zn^{2+} contents were similar in intact non-ulcerated gastric mucosa and in vehicle-control rats with gastric ulcer and those treated with HZnAsp on day 1 and day 3 but in contrast, at day 7 the significant rise in the gastric luminal Zn^{2+} contents was observed when compared to the respective values measured in the vehicle-control rats. In rats with gastric ulcer, a significant decrease in the luminal Zn^{2+} ion concentration on day 7 was recorded in rats with gastric ulcer comparing to respective controls without gastric ulcer (*Fig. 10*). In contrast, the treatment with HZnAsp significantly increased the luminal and mucosal Zn^{2+} contents at day 7 upon ulcer induction (*Fig. 10* and *11*).

DISCUSSION

This study demonstrated that certain trace microelements, such as Zn^{2+} ion could be important for the gastric ulcer healing process. The supplementation of the stomach with experimentally induced gastric ulceration with zinc-containing compound HZnAsp, which increased the Zn^{2+} content in the ulcer area and its luminal concentration in gastric juice, markedly accelerated the ulcer healing and enhanced the gastric microcirculation around the ulcer. One of the major finding of our present study is the direct quantitative evidence for Zn^{2+} deficiency in the ulcerated gastric mucosa, suggesting that the endogenous Zn^{2+} content plays an important role in the process of ulcer healing with respect to zinc supplementation treatment modalities in peptic ulcer disease. The importance of this finding is supported by the fact that this depletion of Zn^{2+} content in the gastric mucosa at the ulcer margin was abolished in animals supplemented with HZnAsp.

Our present study demonstrates that exogenous Zn^{2+}administered in the form of HZnAsp, dose-dependently

accelerated the healing of chronic gastric ulcers and that this ulcer healing action was accompanied by the rise in gastric blood flow at the ulcer margin, the increase in luminal and mucosal Zn^{2+}content and the enhancement in plasma gastrin levels. The importance of endogenous Zn^{2+} in the healing of gastric ulcer is emphasized by the fact that the Zn^{2+} concentration at the ulcer margin was diminished during ulcer healing and this effect was abolished by the supplementation with HZnAsp of animals with gastric ulcer. It is of interest to note that the luminal Zn^{2+} concentration at the ulcer margin was already significantly decreased during the first days of ulcer induction. This fact suggests that the early fall in the concentration of this microelement resulted in the depletion of the ion concentration in both the ulcer area and in gastric juice. This information could significantly influence the natural course of ulcer healing. The mechanism of the Zn^{2+}-induced gastric healing process is not fully explained in this study and further studies are definitely needed to determine whether potent gastroprotective and ulcer healing mediators such as endogenous PG and NO could be involved in the ulcer healing action of zinc-containing compounds.

Our study showed for the first time that the ulcer healing effects of Zn^{2+} were accompanied by a notable increase in the plasma gastrin levels indicating that endogenous gastrin may not only contribute to the to the spontaneous process of ulcer healing as originally proposed (41) but could participate in the acceleration of this healing by zinc-containing compounds. The increase in plasma gastrin observed in rats treated with HZnAsp could contribute to the acceleration of ulcer healing by zinc, because the prolonged hypergastrinemia induced by the administration of exogenous gastrin-17 and cholecystokinin (CCK) throughout the period of 5-15 days, increased cell proliferation at the ulcer margin and accelerated healing of the acetic acid ulcers (36, 40). The mechanism of this increase in plasma gastrin increments in HZnAsp-treated rats is not fully understood but could be attributed to the inhibitory action of HZnAsp on gastric acid secretion as demonstrated in our present study in rats with gastric ulcer that were surgically equipped with gastric fistulas. Interestingly, the treatment with HZnAsp inhibited not only basal gastric acid secretion in animals without gastric ulcer, but also found to be inhibited in rats with gastric ulcer induction using the acetic acid technique. Furthermore, we revealed, for the first time, that plasma gastrin level was significantly elevated in animals with chronic gastric ulcers treated with this zinc-containing formulation. This suggest that zinc may contribute to the ulcer healing action *via* an increase in the plasma level of gastrin, possibly due to its inhibitory effect on gastric secretion. This notion is supported by our previous evidence (31) and present observations that gastric secretion was suppressed during the initial phase of ulcer healing and that following the spontaneous healing of ulcers in the vehicle-controlled animals, the gastric secretion returned progressively to the level recorded in intact animals. In contrast, treatment with HZnAsp resulted in greater inhibition of gastric secretion on day 7 upon ulcer induction when compared to that in vehicle-treated animals with this inhibition persisting up to the end of the study period which was on day 14, with the values still being

