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THE PROTECTIVE EFFECT OF COPPER COMPLEXES AGAINST GASTRIC MUCOSAL ULCER IN RATS

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Abstract—The study examines the anti-ulcer activity of Cu(I)–(nicotinic acid)$_2$Cl [CuCl(HNA)$_2$]. A dose of 8 mg (23 μmol) of complex/kg body mass was suspended in 0.25% Tween-80 in saline solution and administered intragastrically to male Wistar albino rats which had developed gastric ulcers as a result of pyloric ligation (Shay-rat model). Another group of animals received 5 mg (25 μmol)/kg body mass of the copper–glycinate complex Cu(II)(glycinate)$_2$ [Cu(II)(Gly)$_2$]. Both protected as shown by reduction in the ulcer index, inhibition of gastric perforation and death. Significant increases in gastric juice volume and superoxide dismutase (SOD) activity in the gastric mucosa and blood plasma were found with both copper complexes, while the gastric juice prostaglandin E_2 (PGE_2) content was significantly decreased in the Cu(II)(Gly)$_2$-treated group, it was significantly increased in the gastric mucosa of the CuCl(HNA)$_2$-treated group. The copper complex-treated animals, especially those which received Cu(II)(Gly)$_2$ had a marked fall in thromboxane A_2 (TXA_2) levels. These results suggest that intragastric administration of either CuCl(HNA)$_2$ or Cu(II)(Gly)$_2$ produced anti-ulcerogenic activity, with different modes of action.

Gastric and duodenal ulcers, still present one of the most common unresolved medical problems worldwide. In spite of an expanding list of drugs, recurrence after drug withdrawal is common because the disease is multi-causal and acid plays only a minor role [1]. The key step in its pathogenesis is the relation between the destructive and repair processes occurring in the mucosa. Treatment requires drugs with multiple pharmacological activities to counteract the multiple causes of ulcers [1]. Copper complexes may achieve this by activation of copper-dependent enzymes and their physicochemical properties [1, 2]. The present study investigates the anti-ulcer activity and mode of action of Cu(I)–(nicotinic acid)$_2$Cl complex, [CuCl(HNA)$_2$§] in the pyloric ligation-induced gastric ulcer in rats and compares it with the biochemical changes and the mode of action of Cu(II)(glycinate)$_2$ [Cu(II)(Gly)$_2$] a potent anti-ulcer copper complex [2].

MATERIALS AND METHODS

Animals. Male Wistar albino rats, weighing 200–300 g, were kept in plastic cages containing four rats each, and provided with a commercial balanced diet and tap water *ad lib.* After 2 weeks of acclimatization, the Shay rat gastric ulcer was produced according to the method described by Shay *et al.* [3]. Rats were fasted for 72 hr with free access to tap water. Each animal received intragastrically 10 mL of saline to avoid post-operative dehydration. The surgical procedure was performed under light diethylether anaesthesia. An abdominal incision was made, the stomach located, and the pyloris ligated. These rats were randomly divided into three groups. The control group of 25 rats received an intragastric dose of vehicle, 0.5 mL of 0.25% Tween-80 in saline solution. A second group of 20 rats received an intragastric dose of 8 mg (23 μmol) CuCl(HNA)$_2$/kg body mass, in 0.5 mL of vehicle. This complex was prepared as described by Goher [4]. A third group of 20 rats received an intragastric dose of 5 mg (25 μmole) Cu(II)(Gly)$_2$/kg body mass in 0.5 mL of vehicle. The complex was prepared according to Sorenson [5]. The copper content in each dose was 1.473 mg for CuCl(HNA)$_2$ and 1.372 mg for Cu(II)(Gly)$_2$. After administering the complexes, the abdominal incision was closed with sutures. When these animals recovered from anaesthesia, they were individually housed in drop-through cages, without food or water for 19 hr, and then killed.

