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Cisplatin-induced peptic ulcers, vagotomy, adrenal and calcium modulation

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Cytochemical and autoradiographic studies in Wistar rats [Crl:(WI)BR] show that cisplatin treatment (9 mg/kg) inhibits the release of acetylcholine from the axonal endings of the stomach smooth muscle resulting in bloating of the stomach and ulceration. Cisplatin also induces corticosteroid release from the adrenal gland stimulating peptic ulceration. Vagotomy helps ameliorate the effect but not eliminate it. Calcium supplementation restores normal neuromuscular function to gastric smooth muscle, thereby eliminating the gastro-intestinal toxicity due to cisplatin.

Key words: Adrenal, acetylcholinesterase, autoradiography, calcium, [^{3}H]choline, cisplatin-induced ulcers, emesis, gastric emptying, peptic ulcers, vagotomy.

Introduction

Cisplatin (*cis*-dichlorodiammineplatinum II; CDDP), a potent broad spectrum anti-cancer agent, produces severe toxic side effects including kidney damage, nausea and vomiting, loss of hearing, peripheral neuropathy, hypomagnesemia, and hypocalcemia.[1,2] The most severe dose-limiting side effects are kidney toxicity,[3] nausea and vomiting.[4,5] Kidney toxicity can be controlled through slow infusion of the drug[6] and hydration with or without diuretics.[2] Special protective agents like WR2721,[7] GR38032F9 (GRC 507/75),[8] cinnarizin[5] or increased sodium chloride concentrations in the vehicle of administration have proven to be quite effective.[9] Elastase (E La), which has an antiatherosclerotic effect, has been used to protect the kidney from being impaired.[10] Nephroprotective agents like the metal-chelator diethyl dithiocarbamate (DDC) or free-sulfhydryl sodium 2-mercaptoethane sulfonate (mesna) have been used for their high rate of exchange between the chloride ligands of cisplatin and sulfhydryl ligands.[11]

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Nausea and vomiting can be so severe in some patients that cisplatin treatment has to be discontinued. However, this can be controlled to some extent through the use of antiemetic drugs like metoclopramide and dropreridol[12] or ondansetron.[13] Serotonin and serotonin (5-HT$_3$) receptors seem to play an important role in the way chemotherapeutic agents produce nausea and emesis.[14] The 5-HT$_3$ receptor antagonist ondansetron has been shown to control nausea and vomiting produced by cisplatin in both animals[15] and in patients,[16] and has proven to be superior to metoclopramide.[9,17]

In rats cisplatin causes undue distension of the stomach through the inhibition of gastric motility, leading to development of ulcers.[18,19] Adrenal steroidal factors and catecholamines have been implicated in the induction of ulcers in rats and can be blocked by adrenalectomy or bilateral vagotomy.[20] Vagotomy has been used as an alternative for the treatment of chronic ulcer patients[21] and is an effective antiemetic therapy in cisplatin-induced nausea and vomiting,[22] possibly because the vagus nerve also controls the acid secretion by parietal cells in the stomach.[23] The present study is an effort to (i) characterize cisplatin-induced changes in neuromuscular interactions of the stomach smooth muscle, (ii) characterize the neuroendocrine function of the adrenal gland (steroid and catecholamine contents) and (iii) to correlate these changes to the induction of peptic ulcers by the drug cisplatin.

Materials and methods

Animals

Laboratory-bred male Wistar rats [Crl:(WI)BR] (Charles River Breeding Lab, Portage, MI) weighing 200–300 g and approximately 3 months of age were used in these experiments. Animals were housed four per cage at 20 ± 2°C on a 12:12 h

Table 1. Experimental design[a]

Groups	Treatments
1	cisplatin (9 mg/kg; i.p.) in 0.85% sterile normal saline
2	control: (i.p.) 0.85% sterile saline
3	cisplatin (9 mg/kg; i.p.) in sterile normal saline; no food but water
4	cisplatin (9 mg/kg; i.p.) + Ca^{2+} (0.5 ml of 10% calcium gluconate) daily
5	vagotomy alone
6	sham operated
7	vagotomy + cisplatin (9 mg/kg; i.p.)
8	control [sham operated + sterile normal saline (i.p.)]

[a] Each group consisted of at least 10 animals; animals were killed on day 3, 5 and 8 post-treatment.

light : dark cycle with free access to animal feed (Allied Mills, Chicago, IL) and tap water. Animals were divided into groups of 10 animals each (see also Table 1). Animals in group 1 were injected with a single intraperitoneal dose of cisplatin (9 mg/kg) dissolved in 0.85% saline for 3, 5 and 8 days. (This dosage is about 10 times higher than used for humans.) Control animals in group 2 received comparable saline injection. Comparable numbers of cisplatin-treated animals in group 3 were denied access to food but were provided with free access to water. Yet another group of cisplatin-treated animals (group 4) was given daily intravenous injections of calcium (0.5 ml of 10% calcium gluconate or 1.1 ml of 1.3% $CaCl_2$) at 08:00 h on the day of cisplatin treatment and each day thereafter up to the day of euthanasia.

Animals in group 5 were bilaterally vagotomized.[24] Animals in group 6 were sham operated to serve as controls. Vagotomized animals (group 7) were given cisplatin (9 mg/kg) while a similar group (group 8) of control animals received normal saline injections to serve as controls. Animals in various groups were euthanatized by using CO_2 on day 3, 5 and 8 post-treatment. The stomach and the adrenal glands were removed after dissection. The stomach was opened along the greater curvature, emptied of its contents by flushing with 10 ml of 0.15 M NaCl and examined for evidence of ulceration. The diluted stomach contents were measured and their pH was recorded (Table 2).

Tissue handling procedures

Part of the stomach tissue was stretched in 4% buffered (0.05 M cacodylate buffer, pH 7.4) glutaraldehyde for 4 h before processing for light and electron microscopy, while the other part of the tissue was used for frozen sections. Paraffin embedded and frozen sections (7–10 μm) were stained by Masson trichrome staining method, Periodic acid–Schiff method, Ehrlich hematoxylin and eosin, mercuric bromophenol blue and Sudan black B or oil red O methods.[25]

Adrenal glands were cut into two halves. One half was prepared for frozen sections (7–10 μm) while the other half was fixed either in Bouin's fluid and processed for light microscopy or thin slices of adrenal were fixed in 4% buffered (0.05 M cacodylate, pH 7.4) glutaraldehyde and 1% osmiumtetroxide for 1–2 h at 4°C and embedded in araldite after proper dehydration. Thick (1 μm) sections were stained with 1% solution of methylene blue in 1% borax for 60 s at 60°C while the thin sections (500–700 Å) were stained with uranyl acetate followed by lead citrate. Sections were viewed

Table 2. Mean stomach weight (g/100 g body weight) plus contents on day post-treatment cisplatin (9 mg/kg)[a,b]

Treatment	Days post-treatment						
	0	1	2	3	4	5	8
Saline	1.5 ± 1.1	1.3 ± 1	1.4 ± 1.5	1.4 ± 0.9	1.4 ± 0.9	1.5 ± 1.1	1.4 ± 0.7
Cisplatin (9 mg/kg)	1.3 ± 1	3.2 ± 1.6	4.1 ± 0.7	5.3 ± 0.9	7.7 ± 0.8	8.2 ± 0.7	4.1 ± 0.8
Cisplatin + calcium[c]	1.5 ± 0.8	2.1 ± 0.8	2.2 ± 0.8	2.3 ± 0.8	2.2 ± 1.1	2.4 ± 1.0	1.8 ± 1.0
pH Stomach contents + 10 ml NaCl (0.15 M)	4.85 ± 0.4	4.4 ± 0.3	3.9 ± 0.4	3.2 ± 0.5	2.9 ± 0.3	2.6 ± 0.2	3.9 ± 5

[a] Given as single intraperitoneal injection in 0.15 M NaCl on day 0 (24 h before cisplatin treatment).
[b] $n = 10$ per group.
[c] Starting day 0 calcium (5 mg) was administered daily as 1.1 ml of 1.3% $CaCl_2$ (w/v) through the tail vein.

under a Zeiss photomicroscope III and Hitachi HU11E electron microscope operated at 75 kV.

Gastric emptying

Gastric emptying was measured on day 3, 5 and 8 by the phenol red meal method.[26] The meal consisted of a solution of 50 mg phenol red in 100 ml aqueous methylcellulose (1.5% w/v) given by oral intubation of hand-held conscious rats in a dose of 1.5 ml per rat. Gastric emptying was measured 3 h after the meal and their phenol red content was determined.[27] A group of 10 rats was killed immediately after the administration of the meal and the phenol red content of these animals served as zero emptying point. Stomachs and their contents were homogenized in 0.1 M NaOH (100 ml). Proteins were precipitated using 20% trichloroacetic acid, alkalinized using 0.5 M NaOH and assayed using colorimetry at 560 nm. Gastric emptying was calculated for each rat as:

$$\text{Gastric emptying}(\%) = [1 - A/A'] \times 100.$$

A represents absorption at 560 nm by gastric contents at 3 h after the meal and A' represents absorption at zero emptying time.