Table 1. Effect of seven days administration of vehicle (saline) and HZnAsp (65 mg/kg-d i.g.) on gastric acid secretion in rats with gastric ulcers equipped with gastric fistula. Results are means ±SEM of 10-12 rats. Asterisk indicates a significant change as compared with the value obtained in intact animals. Cross indicates a significant increase as compared to the value obtained in vehicle control animals at respective days upon ulcer induction. Asterisk and cross indicate a significant change as compared to the value obtained in vehicle control animals at respective days upon ulcer induction.

Type of test	Gastric acid output	Plasma gastrin
	(μmol/30 min)	(pM/L)
Intact (Control)	137 ± 12	38 ± 3
Day 0		
Vehicle	56 ± 8*	41 ± 2
HZnAsp	51 ± 2*+	46 ± 2*+
Day 3		
Vehicle	65 ± 6*	48 ± 5*
HZnAsp	45 ± 5*+	68 ± 6*+
Day 7		
Vehicle	88 ± 7*	54± 6*
HZnAsp	53 ± 4*+	76 ± 7+
Day 14		
Vehicle	135 ± 9	58 ± 9
HZnAsp	78 ± 8*+	68 ± 6*+

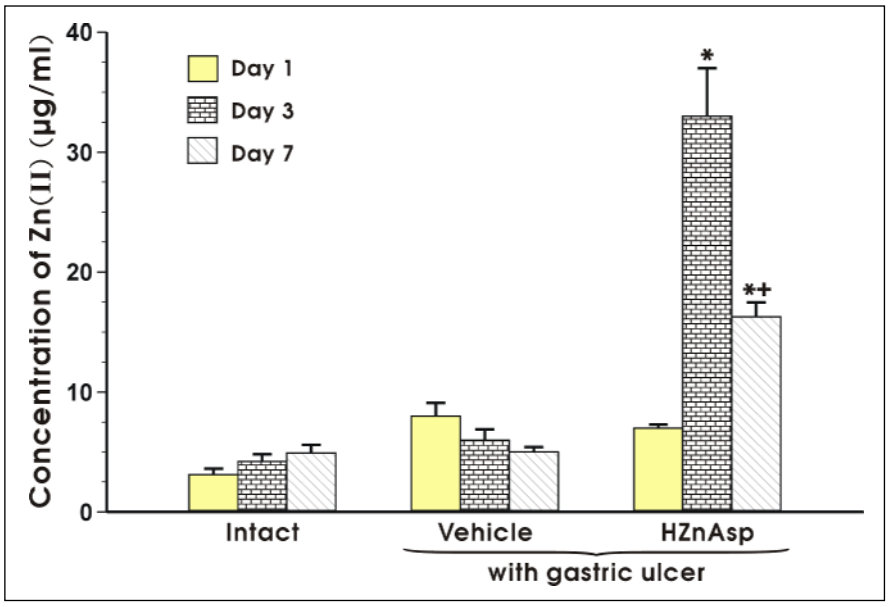


Fig. 10. The changes in the gastric luminal concentration of Zn^{2+} assessed by DP-ASV method in gastric juice collected from intact rats and those with gastric ulcer treated for 7 days with vehicle (saline) and HZnAsp (65 mg/kg-d i.g.). Mean ±SEM of 8-10 rats. Asterisk indicates significant change as compared to the value obtained in intact rats and those with gastric ulcer treated with vehicle. Asterisk and cross indicate a significant change as compared to the values recorded at day 3 in rats with gastric ulcer treated with HZnAsp.