Sample collection. Blood samples were collected from the jugular vein using EDTA as the anticoagulant. Plasma was separated and divided into aliquots and stored at −20°. The animal was then decapitated, the abdomen opened, and the duodenum cut. With the animal in a vertical position, the esophagus was excised and the cardiac opening closed. The gastric contents were collected in a graduated centrifuge tube, the volume recorded and the gastric juice centrifuged. Aliquots of the supernatant were separated and kept frozen at −20°

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§ Abbreviations: CuCl(HNA)$_2$, copper(I)–(nicotinic acid)$_2$Cl complex; Cu(II)(Gly)$_2$, copper glycinate; PGE_2, prostaglandin E_2; TXA_2, thromboxane A_2; SOD, superoxide dismutase; NANA, *N*-acetyl neuraminic acid; BGG, bovine gamma globulin; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

Table 1. Effect of copper complexes on ulceration in Shay rats

Parameter	Control N = 17	Copper(I)–nicotinic acid complex N = 20	Copper–glycinate complex N = 17
Ulcer index*	87.0 ± 4	18 ± 2	12 ± 1†
Severity factors*			
×3	7.0 ± 1	0	0
×2	5.0 ± 4	0	0
×1	57.0 ± 5	17 ± 2	12 ± 1
Prevention index (%)	0.0	78	85
Mortality (%)	17.4	0	0
Gastric perforation	35.0	0	0

* Values are means ± SEM. Severity of ulcer ranges between severe ulcer (necrosis), ×3 and very mild erosion, ×1.

† $P < 0.001$.

until analysed. After gastric juice collection, the stomach was opened and the mucosal surface washed with an ice cold saline solution. The fundic mucosal ulcer area was rapidly scored according to the method described by Peskar *et al.* [6]. The number of necrotic bands of more than 4 mm in length was multiplied by the severity factor 3, the number of lesions of 2–4 mm was multiplied by 2, and the number of lesions of less than 2 mm was multiplied by 1. Hence, the ulcer index is the sum of the total number of lesions times the corresponding severity factor. The efficiency of ulcer inhibition, the preventive index, was calculated according to the equation of Hano *et al.* [7]. The number of gastric perforations per group was recorded. A 50% or greater reduction in ulcer index compared to the control ulcer index indicates anti-ulcer activity. After scoring the ulcer, the fundic mucosal layer was weighed and homogenized in 2 mL phosphate-buffered saline, pH 7.4 containing 0.1 mg/mL (2.7 mM) EDTA and 2.2 mg/dL (60 μM) indomethacin to prevent further prostaglandin biosynthesis, or the thromboxane A_2 (TXA_2) synthetase inhibitor OKY-046 (100 μM), (*E*)-3-[4-(3-pyridylmethyl)phenyl]-2-propenoic acid hydrochloride monohydrate (from ONO Pharmaceutical Companies Japan).

Gastric juice analysis. Total acidity was determined using 0.01 M NaOH as described by Alumets *et al.* [8] and expressed as milliequivalents of H^+ per liter. Hemoglobin content and acid phosphatase activity were measured using kits from BioMerieux France, catalog numbers, 698790 A and 600281 A, respectively. Calcium was determined using an atomic absorption flame-photometer (Shimadzu AA-620). Gastric juice mucus content as *N*-acetylneuraminic acid (NANA, mg/dL) was assayed according to the method of Plucinsky *et al.* [9]. In 13 × 100-mm screw-capped culture tubes, 40 μL of juice and 960 μL distilled water were vortexed and then placed on ice. To each assay tube, was added 1 mL of freshly prepared resorcinol reagent made by the addition of 10 mL of 2% (w/v) aqueous resorcinol to 10 mL of 2.5 mM $CuSO_4$ and the mixture was brought to a final volume of 100 mL with concentrated HCl. Assay tubes were capped, vortexed and placed in boiling water for 15 min, followed by another 10 min in an ice bath. After the addition of 2 mL butyl acetate/*n*-butanol (85:15, v/v) to every assay mixture, tubes were vortexed and centrifuged at room temperature for 10 min at 1200 *g*. The extracted chromophores were recorded at 580 nm against a standard curve of 0–60 mg/dL of NANA (purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.).

