L-arginine protects against ethylene glycol-induced gastric mucosal damage in rats: immunohistochemical and electron microscopic study

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Received: 21.03.2012 • Accepted: 22.11.2012 • Published Online: 16.05.2013 • Printed: 17.06.2013

Abstract: Ethylene glycol is an alcohol widely used in industry as an antifreeze. It affects the normal gastric mucosal defense mechanisms, inducing surface ulcerations. L-arginine is a precursor of nitric oxide that exhibits multiple biological properties; among them is the acceleration of wound healing, especially in the stomach. Caspase-3 immunohistochemistry, and semithin and ultrathin section examinations were used to investigate the possible protective effect of L-arginine on ethylene glycol-induced gastric mucosal damage in rats. Three animal groups were used: group A (control group), group B (received 3.3 mL/kg ethylene glycol for 2 weeks), and group C (received 200 mg/kg of L-arginine and 3.3 mL/kg ethylene glycol for 2 weeks). By examination, group B showed significant increases in caspase-3 positive cells in comparison to the control, semithin sections revealed surface disruption and ulcerations, and ultrathin sections showed multiple pathological changes in cells lining the gastric mucosal glands. In group C significant decreases in caspase-3 positive cells were encountered and the other pathological findings in group B were found to be reversed. Therefore, we conclude that ethylene glycol-induced damage to the gastric mucosa could be prevented by L-arginine administration.

Key words: Ethylene glycol, L-arginine, stomach, caspase-3, immunohistochemistry, ultrastructure, rat

1. Introduction

Gastric ulceration is a multifaceted disease with a complex etiology. Great efforts have been made to determine its possible etiology as well as its proper management and treatment. Many attempts have been applied for achieving proper treatment of gastric ulceration, including single and combination therapies (1).

Ethylene glycol is an alcohol widely used in industry as an antifreeze. Because of its sweet taste, it is intentionally consumed as an inexpensive alcohol substitute or as a suicidal agent. Accidents involving ethylene glycol ingestion often occur in children (2). On ingestion, it is readily metabolized into toxic compounds such as glycoaldehyde, glycolate, and oxalate, which cause many pathological changes. In the stomach, ulceration occurs due to effects on the normal gastric mucosal defense mechanisms, which make gastric mucosa withstand the frequent exposure to substances. Such histopathological changes have also been studied in rats and mice by many investigators (3,4).

Ethylene glycol-induced gastric mucosal damage is an experimental model indicating that the gastric damage produced is a consequence of many interacting factors, each of which can be considered a potential therapeutic target (5).

L-arginine is a glycogenic amino acid that exhibits multiple biological properties; among them is the acceleration of the wound healing process (6). This amino acid serves as a substrate of the constitutive enzyme nitric oxide synthase, which produces a potent vasodilator nitric oxide that possesses many physiological properties in all tissues of the body including the stomach (6). Nitric oxide when released induces gastric smooth muscle relaxation as well as vasodilatation of the gastric microvessels (6). Many experimental studies have also proved the protective effect of L-arginine against gastric mucosal damage induced by a variety of ulcerogens (7–11).

The aim of the present work was to study the protective effect of L-arginine against ethylene glycol-induced gastric mucosal damage in rats using caspase-3 immunohistochemistry to demonstrate apoptosis of the gastric mucosa, toluidine blue staining of gastric mucosal semithin sections, and ultrathin section studies of the different cells lining the gastric mucosal glands.

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2. Materials and methods

2.1. Animal experimental groups
Thirty male Wistar rats from Tanta University animal house weighing 150–200 g were used. They were housed at ordinary room temperature, exposed to natural daily light/dark cycles, fed with standard laboratory diet, and given water ad libitum. The animals were divided into 3 groups (10 rats each): group A animals were untreated and served as a control group, group B animals were orally administered 3.3 mL/kg body weight per day ethylene glycol for 2 weeks, and group C animals were orally administered a daily dose of 200 mg/kg of L-arginine dissolved in distilled water 30 min before the oral dose of ethylene glycol (3.3 mL/kg body weight per day) for 2 weeks. All medications were delivered through an intragastric tube prior to feeding in the morning (5,7).