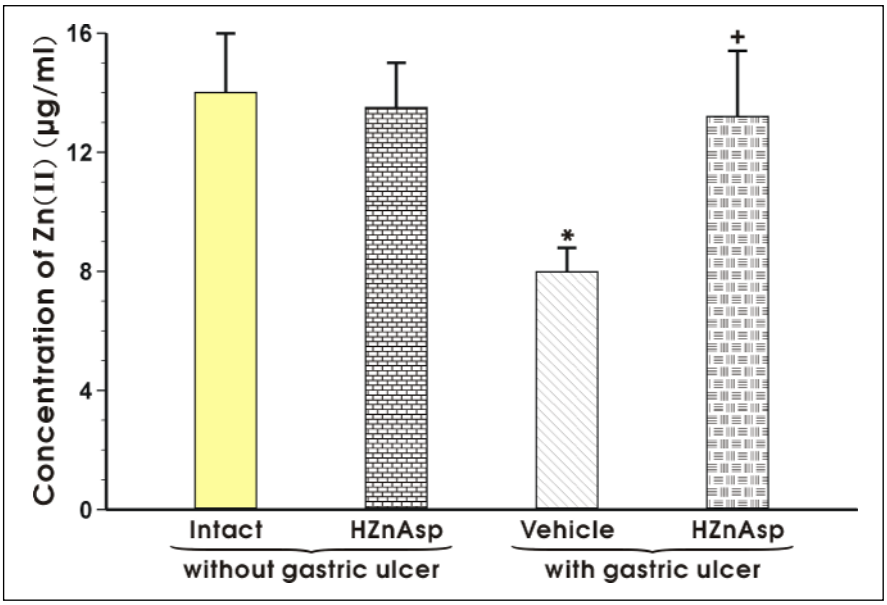


Fig. 11. The changes in the gastric mucosal concentration of Zn^{2+} assessed by DP-ASV method in gastric mucosa of intact rats and those with gastric ulcer treated for 7 days with vehicle (saline) and HZnAsp (65 mg/kg-d i.g.). Mean ±SEM of 8-10 rats. Asterisk indicates significant change as compared to the value obtained in intact rats. Cross indicates a significant change as compared to the values recorded in rats without gastric ulcer.

significantly lower than that measured in vehicle treated animals. Thus, this increase in the plasma gastrin observed in HZnAsp-treated animals could be secondary to the decrease in luminal acidity, due to the reduction in acid secretion caused by this compound. The importance of gastrin in the mechanism of ulcer healing was recently emphasized by the fact that transient hypergastrinemia that caused a trophic response in the gastric mucosa, accelerated healing of gastric ulcers induced by acetic acid and that this acceleration was reversed by administration of anti-gastrin antibodies (41). It is also possible that zinc-containing compounds could influence directly the G-cells to stimulate production of gastrin, but this hypothesis needs to be further explored.

Zinc complexes were originally shown to have antiulcer activity. Zinc-carnosine is an antiulcer drug commonly used in the treatment of gastric ulcers in Japan (22, 23). Of note, the zinc-indomethacin and the zinc-naproxen complexes more significantly attenuated these ulcerogenic effects, when compared with the parent NSAID, without affecting their therapeutic action (42, 43). Mucosal protection by zinc-containing drug, polaprenzinc was attributed to the stimulation of mucus production (24), antioxidant activity (22), membrane-stabilizing action (13), and the induction of heat shock protein (HSP) 70 (44). Besides HSP70, involvement of HSP32, also regarded as heme oxygenase (HO)-1, the potent protective factor against gastric damage and gastrointestinal disorders was reported (43). HO-1 is believed to act as the inducible isoform of HO, which catalyzes the first and rate-limiting step in heme degradation to produce equimolar quantities of biliverdin, carbon monoxide (CO), and free iron. Biliverdin is subsequently converted to bilirubin *via* the action of biliverdin reductase, and free iron is promptly sequestered into ferritin. CO and other toxic agents at low concentrations exert distinctly different effects on physiological and cellular functions; however, the major effect is vasodilatation. Besides vasodilatation CO leads to an inhibition of platelet aggregation (45). Interestingly, the addition of Zn (II) to curcumin resulted in the protection against gastric lesions induced by pylorus ligation by suppressing the expression of mRNA for NF-κB, TGF-1 and IL-8 (46). It was concluded that the antiulcer effect of zinc preparation against experimental pylorus-induced gastric lesions might be attributable to its ability to inhibit gastric acid secretion, and to enhance the mucosal defense mechanism through the suppression of NF-κB-mediated inflammation (46). These findings indicate that Zn(II)-curcumin complex prevents pylorus-ligation-induced damage by a mechanisms, which involves the reduction of NF-κB activation and subsequent production of pro-inflammatory cytokines (46). Previous studies revealed that muco-protective zinc-containing compounds such as polaprenzinc exhibit a potent reactive oxygen species (ROS) quenching effects (47). These compounds exerted various other pharmacological actions, such as induction of the expression of certain HSP proteins including HSP-27, HSP-72 and HO-1 (47). Both, HSP and HO-1 were shown to display cytoprotective and anti-inflammatory effects *via* the inhibition of adhesion molecules on polymorphonuclear leukocytes, or they were noted to inhibit proinflammatory cytokine production by the gastric epithelial cells. Polaprenzinc significantly inhibited the indomethacin-induced apoptosis of RIE-1 cells and the zinc component, rather than L-carnosine, contributed to the inhibition of the indomethacin-induced apoptosis (47). The gastro-protective and ulcer healing properties of zinc is supported by scientific evidence suggesting that zinc inhibited apoptotic proteins such as caspase-1 and caspase-3 signaling, mainly *via* ROS reduction in an ethanol-induced HepG2 cell (a human hepatocellular liver carcinoma cell line) injury model, and also in RIE-1 cells (47).