Analysis of gastric mucosa. Total superoxide dismutase (SOD) activity was assayed in both plasma and gastric mucosa according to the method of Paynter [10]. Prostaglandin E_2 (PGE_2) and TXA_2 levels were determined in the gastric juice and mucosal homogenate after addition of EDTA (0.1 mg/mL) and indomethacin (60 μM) or TXA_2 synthetase inhibitor OKY-046 (100 μM), and extraction with ethyl acetate, using PGE_2–[^{3}H]RIA kit from Advanced Magnetic Inc. (Cambridge, MA, U.S.A.) and tritium labeled TXB_2, the stable degradation product of TXA_2 (BIOTEC, MA, U.S.A.). The radioimmunoassay buffer was a bovine gamma globulin-phosphate (BGG-phosphate) containing 0.01 M phosphate, 0.1% BGG, and 0.1% sodium azide at pH 7.0.

Antibody–antigen complexes were separated from unbound antigen by adding magnetic dextran coated charcoal suspension. At 50% displacement, the cross reactivity of the antisera used in the PGE_2–[^{3}H]RIA kit is reported to be 100% for PGE_2, 1.3% for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and lower than 1.0% for leukotrienes and the other cyclooxygenase dependent arachidonic acid metabolites. The assay detected 8 pg and 50% displacement of [^{3}H]PG occurred with 70 pg of PGE_2. The percent cross-reactivity of the antibody used in the TXB_2-kit is reported to be: 100% for TXB_2, 0.7% for $PGF_{2\alpha}$, 0.33% for prostaglandin D_2 (PGD_2), 0.033% for $PGF_{1\alpha}$, 0.0075% for PGE_2 and less than 0.002% for the other cyclooxygenase dependent arachidonic acid metabolites. The limit of detection for TXB_2 was 5 pg and 50% binding occurred at 50 pg.

Histological examination. Stomach fundic mucosal tissues were prepared and fixed for electron microscopic scanning. Small tissue blocks of the stomach fundic mucosa were fixed in 5% glutaraldehyde for 48 hr then washed in cacodylate buffer and post fixed in 1% osmium tetroxide for 2 hr. The fixed specimens were washed in buffer, dehydrated in ethanol series, then washed in amylacetate for 2 days, and dried in a critical point drier. The specimens were then mounted, sputtered, coated with gold and examined at 25 kV, in JEOL-T 200 scanning electron microscope (JEOL Ltd, Tokyo, Japan).

Statistical analysis. Results are expressed as mean ± SEM. Statistical analysis was performed with an analysis of variance to determine statistical significance among the groups followed by Student's *t*-test to determine significance between groups. A result was considered statistically significant at $P < 0.05$.