Animals were sacrificed 1 day after the last dose using sodium pentobarbitone 100 mg/kg and the stomach was removed. Specimens were immediately taken from the fundus below the limiting ridge of the gastric mucosa of rats.

All experiments were carried out following the guidelines for the care and use of experimental animals in the Tanta Faculty of Medicine with the approval of the Research Ethics Committee of the Faculty.

2.2. Chemicals and reagents
The following chemicals were commercially obtained: ethylene glycol (El Gomhuria Co., Tanta, Egypt), L-arginine (Sigma, St. Louis, MO, USA), sodium pentobarbitone (Abbott Lab., Chicago, IL, USA), anti-caspase-3 antibody (Lab Vision/NeoMarkers, USA), biotinylated goat anti-polyvalent antisera (Dako, USA), anti-caspase-3 antibody (Sigma, St. Louis, MO, USA), sodium pentobarbitone 100 mg/kg and the stomach was removed. Specimens were immediately taken from the fundus below the limiting ridge of the gastric mucosa of rats.

2.3. Immunohistochemical examination
Immunohistochemical staining for caspase-3 was performed on formalin-fixed, paraffin-embedded sections (5 µm) that had been deparaffinized and rehydrated. Slides were then placed in 0.3% hydrogen peroxide/methanol for 20 min, immersed in 10 mL of citrate buffer (pH 6), and microwaved twice for 5 min each. Non-specific protein binding sites were blocked by treating sections with a serum-free protein blocking solution for 20 min at room temperature. Sections were incubated overnight at 4 °C with a 1:150 dilution of rabbit anti-rat caspase-3 antibody directed against the cleaved, activated form of caspase-3. Slides were washed in PBS buffer and incubated with biotinylated goat anti-polyvalent antisera for 10 min at room temperature and then washed again in PBS. Then 1 to 2 drops of diaminobenzidine and chromogen were added to 1 mL of DAB substrate and then applied to the sections for 5–10 min. Sections were then counterstained with Mayer's hematoxylin, dehydrated, and cleared with xylol. Finally the sections were examined by light microscopy (12).

2.4. Electron microscopic examination
Sections from the stomach were cut into 1 mm³ pieces, fixed in 3% glutaraldehyde for 24 h, incubated for 1 h in 1% osmium tetroxide at 4 °C, dehydrated, and embedded in epoxy resin. Then sections were prepared for semithin and ultrathin examination (13).

2.4.1. Semithin examination
Sections were cut (1 µm) and stained with 0.5% toluidine blue (13).

2.4.2. Ultrathin examination
Sections were cut (50 nm) with an LKB ultramicrotome, stained with 2% uranyl acetate and lead citrate, and were examined by JEOL transmission electron microscopy (13).

2.5. Statistical analysis
The number of caspase-3 positive cells of the gastric mucosal gland cells was counted in 10 randomly selected fields from each slide of 10 different animals from each group using 10× objective lenses. Values were represented as mean ± standard deviation. Statistical analysis was performed using a 2-tailed Student's t-test after evaluation with an F-test where the results were considered statistically significant if P < 0.05.

3. Results

3.1. Immunohistochemical results
There was negative caspase-3 immunostaining in slides from group A. In group B, marked caspase-3 reactions were observed in the gastric mucosal gland cells. In group C, decreased caspase-3 immunostaining similar to that in the control group was observed (Figure 1).

3.2. Semithin results
Toluidine blue-stained sections from group A animals revealed a simple layer of pyramidal-shaped surface columnar epithelial cells. Group B showed decreased toluidine blue staining affinities of the surface mucous cells besides surface disruption and ulceration. In group C, the pathological findings of group B were found to be ameliorated (Figure 2).

3.3. Electron microscopic results
Electron microscopic examination of surface mucous cells of group A animals revealed high columnar cells with apical mucinogen granules, rough endoplasmic reticulum, and nucleus. In group B, severe damage was observed in the form of disrupted cell membrane with release of the mucous granules into the gastric lumen and nuclear indentation. Group C showed virtually normal surface mucous cells (Figure 3).