Statistical analysis

Statistical analyses were performed using a one-way analysis variance with Student–Newman–Keals follow-up test.[28]

[^{3}H]Choline uptake experiments

Three days after cisplatin-treatment, 10 rats were injected intraperitoneally with choline chloride (methyl-^{3}H) (New England Nuclear, Boston, MA) at 6 μCi/kg or 60 μCi/kg. Animals were sacrificed at 5 min, 3 and 12 h intervals. Stomachs were removed, emptied of their contents and divided into three parts by cutting along the line separating the cardiac from the pyloric region, and cutting 0.5 cm on both sides of the pyloric sphincter. Each piece was weighed and chopped into small pieces and digested by adding 2.5 ml protosol (New England Nuclear). After digestion for 48 h, 15 ml scintillation cocktail (5 g PPO, 100 g Naphthalene to 1 l Dioxane) was added. Sample radioactivity was measured using a Beckman scintillation counter. Data were statistically analyzed.[28]

Autoradiography

Small pieces (1 mm^3) of tissue from the cardiac stomach were removed from the choline chloride (methyl-^{3}H) injected animals and were fixed in 1% glutaraldehyde and 2% potassium pyroantimonate with 0.05 M cacodylate buffer (pH 7.4) for 12 h at 4°C. Tissues were post-fixed in 1% buffered (0.05 M cacodylate buffer, pH 7.4) osmium for 1 h, dehydrated and embedded in araldite. Autoradiograms were prepared using thick sections (0.5 μm) or thin sections (700–900 Å) and NTB2 or Ilford L_4 emulsions.[29]

Acetylcholinesterase localization

Frozen sections (7 μm) of the pyloric sphincter and the stomach tissue were treated for the localization of cholinesterase activity after a brief fixation (3 min) in 4% buffered formaldehyde (0.05 M phosphate buffer, pH 6.0).[30] Controls included incubating sections in the incubation media without the substrate, acetylthiocholine iodide or in media containing physostigmine sulfate (10^{-4} M) or tetra mono isopropylpyrophosphortetramide (iso-OMPA) (3×10^{-5} M) serving to inhibit acetylcholinesterase or pseudocholinesterase, respectively.[31] For electron microscopy tissues were further treated with 1% osmium tetroxide and processed in a routine manner.

Results

Cisplatin treatment induced gastric bloating mostly through inhibition of gastric emptying (Figure 1). By day 3 the gastric emptying was down to about 40% of the original and it further dropped down to about 1.5% of the total by day 5 when the weight of the stomach along with its contents increased by 5.5-fold (see Table 2) of that of the normal stomach. Daily injections of calcium (1.1 ml of 1.3% $CaCl_2$) reversed this bloating process to almost normal levels. The pH of the stomach contents as diluted with 10 ml of 0.85% saline solution was 4.85 in the normal rats and 2.6 by day 5 of post-cisplatin treatment. Prominent peptic lesions could be detected in the cardiac portion of the stomach after 3 days of cisplatin treatment (see Table 3). No such lesions were observed in the pyloric section of the stomach even when examined histologically in 10 μm thick serial sections. The gastric lesions after cisplatin

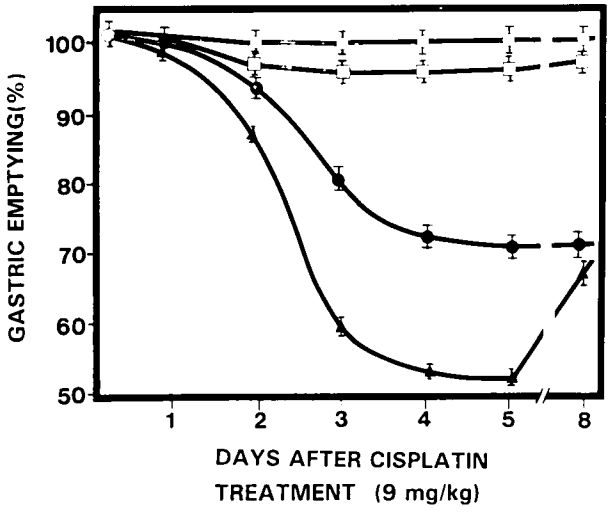

Figure 1. Graph showing inhibition of gastric emptying by more than 90–95% after (▲) cisplatin treatment (9 mg/kg) compared with (○) controls or (□) cisplatin plus calcium treatment. The gastric emptying increased about 40% after cisplatin and vagotomy (●) compared with cisplatin alone.

treatment varied in size from 15 μm appearing as surface erosions to 1–2 mm of bleeding ulcers (Figure 2). The number of these ulcers varied from about seven to more than 40 between day 3 and 5 (Table 3).

Cisplatin-treated animals denied any food but allowed water *ad libitum* did not show any gastric erosions of the mucosal membranes. Vagotomized animals treated with cisplatin also did not show any gastric lesions even after day 8. The ulcers usually started as minute erosions (Figure 2A) in the outermost mucosal layers after day 3 of cisplatin treatment and extended inward into the deeper layers of the mucosa (Figure 2B–D) leading to bleeding ulcers with raised margins (Figure 2E). By day 5 of cisplatin treatment intense granulation in the outermost layers of the mucosa was very prominent after hematoxylin and eosin staining (Figure 3). Such granulation was not observed in the untreated rat stomach mucosal layers. This possibly results from increased gastrin/pepsin and parietal cell secretory activity as a result of cisplatin treatment.

Acetylcholinesterase localization

Acetylcholinesterase was histochemically localized in the gastric tissues from the normal and cisplatin-treated rats, and the staining intensities and distribution were compared. The muscularis mucosa in the cardiac or the pyloric regions was negative whereas the circular and the longitudinal muscle layers were intensely positive only in localized places both in the normal and cisplatin-treated tissues (Figure 4). These areas included mostly sections of nerve fibres, nerve fascicles and ganglia. There was no significant detectable difference between the normal and cisplatin-treated animal tissues from the cardiac or the pyloric portions of the stomach. Similarly, the muscularis of the sphincter in the normal and cisplatin-treated rats demonstrated no significant differences in staining intensities for acetylcholinesterase. Using electron microscopical methods acetylcholinesterase was demonstrated to be associated with the membranes of the axonal endings and the smooth muscles in a uniform fashion (Figure 4). There was no difference in the distribution or its concentration between the treated or untreated tissues.

[^{3}H]Choline uptake

In order to determine if acetylcholine metabolism in the enteric nervous system was altered by cisplatin treatment, [^{3}H]choline uptake into the stomach tis-

Table 3. Peptic ulcers after various treatments in male Wistar rats

Treatment	Days post-treatment						
	0	1	2	3	4	5	8
Saline (0.15 M)	0	0	0	0	0	0	0
Cisplatin (9 mg/kg) with food and water	0	0	0	7 ± 3	12 ± 1	40 ± 12	0
Cisplatin (9 mg/kg) without food but with water	0	0	0	0	0	0	0
Cisplatin (9 mg/kg) + calcium[a] with food and water	0	0	0	0	0	0	0
Cisplatin (9 mg/kg) + vagotomy with food and water	0	0	0	0	0	0	0

[a] Starting day 0 (24 h before cisplatin treatment) calcium (5 mg) was administered daily as 1.1 ml of 1.3% $CaCl_2$ (w/v) through the tail vein.