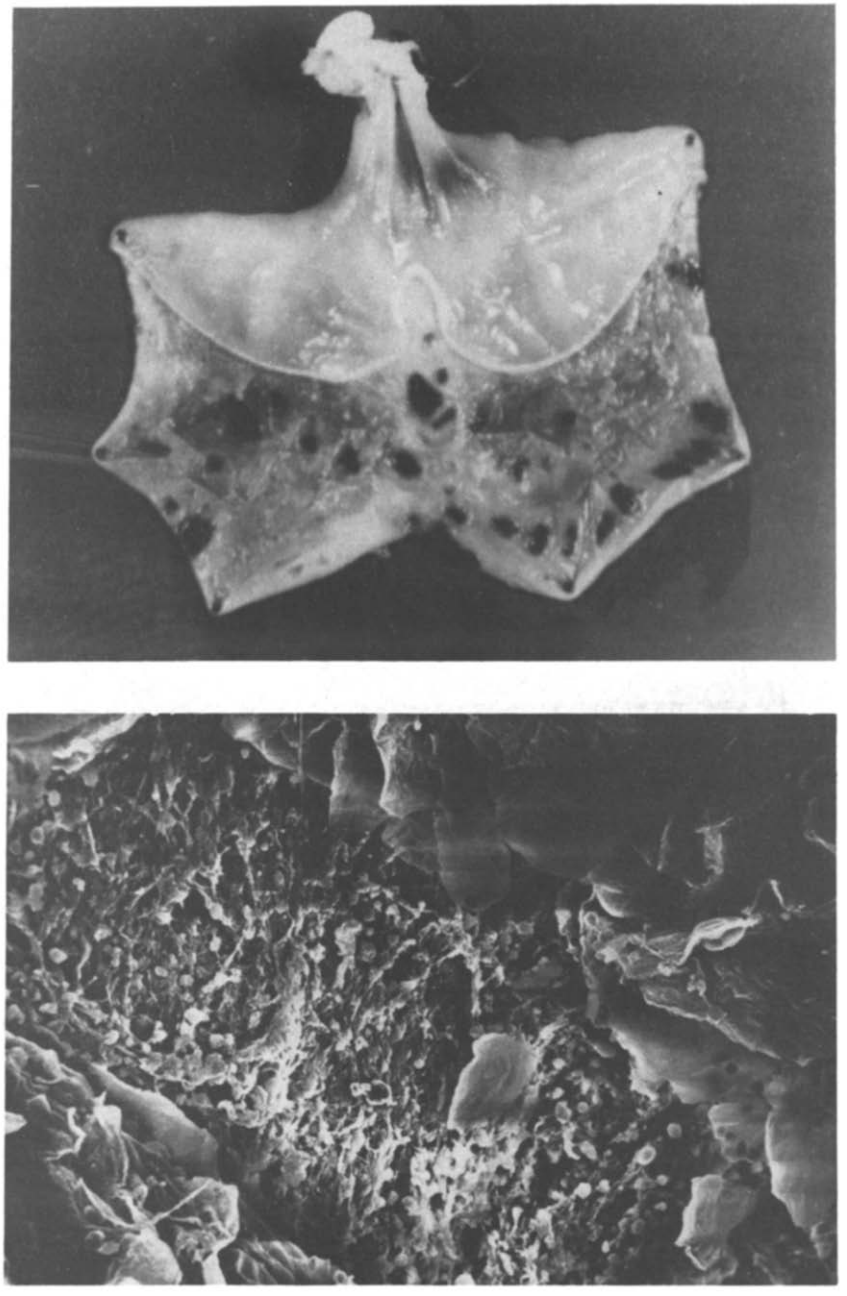

Fig. 1. (a) Stomach mucosal surface of a rat from the control group. It shows the mucosa studded with a number of ulcerations in the fundic area. Ulcerations are massive and deep. Some perforated the stomach wall. (b) Scanning electron micrograph of the stomach mucosal surface of the same experimental animal showing the stomach fundic mucosa with an ulcer that exposes the subepithelial lamina propria due to the shedding of the epithelial covering (magnification 350 × 2.7).

RESULTS

Table 1 shows that significant reduction in the ulcer index was produced by either $CuCl(HNA)_2$ or $Cu(II)(Gly)_2$ but that $Cu(II)(Gly)_2$ was more potent than $CuCl(HNA)_2$. Death during the post-operative 19-hr period due to gastro-esophageal perforation was only found in the control group. Figure 1a and b shows gross histopathology and a scanning electron micrograph of the mucosal surface of a control animal. By gross histopathology (Fig. 1a) massive and deep ulcerations are restricted to the fundic area of the mucosa. The scanning electron micrograph (Fig. 1b) shows one ulcer where the epithelium is completely detached and exposes the underlying lamina propria. On the other hand, Fig. 2a is a photograph of the stomach treated with $CuCl(HNA)_2$. The mucosal surface is glistening and