Electron microscopic examination of mucous neck cells of group A revealed low columnar cells with apical...
numerous secretory granules, rough endoplasmic reticulum, and nucleus. Samples from group B showed disrupted cell membrane, coalescence of the mucous granules, and release of granules to the gastric lumen. Giant secondary lysosomes were also encountered. Examination of group C samples showed normal cells (Figure 4).

Electron microscopic examination of parietal cells of group A revealed large pyramidal cells with long apical microvilli, prominent C-shaped intracellular canaliculi lined by numerous microvilli, electron dense mitochondria, and central nucleus. In group B, marked cytoplasmic vacuolation, distorted nuclei, mitochondrial

**Figure 1.** Caspase-3 immunoreactivity of the gastric mucosal cells of the rats. (a) control group showing negative reaction (→) (×100); (b) ethylene glycol treated group showing positive reaction (→) (×100); (c) ethylene glycol and L-arginine treated group showing apparent reduction in caspase-3 activity (→) (×200).

**Figure 2.** Toluidine blue-stained sections from the gastric mucosa of the rats. (a) control group showing normal epithelial lining (→) (Toluidine blue ×1000); (b) ethylene glycol treated group showing disrupted, ulcerated surface (→) (Toluidine blue ×1000); (c) ethylene glycol and L-arginine treated group showing nearly normal mucosal surface (→) (Toluidine blue ×1000).
Figure 3. Electron micrographs of surface mucous cells from the gastric glands of the rats. (a) control group showing high columnar cells with apical mucinogen granules (→), rough endoplasmic reticulum (r), and nucleus (n) (×2500); (b, c) ethylene glycol treated group showing disrupted cell membrane with release of the mucous granules into the gastric lumen (→) and nuclear indentation (n) (×2000 and 3000, respectively); (d) ethylene glycol and L-arginine treated group showing nearly normal cell (×3000).

Figure 4. Electron micrographs of mucous neck cells from the gastric glands of the rats. (a) control group showing low columnar cell with apical numerous secretory granules (→), rough endoplasmic reticulum (r), and nucleus (n) (×3000); (b, c) ethylene glycol treated group showing disrupted cell membrane (→), coalescence of the mucous granules (c), and giant secondary lysosomes (→) (×3000 and 2000, respectively); (d) ethylene glycol and L-arginine treated group showing reversal of the previous pathology (×3000).

disruption and dilated intracellular space were observed. For group C, most parietal cells were observed to be similar to those in control samples (Figure 5).

Electron microscopic examination of chief cells of group A revealed pyramidal cells with apical zymogene granules, rough endoplasmic reticulum, numerous
mitochondria, and basal nucleus. In group B, we observed dilated rough endoplasmic reticulum, swollen disrupted mitochondria, secondary lysosomes, cytoplasmic vacuolation, and hyperchromatic nuclei. In group C, the chief cells were virtually normal in morphology (Figure 6).

Electron microscopic examination of enteroendocrine cells of group A revealed ovoid cells with basal electron-dense granules, mitochondria, and central nucleus. Group B sections revealed total degranulation, nuclear indentation, swollen degenerated mitochondria, and cytoplasmic vacuolation. In group C, pyramidal cells were seen, similar to those in the control group (Figure 7).

3.4. Statistical results
Regarding the statistical analysis of results from the gastric mucosa, there was a significantly higher number of caspase-3 positive cells in group B (ethylene glycol) when compared to the control group. In group C (ethylene glycol + L-arginine) there was a significantly lower number of caspase-3 positive cells when compared with group B (ethylene glycol group) (Figure 8).

4. Discussion
The present results showed that L-arginine protected the gastric mucosa against ethylene glyco-induced alterations and damage in rats, which was shown by the significant decrease in the immunohistochemical results as well as in the reversal of semithin and ultrathin histopathological findings. This is inconsistent with the clinical findings of Anton et al. (14), who reported that a sublethal dose of ethylene glycol induced pathological changes in the cells lining the gastric mucosa.