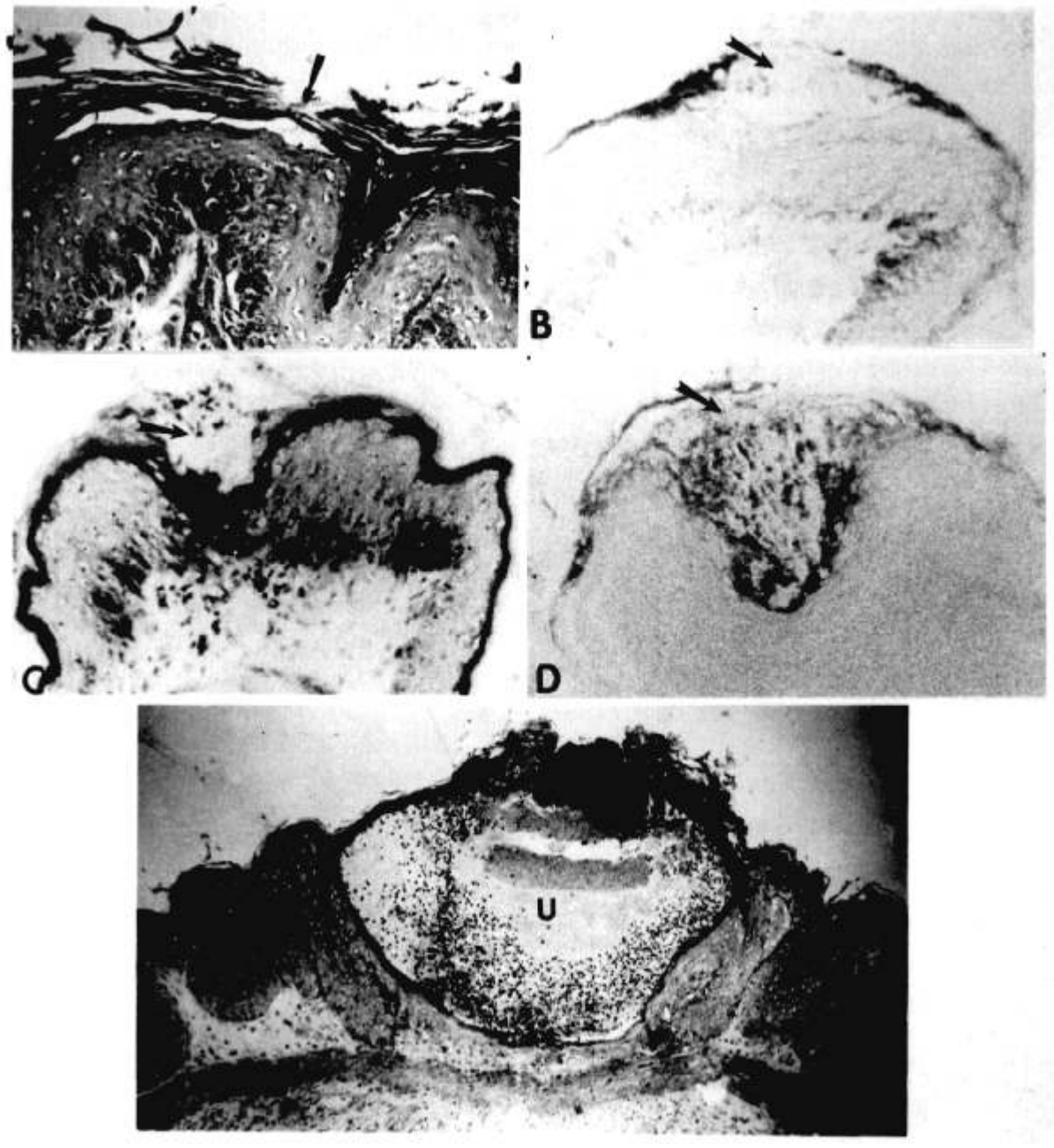

Figure 2. Light micrographs of cross-sections through the cardiac portion of the rat stomach illustrating the sequence of gastric erosions and ulceration after cisplatin (9 mg/kg) treatment of 3 and 5 days. (**A**) Initial gastric lesion in the form of an abrasion (arrow) in the outer mucosal epithelium. ×250. (**B**) Focal necrosis in the gastric epithelium (arrow). ×75. (**C**) Deep foci of necrosis almost involving the full thickness of the mucous membrane (arrow). ×75. (**D**) Deep ulceration showing signs of an acute ulcer with minimal fibrosis reaction (arrow). ×75. (**E**) A fully developed acute ulcer with cellular debris, inflammatory cells in the large ulcer crater (U), with surrounding intact mucosa showing inflammation and hyperplasia. Note the marked necrosis. Hematoxylin, ×125.

sue of the normal rats was compared with that of cisplatin-treated rats. Radioactivity was measured in the cardiac, pyloric and pyloric sphincter regions of the stomach at 5 min, 3 and 12 h after [^{3}H]choline injections. In the cardiac region of the stomach the radioactivity was significantly higher in the cisplatin-treated animals than in controls at 5 min, 3 and 12 h (Figure 5). There was a significant increase in the radioactivity with time in both the cisplatin-treated and non-treated rats. However,

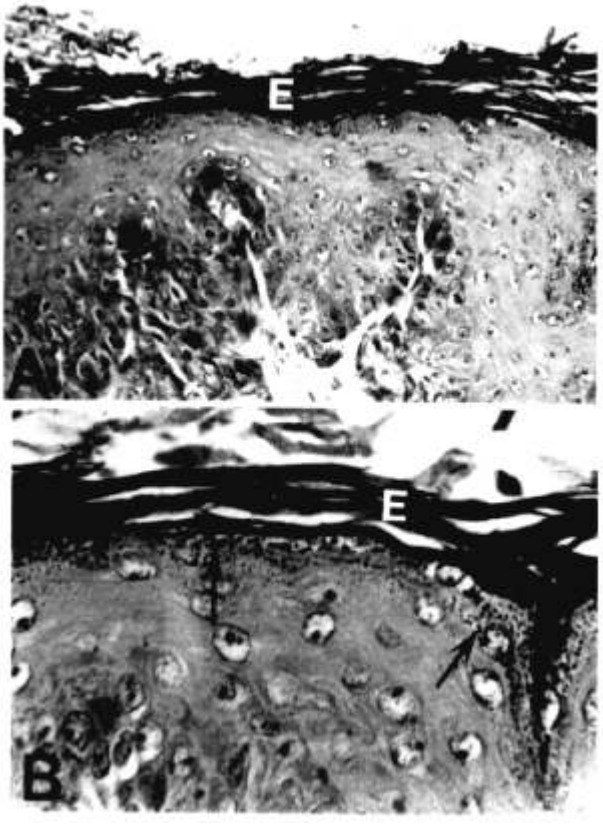

Figure 3. Light micrographs of cross sections through the cardiac portion of the rat stomach before (**A**) and after (**B**) cisplatin (9 mg/kg) treatment for 5 days. Note the increased granulation in the outer epithelial mucosal layers (arrows) after cisplatin treatment. E, Keratinized outer epithelium. Hematoxylin. A, ×90; B, ×180.

there was no significant difference in the [^{3}H]choline incorporation in the pyloric region of the stomach between normal or cisplatin-treated rat tissues nor was there a significant change in the tissue radioactivity over time (Figure 6). In the pyloric sphincter (Figure 7) [^{3}H]choline incorporation in the cisplatin-treated rats was significantly higher compared with controls after 5 min and 12 h, and this activity decreased over time in both the normal and cisplatin-treated animals. Thus when measured as a function of time following [^{3}H]choline injection, the cardiac region of the stomach demonstrated an increase in radioactivity over time whereas in sphincter tissue there was a decrease.

Autoradiography

Autoradiography was performed to determine the specificity of [^{3}H]choline uptake. Light and electron microscope autoradiographs show silver grains mostly localized over the cytoplasmic membranes of the smooth muscle cells and over the synaptic endings (Figure 8). Because of the large size of the silver grains relative to the dimensions of organelles within synaptic endings, it was difficult to attribute these grains to neurotransmitter vesicles or mitochondria.

Ultrastructure of gastric smooth muscle and axonal endings

Two types of cells were easily recognized in the gastric smooth muscle tissue after cisplatin treatment for 5 days. There were cells with highly electron dense microfilaments and others that were quite electron lucent with swollen mitochondria (Figure 9). The axonal endings clearly demonstrate an increase in the synaptic vesicles after only three days of cisplatin treatment (Figure 10) indicating a possible block in the release of the neurotransmitter. By day 5 these synaptic vesicles and the associated mitochondria break down leaving large axonal endings with no cellular organelles but membranous debris in them (Figure 10).

Adrenal gland morphology

The adrenal gland consists of two regions, the cortex and the medulla (Figure 11). The cortical region is divided into three zones: zona glomerulosa, zona fasciculata and zona reticularis which occupy 15, 70 and 7% of the total volume of the gland, respectively. The normal morphology of the gland is well known and will not be repeated here in any detail.[32] Adrenal cortex is actively involved in the process of steroidogenesis while the adrenal medulla is responsible for catecholamine synthesis.[33] The zona glomerulosa of the adrenal cortex secretes principally the mineralocorticoid aldosterone and the zona fasciculata produces glucocorticoids such as corticosterone. The zona reticularis secretes the sex hormones including dehydroepiandrosterone. Lipid granules are abundant throughout the cortical regions especially in the zona fasciculata and zona reticularis while being undetectable in the medulla (Figure 12). Lipid granules containing precursors for steroid hormones[34] have different sizes and are limited by a boundary membrane. These lipid granules often appear as highly electron dense globules under the electron microscope (Figure 13). Mitochondria in the adrenal cortical region are from elongated (zona glomerulosa), to rounded (zona