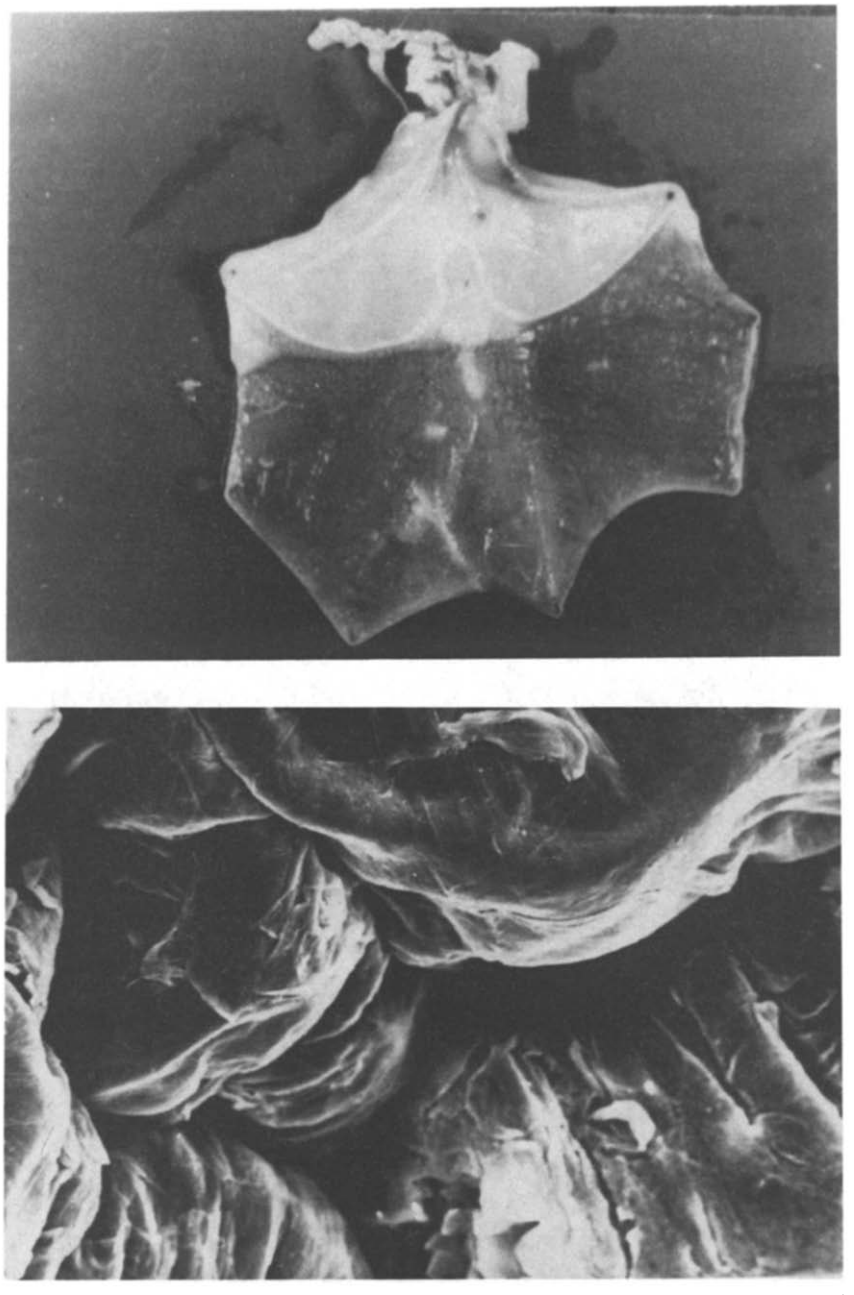

Fig. 2. (a) The stomach mucosal surface of a rat treated with $CuCl(HNA)_2$. Mucosal surface glistening, covered with mucus and studded with brownish erosions, restricted to the fundic area. (b) Scanning electron micrograph of the same experimental rat stomach. Fundic mucosa shows normal epithelium covered with a thick mucus layer (magnification 750 × 2.7). A typical gross picture and electron micrograph were obtained from rats treated with $Cu(II)(Gly)_2$.

studded with brownish erosions which are confined to the fundic area (Fig. 2a); whereas the scanning electron micrograph (Fig. 2b) shows normal epithelial lining covered by a thick mucus layer. A typical gross picture and an electron micrograph were obtained from rats treated with $Cu(II)(Gly)_2$.