Zinc could inhibit oxidative stress *via* protection of cellular membranes and macromolecules, by its stabilizing effects of lipids and proteins, and preservation of sulfhydryl groups for proteins, preventing oxidation by forming strong thiolate complexes. Moreover, these protective and anti-apoptotic actions of zinc were shown to be mediated by the smac/DIABLO signaling in indomethacin-induced apoptosis of RIE-1 cells (47).

Our study concurs with these past reports by demonstrating that intragastric supplementation with zinc in animals with gastric ulcers led to a considerable enhancement in the mucosal and luminal Zn^{2+}content, ultimately leading to the acceleration of ulcer healing. Furthermore, we found that the healing effect of zinc hydroaspartate involves hyperemia at the ulcer margin, and this microcirculatory effects could be attributed to zinc. However, the hypothesis that zinc induced an increase in the gastric blood flow at the ulcer area and in the ulcer bed which could be mediated by others vasoactive mediators such as NO originating from the vascular endothelium, gastric epithelium or from the capsaicin-sensitive nerve endings could not be ruled out.

Zinc and zinc-containing proteins are involved in nearly every stage of cutaneous wound repair due to their profound role in the modification of the ECM component, cell migration, protein synthesis and anti-inflammatory properties (48, 49). This healing action by zinc could also be attributable to its protective action on visceral organs by demonstrating that zinc, which is considered to be an active component of polaprenzinc, exerts inhibitory effects on proinflammatory NF-κB activation (50, 51). This is in keeping with the observation that the Zn^{2+} ion administered in the form of $ZnSO_4$, inhibited NF-κB activation and TNF-α-induced impairment of gastric integrity, whereas L-carnosine had no significant effect. Thus, this suggests that the inhibitory effect of polaprezinc on NF-κB activation is likely to be mediated by its active component, zinc. Connell *et al*. (52) reported that zinc protects endothelial cells against cytokine-mediated activation of NF-κB and AP-1, that caused an up-regulation of inflammatory cytokines, and endothelial dysfunction.

The selection of compound carrying an active ingredient such as zinc seems to be significant since the most important considerations require balancing the solubility of the specific zinc salt used while at the same time maximizing the bioavailability of the zinc ion. We have employed zinc hydroaspartate to deliver zinc to ulcer area. This is based on the high efficacy of this compound to pass cellular membranes including the blood-brain barrier (53). It was reported that the soluble salts have the potential to be irritating, whereas sparingly soluble ones such as zinc oxide and zinc carbonate are not (53). Sparingly soluble forms of zinc may be thought to have limited bioavailability. Topical zinc oxide has been shown to be effective in several wound healing applications suggesting that zinc oxide seems to be more effective than soluble zinc salts. Pharmacokinetic studies revealed that sparingly soluble forms may offer the benefit of a long-lasting, slow-release form of zinc. Moreover, the patients suffering from ulcerative colitis, exhibited depleted content of zinc in the colonic mucosa which was associated with an increase in the excessive formation of reactive oxygen intermediates under these conditions (54).

In conclusion, this study serves as primary evidence that oral administration of zinc-containing compound such as zinc hydroaspartate accelerates ulcer healing as reflected by the reduction of the ulcer size in acetic acid induced ulcers, which was determined by macroscopic and microscopic assessment *via* mechanism involving an enhancement of the gastric microcirculation, the inhibition of gastric acid secretion and the subsequent rise in the plasma gastrin levels. We provided evidence that the healing action of zinc hydroaspartate is accompanied by an increase in the mucosal and luminal Zn^{2+} contents as well as a rise in the gastric microcirculation around the ulcer including ulcer margin and ulcer bed. These results indicate that the ulcer healing and microcirculatory activity of zinc could be a promising approach to counteract inflammation due to its anti-oxidizing and anti-secretory action.

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