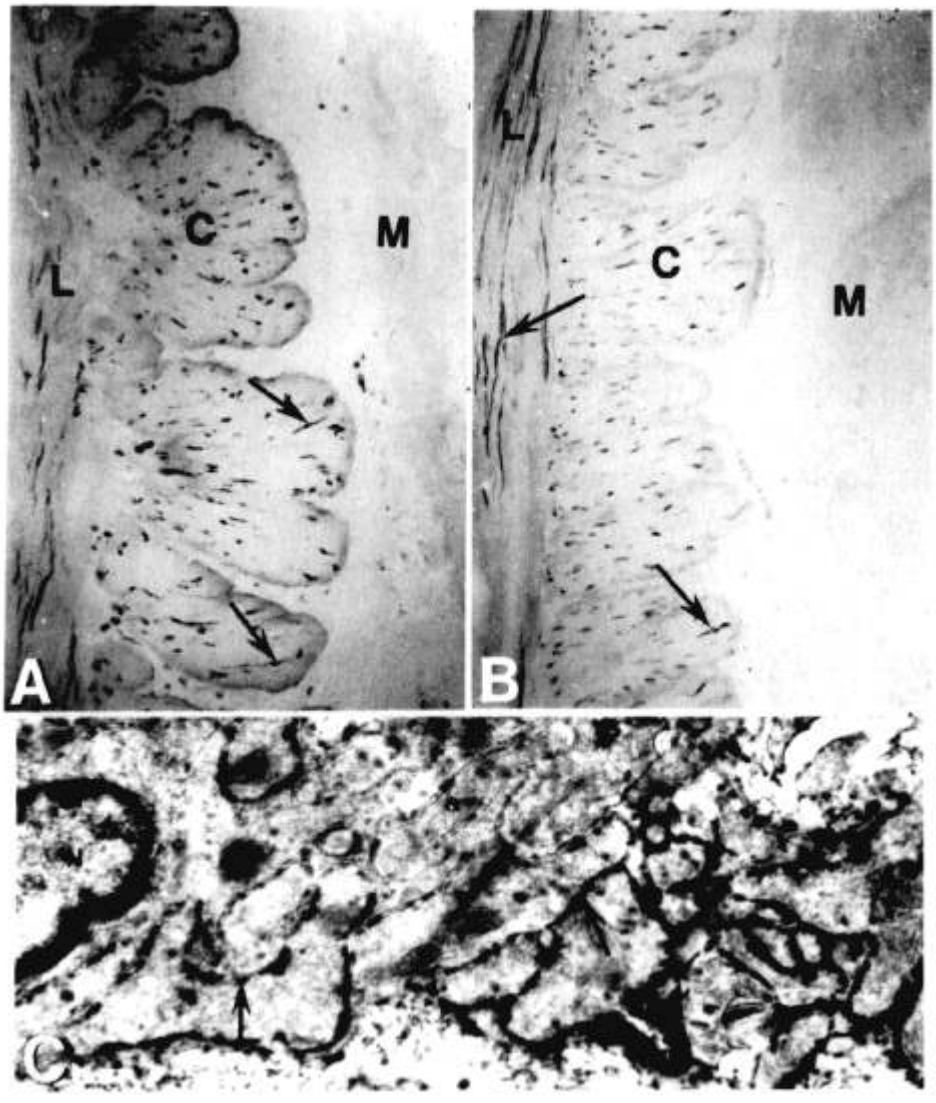

Figure 4. Light micrographs showing acetylcholinesterase staining in the cardiac region of the stomach from a normal (**A**) rat and a 3 day cisplatin-treated (**B**) rat. Structures most heavily stained are the nerve fibers (arrows) in the muscularis. ×170. (**C**) Electron micrograph showing the localization of acetylcholinesterase mostly associated with the membranes of the axonal endings and the smooth muscle cells (arrows). M, muscularis mucosa; N, nucleus; C, circular muscle layer; L, longitudinal muscle layer. ×17 000.

fascicularis) and ovoid (zona reticularis) with tubular or vesicular cristae. In cells of the zona fasciculata and the zona reticularis of the normal rat adrenal the outer nuclear membrane often shows blebbing (Figure 13). Sometimes the electron dense lipid globules may show lamellar configurations enclosed by their boundary membrane. Such regions are usually quite electron transparent (Figure 13). Catecholamines (norepinephrine and epinephrine) are produced in the medullary region in two different cell types (Figure 14). Norepinephrine granules are more electron dense, smaller and their contents are irregular in shape compared with the epinephrine granules that are less electron dense, are larger and their content fills the entire granule. There is more variation in the density of the epinephrine granules than the norepinephrine granules. There is a membrane surrounding both types of granules and it is more evident around the epinephrine granules than the norepinephrine granules. Mitochondria are elongated or round with tubular cristae (Figure 14).

Cisplatin treatment (9 mg/kg) of 3 days induced a sharp decrease in the lipid contents of the cortex (Figure 15) compared with the normal (Figure 12). Lipid granules are almost absent from the corticomedullary region after cisplatin treatment (Figure 15). Telangiectasia was very noticeable after 3 days of cisplatin treatment in both the cortical and the medullary regions (Figure 16). Cisplatin caused an abnormal swelling of the mitochondria and complete disruption of their cristae (Figure 17). The medullary region was significantly atrophied after 3 days of cisplatin treatment (Figure 16) with mitochondria being most affected as in the cortex while their number seemed to be greatly reduced. Norepinephrine granules demonstrated abnormal swelling (Figure 18; see also Figure 14 for comparison).

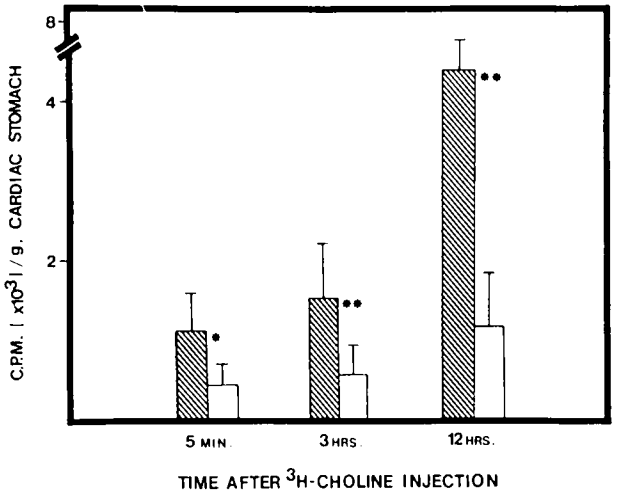

Figure 5. Radioactivity counts (mean + SD) in the cardiac region of the stomach of rats at 5 min, 3 and 12 h intervals after [^{3}H]choline injection. Tissues from cisplatin-treated rats show greater radioactivity incorporation than the controls at all time periods ($^*p \leq 0.02$, $^{**}p \leq 0.01$). Each data point is based on counts obtained from an average of 10 animals. ■, CDDP; □, control.

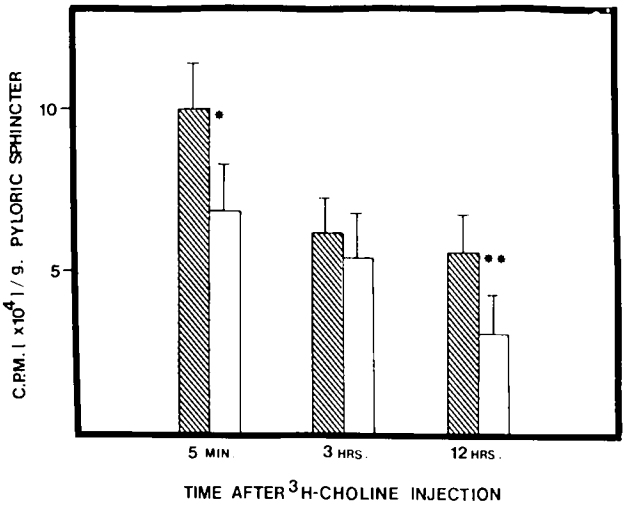

Figure 7. Radioactivity counts (mean + SD) in the pyloric sphincter of rats at 5 min, 3 and 12 h intervals after [^{3}H]choline injection. Tissues from cisplatin-treated rats show greater radioactivity counts than the controls at 5 min and 12 h intervals ($^*p \leq 0.5$; $^{**}p \leq 0.01$). Each data point is based on counts from an average of 10 animals. ▧, CDDP; □, control.