The destructive parameters are shown in Table 2. The gastric juice content of total acid, calcium, acid phosphatase and hemoglobin were significantly lower in $CuCl(HNA)_2$ and $Cu(II)(Gly)_2$ treated animals than the control group. However, no significant differences were noticed between the two different copper complexes.

The therapeutic effects are presented in Table 3. Both $CuCl(HNA)_2$ and $Cu(II)(Gly)_2$ treatments increased the secretion of watery viscous juice NANA (mg/dL) and the mucus content for both $CuCl(HNA)_2$ and $Cu(II)(Gly)_2$ groups was

Table 2. Effect of copper complexes on the Shay ulcer

Parameters	Control	Copper(I)–nicotinic acid complex	Copper–glycinate complex	P Value
Gastric juice acid content (mEq/L)	58.59 ± 10.30	16.05 ± 2.2	18.24 ± 3.80	<0.001
Gastric juice Ca(II) content (μg/mL)	63.88 ± 4.60	34.40 ± 2.7	37.12 ± 2.10	<0.001
Gastric juice acid phosphatase (unit/L)	3.87 ± 0.37	2.63 ± 0.3	2.75 ± 0.22	<0.05
Gastric juice hemoglobin content (mg/dL)	733.30 ± 67.90	193.50 ± 21.0	192.18 ± 15.40	<0.001

N = 20. Values are means ± SEM.

Table 3. Effect of copper complexes on reactive changes

Parameters	Control	Copper(I)–nicotinic acid complex	Copper–glycinate complex	P Value
Gastric juice vol. (mL)	12.52 ± 1.0	18.50 ± 0.85	16.90 ± 1.00	<0.01
Gastric juice mucus content, NANA (mg/dL)	21.10 ± 1.7	10.36 ± 0.75	13.33 ± 0.64	<0.001
Gastric juice PGE_2 (pg/mL)	583.00 ± 43.0	520.00 ± 47.00	348.00 ± 45.00	<0.01
Gastric mucosal PGE_2 (pg/mg wet wt)	387.00 ± 36.0	699.00 ± 81.00	431.00 ± 27.00	<0.001
Gastric mucosal TXA_2 (pg/mg wet wt)	291.00 ± 31.0	201.00 ± 43.00	153.00 ± 51.00	<0.005
Gastric mucosal SOD activity (unit/mL)	770.00 ± 65.1	960.00 ± 59.20	1006.30 ± 66.10	<0.05
Plasma SOD activity (unit/mL)	48.00 ± 5.0	85.40 ± 5.50	68.10 ± 6.5	<0.001

N = 20. Values are means ± SEM.

significantly lower than the control. However, NANA was significantly higher in the $Cu(II)Gly_2$ group than in the $CuCl(HNA)_2$ treated group. Yet the gastric juice of these treated groups was viscous compared to the watery juice of the control. While the gastric juice PGE_2 was significantly lower in the group treated with $Cu(II)(Gly)_2$, it was significantly higher in the gastric mucosa of the $CuCl(HNA)_2$ group. However, the copper complex treated animals, especially those received $Cu(II)(Gly)_2$ showed a strong decline in TXA_2 levels (Table 3). Gastric mucosal SOD activity in both copper complexes groups showed a significantly higher activity than the control; whereas, the plasma level was the highest in the $CuCl(HNA)_2$ group, and the lowest in the control one.

DISCUSSION

The Shay rat gastric ulcer model [3], used in the present study, was reported to be simple, uniformly reproducible and highly predictable [11]. The Shay model utilizes neither exogenous ulcerogens nor induces endogenous interfering factors. Moreover, this model has been used to test the efficiency of the anti-ulcer drugs for human use [12]. The gastric ulceration developed in this model was restricted to the fundic area. This may be attributed to the lower content of the cytoprotective prostaglandins of this area compared to other gastric mucosal regions [13]. To our knowledge, most of the parameters studied in the present investigation were not tested previously in the "long term" Shay rat gastric ulcer model, which has a 72 hr pre-operative and a 19 hr post-operative fasting period. In the present study, the intragastric administration of $Cucl(HNA)_2$ and $Cu(II)(Gly)_2$ complexes strongly suppressed gastric ulcers in the Shay rat model. The reduction was 78 and 85% for $CuCl(HNA)_2$ and $Cu(II)(Gly)_2$, respectively, compared to untreated animals. Moreover, both complexes inhibited gastric perforation and death during the post-operative fasting period and, the existing ulcers, if any, were reduced in severity (Table 1). The lesion that developed healed within 1 hr, which is similar to that previously reported by Szabo and Vattay [14]. However, the untreated animals developed 35% gastric perforation and 17% mortality.