The apoptosis that occurred in the current study can be explained by activation of the intrinsic mitochondrial pathway with the release of cytochrome c and other apoptogenic factors into the cytosol with opening of the permeability transition pore that allows solutes to diffuse across the inner mitochondrial membrane, leading to depolarization, inhibition of oxidative phosphorylation, and ATP depletion, resulting in apoptotic cell death (15,16).

Mucosal disruption and ulceration were found in the ethylene glycol treated groups. This was due to impairment of the mucosal repair process and inactivating several growth factors (e.g., fibroblast growth factor) that were important for gastric mucosal defense and repair. In addition, the discontinuity of the mucosal surface was due to dissociation of cell adhesion molecules between surface cells, resulting in surface disruption (17,18).

Histopathological findings of the different cells lining the gastric mucosa were explained by Bigaillon et al. (19). They reported that ethylene glycol metabolites inhibit protein synthesis, deoxyribonucleic acid replication, ribosomal ribonucleic acid synthesis, and serotonin metabolism. In addition, ethylene glycol inflicts gastric damage through accumulation of oxygen free radicals.

Figure 5. Electron micrographs of parietal cells from the gastric glands of the rats. (a) control group showing pyramidal cells with long microvilli (→), prominent C-shaped intracellular canaliculi (i), mitochondria (m), and nucleus (n) (×1500); (b,c) ethylene glycol treated group showing cytoplasmic vacuolation (v), distorted nuclei (n), mitochondrial disruption (m), and dilated intracellular space (i) (×2500 and 4000, respectively); (d) ethylene glycol and L-arginine treated group showing electron microscopic image similar to the control group (×2000).
Figure 6. Electron micrographs of chief cells from the gastric glands of the rats. (a) control group showing pyramidal cell with apical granules (→), rough endoplasmic reticulum (r), mitochondria (m), and nucleus (n) (×2000); (b, c) ethylene glycol treated group showing dilated rough endoplasmic reticulum (r), swollen disrupted mitochondria (m), secondary lysosomes (→), cytoplasmic vacuolation (v), and hyperchromatic nucleus (n) (×2500 and 2500, respectively) (d) ethylene glycol and L-arginine treated group showing nearly normal cell (×1500).

Figure 7. Electron micrographs of enteroendocrine cells from the gastric glands of the rats. (a) control group showing ovoid cells with spherical electron-dense granules (→), mitochondria (m), and nucleus (n) (×2500); (b) ethylene glycol treated group showing total degranulation (→) and nuclear indentation (n) (×2000); (c) ethylene glycol treated group showing swollen degenerated mitochondria (m) and cytoplasmic vacuolation (v) (×5000); (d) ethylene glycol and L-arginine treated group showing electron microscopic image similar to the control group (×5000).

Secondary to direct action on mucosal cells and impairment of the tight junctions between them with subsequent lipid peroxidation (20,21). Esfahlan and Jamei (22) mentioned that the accumulation of oxygen free radicals could lead to oxidative damage to lipids, proteins, and nucleic acids, resulting in cell injury.
As regard our results, the administration of L-arginine prior to ethylene glycol ameliorates its pathological effects. There are different mechanisms that could explain L-arginine gastric mucosal protection. L-arginine is the precursor of nitric oxide, which is a potent vasodilator playing an important role in the regulation of gastric vascular tone and permeability. Nitric oxide stimulates guanyl cyclase, which induces smooth muscle relaxation and vasodilatation with an increase in gastric mucus secretion (23). Moreover, nitric oxide has an antioxidant property associated with lowered DNA damage and diminished lipid peroxidation that could effectively protect the cytoplasm of the gastric mucosal cells against oxidative damage (23–25).

Based on the current results and previous studies, it was found that L-arginine prevents gastric mucosal damage induced by ethylene glycol through its role as a precursor of nitric oxide, and it could be beneficial in the treatment of gastric ulceration.

Acknowledgements
The authors would like to thank the Electron Microscopy Unit, Faculty of Medicine, Tanta University, Egypt. Great thanks are due to the Faculty of Applied Biological Sciences, Gifu University, Japan, for technical support.

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