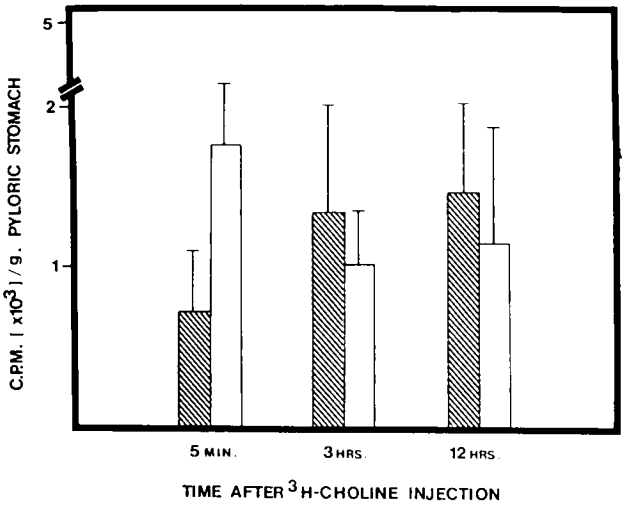

Figure 6. Radioactivity counts (mean + SD) in the pyloric region of the stomach of rats at 5 min, 3 and 12 h intervals after [^{3}H]choline injection. There are no significant differences between values obtained from cisplatin-treated rats and control rats. Each data point is based on counts obtained from an average of 10 animals. ▧, CDDP; □, control.

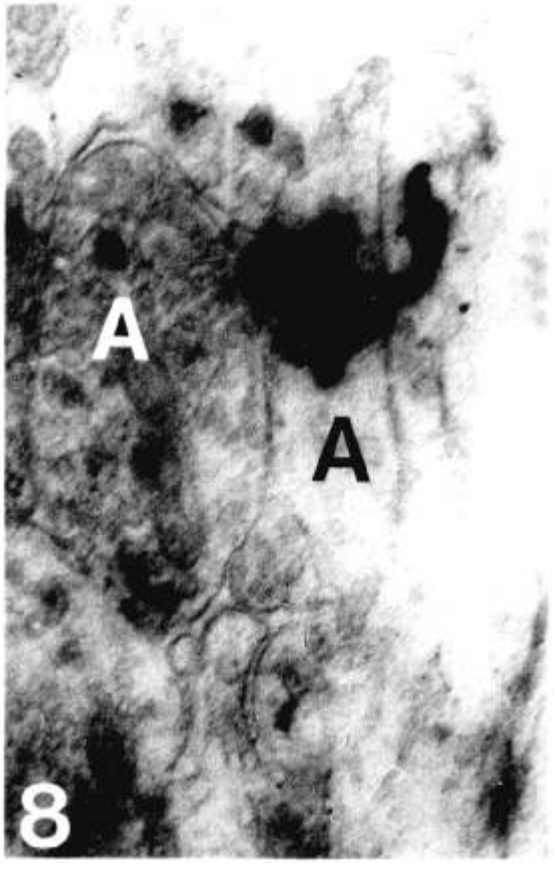

Figure 8. Electron microscope autoradiogram indicating [^{3}H]choline incorporation into the axonal endings (**A**). This section is of tissue from the gastric muscularis of a cisplatin-treated rat injected with [^{3}H]choline and sacrificed 5 min later. ×16 000.

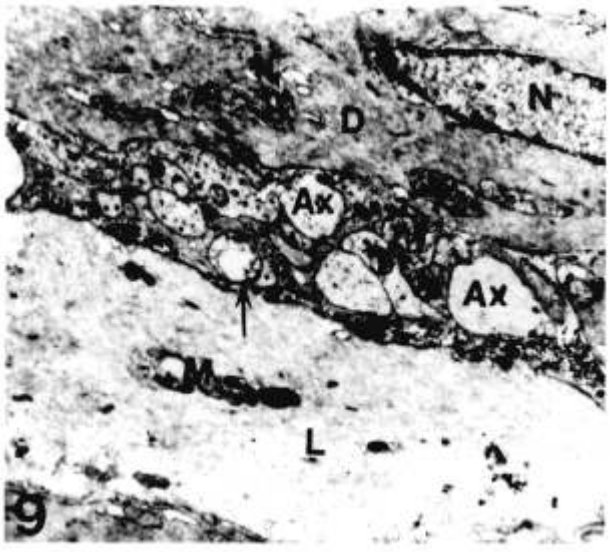

Figure 9. Electron micrograph showing two types of smooth muscle cells [electron dense cells (D) with normal elongated mitochondria and electron lucent cells (L) with swollen mitochondria] on either side of a nerve ending in the gastric muscularis of a rat after 5 days of cisplatin treatment (9 mg/kg). Note most of the cholinergic endings (Ax) are without any synaptic vesicles and have swollen mitochondria (arrow). Ax, axonal endings; M, mitochondria; N, nucleus. ×18 000.

After bilateral vagotomy we observed an overall hypertrophy in the gland (Figure 19) with an increase in the lipid droplets of both the zona reticularis and the zona fasciculata. Accumulation of these lipids after vagotomy was taken as an indication of steroid hormone precursor accumulation, in turn reflecting a depression in the functional activity of the two zones. There was considerable telangiectasia, especially in the zona reticularis region of the cortex. The number of mitochondria per unit area also was much less (Figure 20) compared with the normal glands (see Figure 13 for a comparison). The medullary region was also hypertrophied (Figure 19) within 3 days of vagotomy. The norepinephrine granules were abnormally enlarged with some vesicles appearing completely empty. Some mitochondria appeared swollen and lacking organized cristae (Figure 21).

Discussion

Various chemotherapeutic and noxious agents like aspirin, cisplatin, mercuric chloride and alcohol are known to induce gastric lesions leading to ulceration.[19,20] In order to develop treatment for gastric ulcers, it is essential to understand the causes or mechanisms of induction of such ulcers. Different animal models have been developed through pyloric ligation, stress induction restraint, cold, and use of agents like alcohol, tobacco smoke, bile acids, corticosteroids, lysolectins and aspirin.[20] All disrupt the continuity of the epithelial surface inducing increased shedding of the cells and possibly contribute to the development of the gastric mucosal erosions or ulcers. Various chemical agents affect different regions of the gastro-intestinal system. Some induce gastric lesions in the cardiac portions, others the pyloric region and yet others the duodenal portion of the stomach.[35]

A variety of measures, mostly poorly understood, have been employed to protect the gastric mucosa in animal models. Adequate mucosal blood flow, normal levels of mucosal cells with carbonic anhydrase, prostaglandins, sulfhydryl-containing drugs (dimercaprol, cysteanine, dimercaptosuccinic acid), non-protein thiols, natural amino acids (L-cysteine, methionine) and epidermal growth factor are all potential protectors of mucosal cells against erosive acid-peptic secretions.[36,37] Therapeutic agents that stimulate mucus output also seem to help in healing peptic ulcers.[38]

Cisplatin, a broad spectrum potent chemotherapeutic agent, causes gastric lesions in the cardiac portion of the stomach in Wistar rats within 3 days.[18,19] Its use at a chemotherapeutic dose of 9 mg/kg body weight leads to hemorrhagic gastric ulcers after day 5. Cisplatin treatment induces a release of corticosteroids from the zona fasciculata and the zona reticularis, while the mineralocorticoids in the zona glomerulosa remain unaffected. Corticosterones have been shown to be essential in the induction of gastric lesions requiring the parasympathetic nervous system.[20] Adrenalectomy completely prevents the inhibition of gastric emptying by sauvagine and corticotropin-releasing factor (CRF).[39] Corticosterone administration, however, causes these peptides to recover their inhibitory activity thus demonstrating the important role of corticosterones in gastric emptying and induction of ulcers.

In some cases, vagotomy is effective in controlling the release of corticosterones and in mitigating gastric ulceration. Vagotomy has been utilized as a method to control the gastric emptying and gastric lesions after various drug treatments, like Bombesin,[40] Dermorphin[27] or both, in animals[27,41] and in clinical situations.[42] It has been demonstrated that after vagotomy the adrenal cortex shows hypertrophy and there is an accumulation of lipids, which has been interpreted as a morphological sign of depressed glandular activity.[43] The medullary portion of the adrenal shows hypertrophy after vagotomy.