The intragastric administration of either $CuCl(HNA)_2$ or $Cu(II)(Gly)_2$ stimulated the pro-

duction of gastric juice, however the NANA content was decreased. This could be attributed to the increased blood content in the gastric juice mucus of the untreated animals since blood is rich in sialic acids [9]. These results are consistent with retention of mucoids on the protected mucosa while necrosis triggers the liberation of mucosal glycoprotein- and/or glycolipid-bound neuraminic acid. The electron microscopic scanning micrographs show a thick mucus covering on the two treated mucosa, compared to the non-treated mucosa. Enhanced fluid flux by the gastroprotective agents may contribute to mucosal protection by diluting the injurious agents in the vicinity of gastric mucosa and/or lumen [15]. Furthermore protein in the extravasated fluid may scavenge free radicals [1]. The fluid efflux is an indicator of enhanced blood flow which is necessary for anti-ulcer action [9].

Lysosomal enzymes, such as acid phosphatase and hemoglobin, are used to assess the extent of mucosal damage [15, 16] but it is difficult to establish whether they are the cause, or the result of mucosal damage. Once these enzymes are released, they trigger further lesions and contribute to chronic changes [15]. Our results show that treatment with copper complexes decreases the content of gastric juice acid phosphatase and hemoglobin and indicate that copper complexes stabilize the lysosomal membranes and stimulate blood flow which in turn prevents gastric congestion and capillary damage.

Pyloric ligation stimulates acid secretion due to the activation of pressure receptors in the pyloric region. However, vagal stimulation is involved rather than histaminergic mechanism [8]. A significant reduction of gastric acid content [70%] was noticed for both copper complexes. Since copper complexes were shown to have no significant anti-cholinergic activity [17] and, since copper–histamine complex was claimed to have a potent anti-ulcer and anti-secretory activity [2], it was thought that the formation of copper–histamine complex *in vivo* may account for the observed anti-ulcer and anti-secretory activities of these agents.

Studies that correlate abnormal calcium mobilization with ulcer pathogenesis are few. Calcium channel blockers were reported to prevent ulceration against chemically induced gastric acid secretion [18]. Our results demonstrated that treatment with copper complexes produced more than 40% reduction in lumenal calcium efflux along with the anti-ulcerogenic activity of these agents. The pathogenesis of oxidative injury was reported to be mediated through lipid peroxidation, changes in calcium release and glutathione depletion [19]. Therefore, the best approach to protect the cells against oxidative injury is a combination of antioxidants and calcium modulators, which is accomplished by $CuCl(HNA)_2$ administration as well as $Cu(II)(Gly)_2$ in our study.