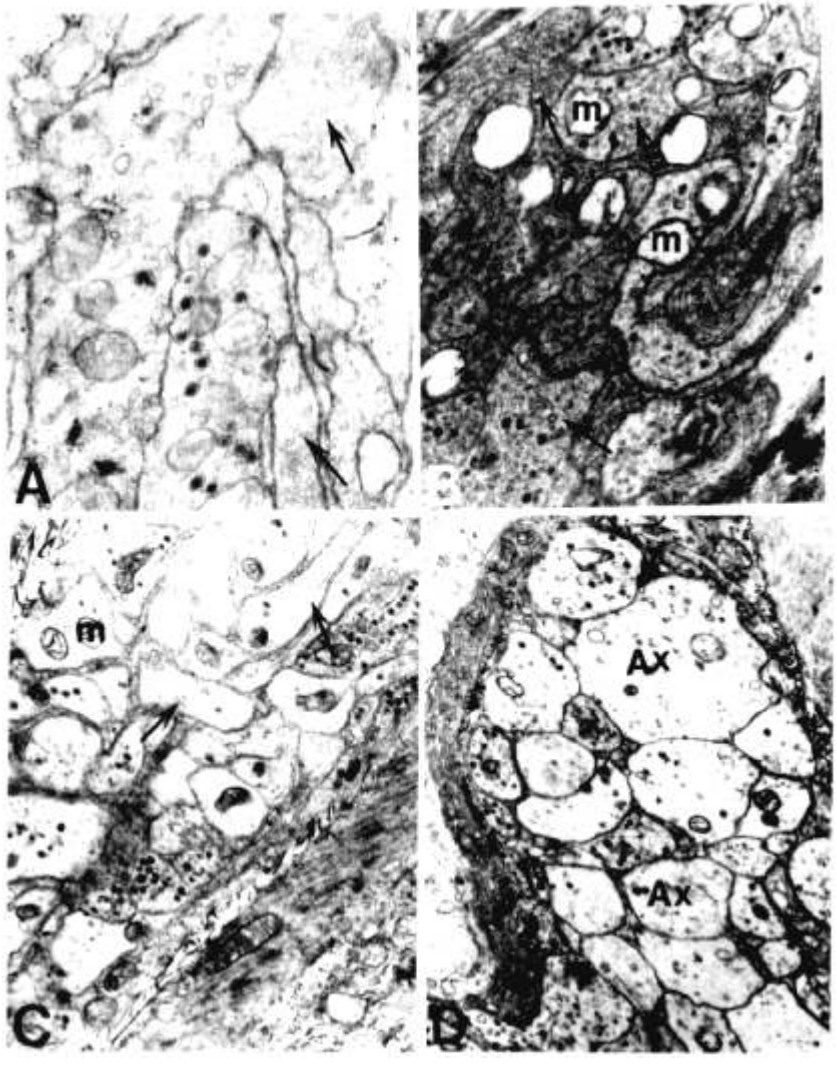

Figure 10. Electron micrographs of the axonal endings from (**A**) normal rat gastric muscularis showing the number and distribution of synaptic vesicles (arrows). (**B**) Axonal endings from the cisplatin-treated (9 mg/kg) rat gastric muscularis of 3 days showing an increase in the number of synaptic vesicles (arrows) and abnormally swollen mitochondria (m) without any cristae. (**C**) Axons and axonal endings from a normal rat gastric muscularis showing well organized microtubules (arrows) and normal looking mitochondria (m). (**D**) Abnormally enlarged axonal endings (Ax) from the cisplatin-treated gastric muscularis after 5 days. Note the complete disorganization of structures with no synaptic vesicles, microtubules or mitochondria. ×16 000.

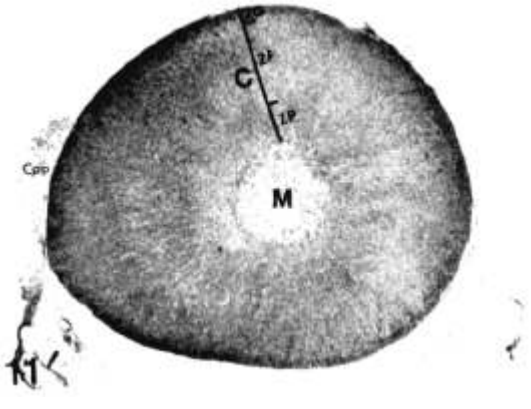

Figure 11. Light micrograph of a cross section (10 μm) of the adrenal gland from a normal rat showing the normal morphology of the gland after staining with hematoxylin. C, cortex; Cap, capsule; M, medulla; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis. Arrows point towards the capillaries in the gland. ×56.

Various drugs are known to induce destructive, degenerative (senile nodular hypertrophy, telangiectasia, focal fatty deposits) changes in the adrenal gland.[44] Cisplatin causes severe telangiectasia and loss of lipoidal vesicles from the zona fasciculata, and the zona reticularis cells, which are reflective of the corticosteroid changes.[45] In cisplatin-treated rats, the medullary region of the adrenal demonstrates a similar loss of contents after 3 days and a swelling of the secretory granules as in vagotomized animals.

The mitochondria of the adrenal cells are most affected by cisplatin treatment. These are abnormally swollen with an enlarged matrix and their inner membranes are completely disrupted. Hydroxylated cisplatin uncouples oxidative phosphorylation in isolated mitochondria. Mitochondria seem to be the initial target for cisplatin and have been implicated in some toxicities, especially nephrotoxicity

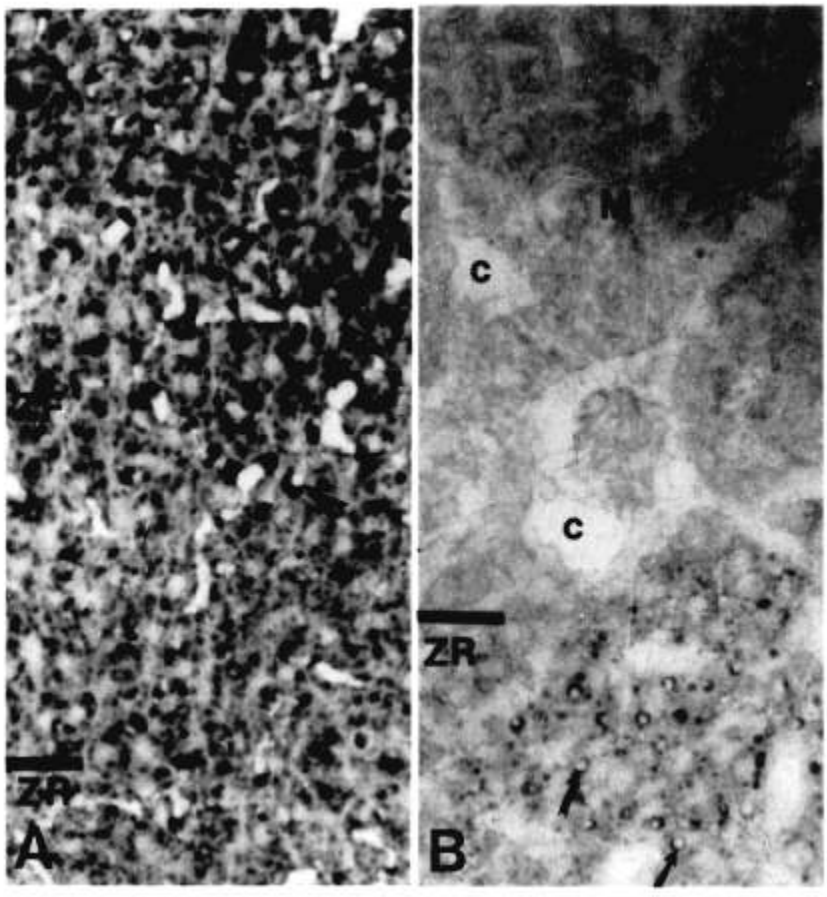

Figure 12. Light micrographs of a fresh frozen section (10 μm) of the adrenal gland from a normal rat stained with Sudan black B. (**A**) Showing the normal distribution of lipids (arrows) in the zona fasciculata (ZF) and zona reticularis (ZR). (**B**) Cortico-medullary region of the adrenal gland. Note the absence of lipids in the medullary region (M). Arrows point towards the lipid inclusions in the zona reticularis (ZR). c, capillaries. ×1040.

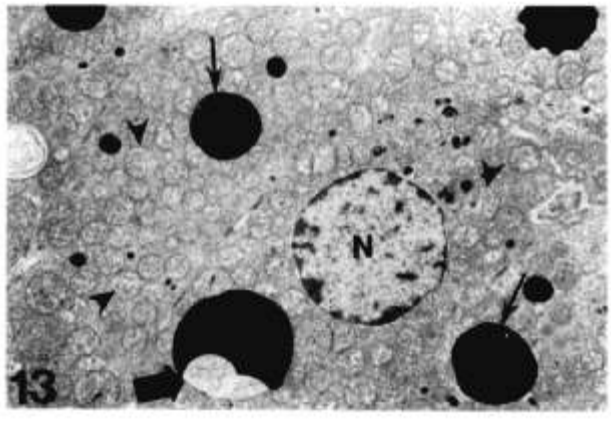

Figure 13. Electron micrograph of a section through the zona reticularis region of an adrenal gland from a normal rat showing the distribution of the lipid globules (arrows) and the mitochondria (arrowheads). Note the lamellar configurations in one of the lipid globules (heavy arrow). The outer nuclear membrane shows extensive blebbing. N, nucleus. ×5000.

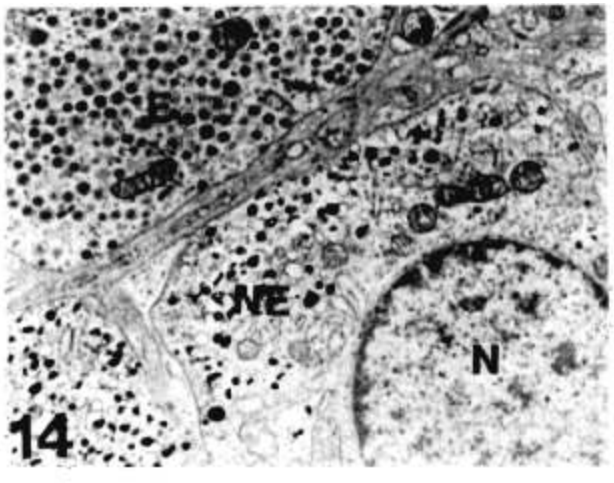

Figure 14. Electron micrograph of a section through the medullary region of an adrenal gland from a normal rat showing the epinephrine (E) and norepinephrine granule (NE) containing cells. m, mitochondria, N, nucleus. ×6500.

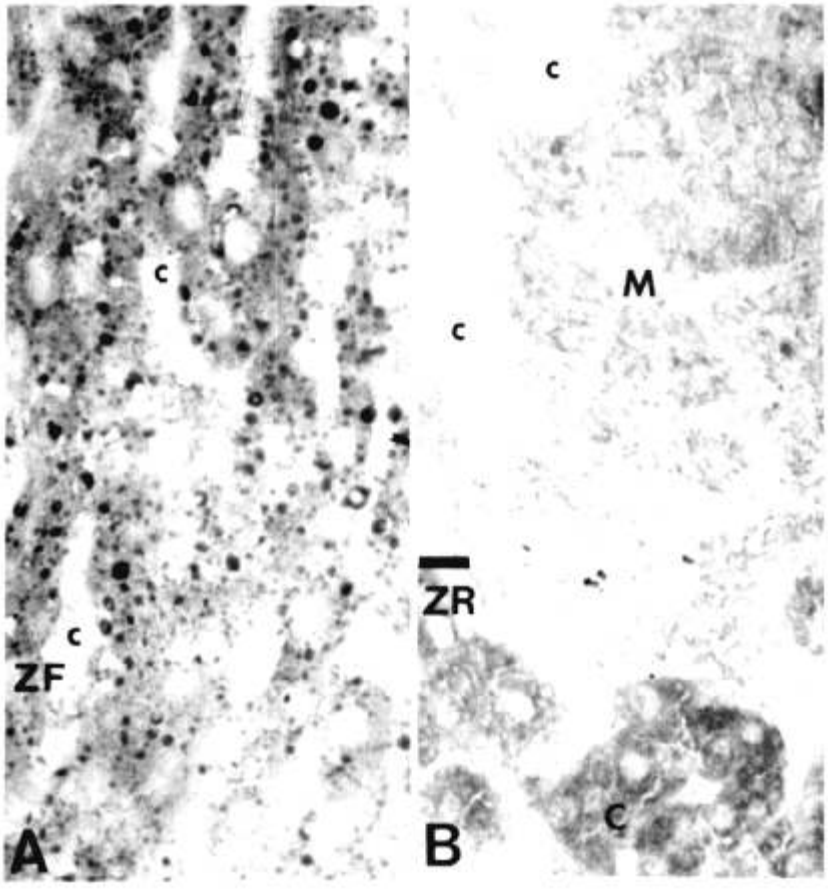

Figure 15. Light micrographs of a fresh frozen section (10 μm) of an adrenal gland after 3 days of cisplatin treatment (9 mg/kg) stained with Sudan black B. (**A**) Showing the general distribution of lipids in the zona fasciculata of the cortex and the distended capillaries (c). Note the smaller number of lipid inclusions (arrows) in the cortex after cisplatin treatment (see also Figure 12 for comparison). (**B**) Showing an apparent absence of lipid inclusions in the cortico medullary region (zona reticularis; medulla) after cisplatin treatment. C, cortex; M, medulla; ZF, zona fasciculata; ZR, zona reticularis. ×1040.

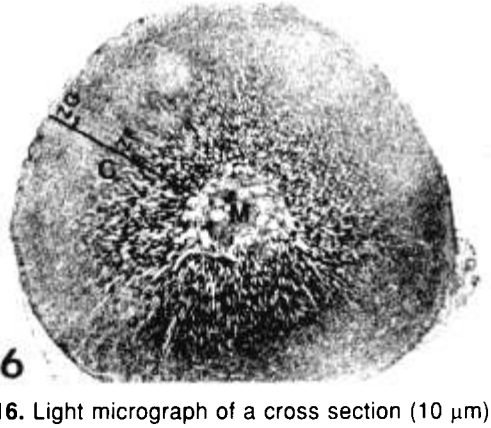

Figure 16. Light micrograph of a cross section (10 μm) of an adrenal gland from a rat treated with cisplatin (9 mg/kg) for 3 days demonstrating telangiectasia of the cortex (C) and the medulla (M). Note the atrophy of the medulla. ZG, zona glomerulosa, ZF, zona fasciculata; ZR, zona reticularis. Hematoxylin stained. ×56.

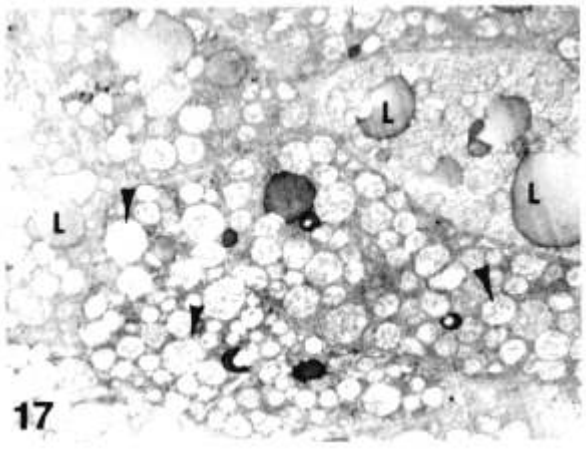

Figure 17. Electron micrograph of a section through the cortical region (zona reticularis) of an adrenal gland from a 3 day cisplatin-treated (9 mg/kg) rat. Note the swollen mitochondria (arrowheads) with highly irregular cristae. L, lipids. ×6270.

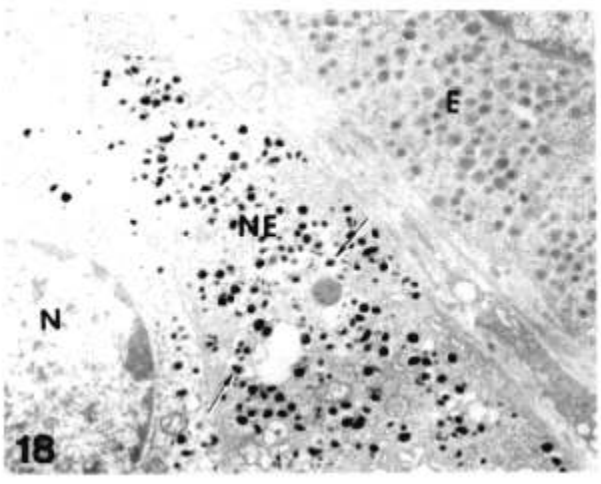

Figure 18. Electron micrograph of the medullary region of an adrenal gland after 3 days of cisplatin treatment. Note the abnormal swelling of the norepinephrine (NE) vesicles (arrows) but apparently normal epinephrine vesicles exist in the same section (E). N, nucleus. ×6600.

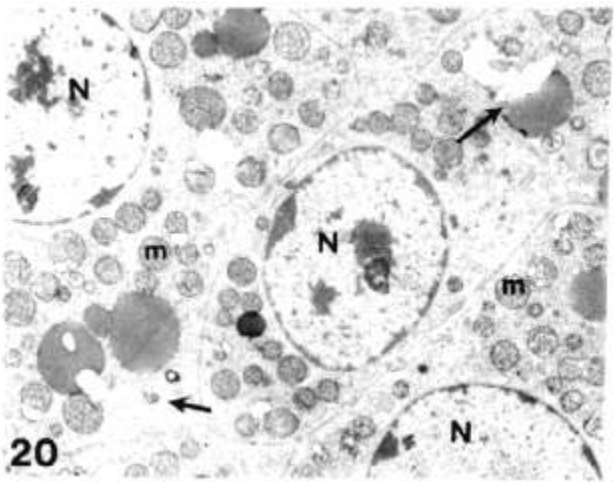

Figure 20. Electron micrograph showing the cells of the cortical region (zona reticularis) in a vagotomized rat after 3 days. Note the density of mitochondria (m) per unit area is much less compared with the normal gland sections (see Figure 13). Some of the lipid globules do show lamellar configurations (arrows). N, nucleus. ×6400.

where energy-dependent membrane filtration is involved.[46] Steroid hormones in the adrenal are synthesized by intimate contact between smooth endoplasmic reticulum, mitochondria and lipids which facilitate the exchange of enzymes and intermediate products in their synthesis.[34] If the mitochondria are damaged then probably the synthesis of corticoids is also affected.