Ulceration, either in human or animal models, correlates with lower levels of prostaglandins and/or increased ulcerogenic thromboxane and other lipoxygenase products [20, 21]. Inhibition of thromboxane synthesis prevented gastric mucosal damage caused by absolute ethanol, serotonin or aspirin in rats, which suggests that endogenous TXA_2 is ulcerogenic [22]. In the present study, treatment with $CuCl(HNA)_2$ stimulated the fundic mucosal PGE_2 content, whereas $Cu(II)(Gly)_2$ caused a significant decrease in the mucosal TXA_2 content. Although $Cu(II)(Gly)_2$ did not enhance PGE_2 content, it showed potent anti-ulcerogenic activity which is consistent with the result reported by Kishore *et al.* [2]. Therefore, $Cu(II)(Gly)_2$ may exert the gastroprotective action observed in our study, by lowering the ulcerogenic eicosanoid product, TXA_2; a mechanism that is consistent with the proposal by Tsujii *et al.* [20]. Most of the effective ulcer managing drugs increase or maintain the normal PGE_2 level, which has been accomplished by $CuCl(HNA)_2$ in the present investigation. Increase in endogenous PGE_2 protects the gastric mucosa by relaxing muscular spasms, strengthening the mucosal barrier components and stimulating the cellular cAMP content [14, 21]. Therefore, PGE_2 enhances macromolecular biosynthesis which in turn enhances cellular regeneration and heals the gastric mucosa [21]. PGE_2 also inhibits calcium mobilization, superoxide radical generation and lysosomal enzyme release [21]. Prostaglandin biosynthesis is a process of lipid peroxidation of arachidonic acid and $CuCl(HNA)_2$ has been found to mediate the oxidation of arachidonic acid possibly through the activation of cyclooxygenase and other oxidases [23, 24]. Furthermore, $CuCl(HNA)_2$ may act as a coenzyme for several oxidoreductases by contributing to the formation of pyridine nucleotides, NAD- and NADP-like structures as reported by Megalla *et al.* [25]. Therefore, $CuCl(HNA)_2$ may initiate the arachidonic acid cascade, through activation of the cyclooxygenase by furnishing the necessary active coenzymes, NAD and NADP similar structures and/or regulating the essential glutathione through the redox-active couple of the pyridine nucleotides [26]. However, $Cu(II)(Gly)_2$ did not show the same effect. These results suggest that, although both copper complexes decreased ulceration, PGE_2 and TXA_2 measurements proved that different modes of action were responsible.

The abnormal increase of lipid peroxides irreversibly inactivates the cyclooxygenase activity [27] and shifts the arachidonic acid cascade into the ulcerogenic products, i.e. leukotrienes and thromboxanes at the expense of the cytoprotective PGE_2 [28]. The involvement of lipid peroxidation in gastrointestinal ulceration is a subject of controversy. Szabo [16] minimized the role of lipid peroxidation in chemically induced ulceration, whereas, others [1, 29] implied free radical initiated lipid peroxidation in the anti-ulcer effect of non enzymatic and enzymatic anti-oxidants.

In ethanol and stress induced acute ulceration, exogenous SOD, as a free radical scavenger, prevented the associated increase of lipid peroxides and ulceration [30]. Our results show that both copper complexes increased SOD or SOD-mimetic activity in both the gastric mucosa and plasma. SOD and copper complexes modulated other anti-oxidants such as thiols and ceruloplasmin [1]. Gastric mucosal SOD activity was increased after 4 hr of pyloric ligation-induced gastric ulceration [31]. The high activity of SOD was suggested to be a defensive

reaction [32]. Therefore, the increase in the SOD activity observed with $CuCl(HNA)_2$ may be due to the enzyme induction under the effect of the complex which in turn prevents lipid peroxidation. On the other hand, the SOD-like activity of a series of coordination complexes, e.g. Cu-(nicotinate)$_2$ and Cu-(1,2-diamino-2-methylpropane)$_2$ has been established [33]. This in part explains the elevated levels of plasma SOD activity detected after administration of $CuCl(HNA)_2$. The SOD induction by $Cu(II)(Gly)_2$ is possibly due to copper mobilization [34] since this complex is liable to ligand exchange.

In conclusion, the intragastric administration of either $CuCl(HNA)_2$ or $Cu(II)(Gly)_2$ complex showed anti-ulcerogenic activity through mechanisms involving previously described biochemical changes and by electron microscopic histological examination of the mucosa. $CuCl(HNA)_2$ exhibited its effect possibly through its SOD-like activity and also by enhancing the production of PGE_2, an integral cytoprotective agent. Whereas, $Cu(II)(Gly)_2$ showed strong anti-ulcer activity with a different mode of action possibly through mimicking the activity of SOD and lowering TXA_2, a known mechanism for cytoprotection.

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