Cisplatin is known to cause hypocalcemia and hypomagnesemia.[19] Calcium is required for the release of acetylcholine from the synaptic vesicles by their fusion with the axonal ending membrane.[47] Hypocalcemia is probably the reason for the inhibition of acetylcholine release from the axonal endings of the gastric smooth muscle and the pyloric sphincter; however, the amount of acetylcholinesterase seems to be unaffected by cisplatin, thus any acetylcholine released in drug-treated muscle should be hydrolyzed normally. Inhibition of acetylcholine release would lead to a chronic/spasmogenic effect on the pyloric sphincter, thus inhibiting gastric emptying resulting in bloated stomachs. The starved, cisplatin-treated animals did not develop stomach bloating or ulcers. Bloating

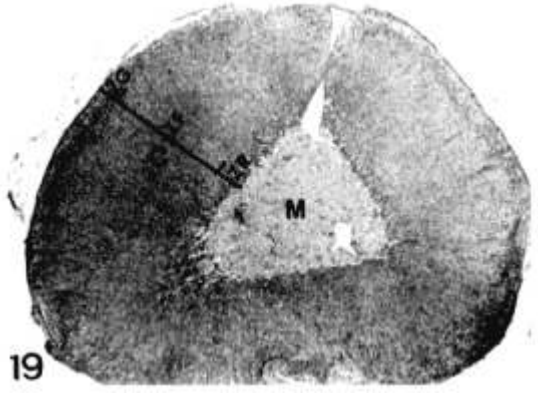

Figure 19. Light micrograph of an adrenal gland from a vagotomized rat of 3 days showing the hypertrophy of the cortex (C) and the medulla (M). Note the distended capillaries in the zona reticularis. ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis. Hematoxylin stained. ×56.

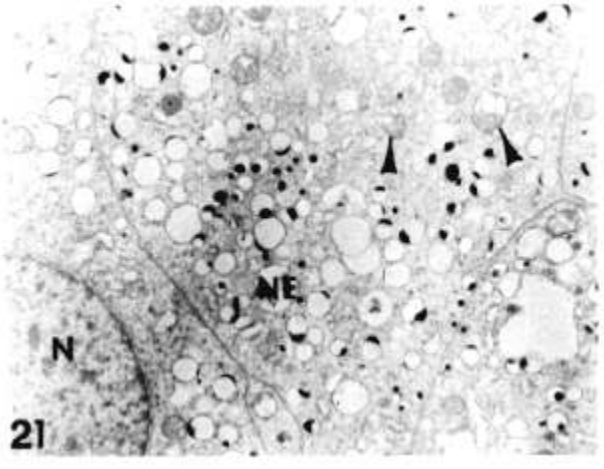

Figure 21. Electron micrograph of a section through the medullary region of an adrenal gland from a vagotomized rat after 3 days showing abnormal swelling of the norepinephrine vesicles (NE) and the mitochondria (arrowheads). N, nucleus. ×6700.

of the stomach in this case seems to be a requisite for ulceration, and has been associated with increased gastric acid and gastrin/pepsin secretion.[18]

Calcium is a necessary component in the process of gastric secretion and may influence parietal cell stimulus–secretion coupling.[48] The common denominator for acetylcholine-induced gastric acid response is calcium. Extracellular calcium seems to control the secretory response to acetylcholine and pentagastrin, which is decreased in its absence.[49]

Calcium supplements seem to alleviate gastric bloating and prevent gastric lesions.[19] Calcium is responsible for initiating muscle contractions,[37] synthesis and secretion of neurotransmitters, release of hormone,[50] and regulation of certain enzyme activities and membrane permeabilities.[51] Calcium (calcium gluconate or calcium chloride) has been shown to ameliorate the nephrotoxicity and gastrointestinal toxicity due to cisplatin treatment.[19] Calcium gluconate induces a transient rise in Ca^{2+} concentration in the glandular muscle layer and serum, while preventing any ulceration. Calcium is probably responsible for the release of acetylcholine from the vagus nerve fibres, inducing relaxation of the pyloric sphincter and contraction of the gastric smooth muscles. *In vitro*, fundal strips from cisplatin-treated rats have been demonstrated to be hypercontractile to acetylcholine and serotonin in calcium-free Tyrode solution, but contract normally in Tyrode solution with calcium, clearly demonstrating the important role of altered calcium homeostasis in cisplatin-treated smooth muscle contraction.[52]

Distension stimulus is known to involve some well recognized pathways, including vagal reflexes and intramural reflexes. In addition, there may be direct mechanical effects on the epithelium that enhance gastric acid secretion without the activation of nerves or known physiological ligands like gastrin, histamine or acetylcholine.[53] Acetylcholine, gastrin and histamine stimulate K^+/H^+-ATPase (parietal cells) to induce acid secretion.

Acetylcholine exerts an acute and direct stimulative effect on the adrenocortical function by stimulating the production of 17-hydroxylated corticosteroids, while atropine blocks the acceleration of steroidogenesis by acetylcholine.[54,55] Acetylcholine transmits the nerve excitation and is a direct stimulant of the adrenal cortex in terms of hormone output. Adrenalectomy, but not vagotomy, reverses the worsening effect of Ca^{2+} blockers on ethanol-induced gastric lesions in rats.[39] Compounds with calcium blocking properties, e.g. verapamil, nifedipine and WY47037, have been used to reduce stress-induced ulceration.[38]

Adrenalectomy antagonizes ethanol lesion aggravation by nitrendipine or verapamil when it becomes gastroprotective, while dexamethasone restores the lesion-enhancing effects of both Ca^{2+} channel blockers. Ulcers have also been attributed to histamine and 5-hydroxytryptamine released chiefly by stomach wall mast cell degranulation. Verapamil interferes with this degranulation and may be the way it acts as a protective agent.[56]

Stressful life events cause increased acid secretion, ulceration or symptoms.[57] Ulcers, if caused by gastric acid, may be prevented by antiacids, anticholinergic agents or vagotomy.[58] Yombine (α-blockers), propranolol, MJ 1999 and alprenolol (β-blockers) do show inhibition of ulceration and hemorrhage.[59] Prostaglandins (PGE_2) as well as certain methyl analogs of PGE_2 inhibit gastric secretion in animals and humans,[60] prevent ulcer formation in animals and accelerate ulcer healing in humans.[61] Most non-steroidal anti-inflammatory compounds like aspirin, indomethacin, phenylbutazone, naproxene and ibuprofen can produce gastric damage in animals,[62] and humans.[63] The presence of acid plays a crucial role in ulcer pathogenesis.

Serotonin and serotonin 5-HT_3 receptors also seem to play an important role in the way chemotherapeutic agents produce nausea and vomiting.[14] Of the antiemetic agents, ondansetron, a selective 5-HT_3 receptor antagonist, has been proven to be effective in controlling nausea and vomiting in both laboratory animals[15] and patients.[16,17,64] Release of large amounts of 5-HT from enterochromaffin cells during the first 6 h of cisplatin treatment is thought to be a crucial factor in the initiation and maintenance of the vomiting reflex.[65] Ondansetron has proven to be superior as a 5-HT_3 receptor blocker to metoclopramide.[14] In our present studies, accumulations of gastric acid through non-emptying of the stomach contents, or both, is hard to establish although a drop in the pH of the stomach contents after cisplatin treatment does suggest so. We did not monitor the blood flow, but decreased blood flow has been implicated in the rise of gastric acid.[66] Pharmacologically-induced increases in mucosal circulation have been shown to prevent ulceration induced by bile salts and shock, thus the role of the circulation sweeping away excessive quantities of acid becomes very clear.[67]

So far it seems that every effort has been directed towards controlling the symptoms of various toxicities associated with cisplatin treatment[10] and not

the causes of these toxicities. Our studies clearly show that hypocalcemia and depletion of membrane associated calcium are responsible for the significant toxicities due to cisplatin treatment. It is important to note that simple calcium supplements given before cisplatin administration can be used to control various toxicities, especially emesis, diarrhea and nephrotoxicity.[1,19]

Conclusion

In conclusion, the induction of gastric lesions in cisplatin-treated rats seems to be due to hypocalcemia. This should result in the inhibition of acetylcholine release, which in turn would cause spasm of the pyloric sphincter. This effect on the pyloric sphincter causes bloating of the stomach by inhibiting gastric emptying, thereby producing excessive accumulation of gastric acid, gastrin and pepsin, and possible reaction with food. These substances are responsible for the mucosal injury leading to ulceration. Calcium injections seem to raise the serum calcium levels which in turn influence the release of acetylcholine. Acetylcholine release reverses the pyloric sphincter stenosis and induces gastric smooth muscle contractility, stimulating gastric emptying and gastric blood flow. Cisplatin treatment also induces corticosterone release from the adrenal glands which is responsible for mucus destruction. Subphragmatic vagotomy or calcium supplements seem to inhibit these effects.

Acknowledgments

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