Ingestion of elevated amounts of ethanol in humans and rodents induces hemorrhagic gastric lesions, at least in part by increasing oxidative stress. The present study was undertaken in order to evaluate the influence of a bicarbonate-alkaline mineral water (Uliveto®) on ethanol-induced hemorrhagic gastric lesions in mice. Lesions were evaluated by both macroscopic and microscopic analysis. In a first set of experiments, mice were allowed to drink Uliveto® or reference water ad libitum until 3 h prior to intragastric (i.g.) ethanol (23 ml/kg) administration. Neither Uliveto® nor reference water did afford any protection. In a second set of experiments, acute exposure to reference water (35 ml/kg, i.g.), given 30 min before ethanol, did not inhibit gastric lesions. However, administration of the same amount of Uliveto® caused a remarkable reduction in ethanol-evoked gastric lesions. Ethanol administration increased 4-hydroxy-2-nonenal levels, a byproduct of oxidative stress, in the luminal part of the gastric mucosa. This response was substantially reduced by about 70% by Uliveto®, but not by reference water. Reference water, added with the bicarbonate content, present in the Uliveto® water, protected against ethanol-induced lesions. Thus, acute pre-exposure to bicarbonate-alkaline mineral water (Uliveto®) protects from both oxidative stress and hemorrhagic gastric lesions caused by ethanol. The elevated bicarbonate content of Uliveto® likely accounts for the protection against ethanol-induced gastric injury.

**Key words** bicarbonate-alkaline mineral water; ethanol; gastric lesion; 4-hydroxy-2-nonenal

Peptic ulcers are chronic lesions that occur in gastroduodenal mucosa exposed to aggressive action of acid-peptic juices. An imbalance between mucosal defense mechanisms and damaging forces produces the lesions. The mechanism of peptic ulcers appears to be multi-factorial, with an important contribution by *Helicobacter pylori*. A variety of strong topical irritants and obnoxious agents, including hyperosmolarity solutions, strong acids (e.g., 0.6 N HCl), bases (e.g., 0.2 N NaOH), concentrated bile and boiling water are well known inducers of gastric lesions in experimental animals. In addition, behavioral factors or some drugs (e.g., non-steroidal anti-inflammatory drugs) may cause or worsen gastric lesions. Hemorrhagic ulceration of the stomach in humans and experimental animals is also caused by ingestion of elevated amounts of ethanol. Main features of ethanol-induced gastric damage are epithelial cellular loss, mucosal edema and sub-epithelial hemorrhage. The mechanism of toxic and damaging action of ethanol, although incompletely understood, seems to involve byproducts of oxidative stress. Several endogenous lines of defense may be activated to protect the stomach from injurious agents. The most important of these protective mechanisms include the secretion of bicarbonate and protective mucus, mucosal hydrophobicity, gastric microcirculation, generation of protective prostaglandins within the gastric mucosa, increase in the mucus sulfhydrlys and release of vasoactive neuropeptides from sensory nerve terminals. Protective mechanisms may be reinforced or supplied by the exogenous administration of defensive agents. In fact, protective alimentary habits or specific antacid drugs have been reported to produce from mild to very effective protection in patients with peptic ulcers.

The first line of mucosal defense against gastric tissue damage is constituted by the mucus-bicarbonate-phospholipids “barrier,” that is formed by mucus gel, bicarbonate, and surfactant phospholipids. A protective pH gradient in the adherent mucus gel layer at the epithelial surface in the stomach, made up by the secretion of HCO₃⁻ defends the mucosal surface against luminal acid. In acid-secreting gastric mucosa HCO₃⁻ is exported from the surface epithelium at rates of only 10% of the acid secretion rate. However, mucus gel minimizes luminal loss of HCO₃⁻ sufficiently to maintain a neutral pH at the apical cell surfaces. Antacid medicines have been used for a long time to heal the pain and tissue damage caused by gastric mucosa injury. Exogenous HCO₃⁻ may also contribute to this defense mechanism, although its protective action may be transient. Mineral water is more and more used as an alimentary habit in developed countries, and mineral waters may differ significantly in their solute content, thus conferring specific and variable properties to each individual water. Here, we have investigated whether a bicarbonate-alkaline mineral water (Uliveto®) protects from ethanol-induced hemorrhagic gastric lesions in mice. Results show that Uliveto®, but not a reference water with a minimal bicarbonate content, affords protection against ethanol-induced gastric lesion, and that the protection is due to the elevated bicarbonate content of the Uliveto® water.

**MATERIALS AND METHODS**

**Animals** Male Swiss mice (BALB/c) (ca. 25 g) were from Morini Laboratories (Reggio Emilia, Italy). Experiments were conducted according to the Italian guidelines for animal experimentation. Animals were sacrificed with an overdose of sodium pentobarbitone (200 mg/kg, intraperitoneally (i.p.)).

**Experimental Procedure** In a first set of experiments,
mice had free access to standard laboratory tap water (vehicle), reference water or Uliveto® water (see composition Table 1) for 2 d. The access to water was stopped 3 h before the challenge with an intragastric bolus of ethanol 100% (23 ml/kg). In a second set of experiments, mice were allowed free access to standard laboratory tap water until 3 h before the challenge. Mice, then, received by gavage (35 ml/kg, intragastric (i.g.)) either standard laboratory tap water (vehicle), reference water or Uliveto® water 30 min before ethanol administration. The experimental design adopted in the second set of experiments was also used before collecting gastric tissue for the measurement of prostaglandin E2 (PGE2) levels or 4-hydroxy-2-nonenal (HNE) adducts. Finally in a third set of experiments mice had free access to standard laboratory tap water until 3 h before the challenge. Then, 30 min before the ethanol challenge mice received the reference water added with different solutes at the same concentrations present in the Uliveto®. Mice were sacrificed 60 min later. In some experiments reference water was added with the solutes contained in the Uliveto® in excess to the content present in the reference water (LiCl 1.3 mg/l, Na2SO4 82.4 mg/l, NaCl 237.5 mg/l, KCl 21.7 mg/l, MgSO4 282.9 mg/l, SrSO4 1.8 mg/l, CaCl2 557.1 mg/l, NaNO3 6.8 mg/l, SiO2 1.2 mg/l) for 10 min at 4 °C and the supernatants were collected for PGE2 determination. Gastric PGE2 levels was determined by enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to the manufacturer’s instructions.

**Histology and Immunohistochemistry** For histopathological analysis, the oxyntic region of the stomach was fixed in 10% buffered formalin and embedded in paraffin, and 5-µm sections were stained with hematoxylin and eosin. For immunohistochemistry 4-µm sections were dewaxed in BioClear (Bio-Optica, Milan, Italy) and hydrated with graded ethanol concentrations. Antigen retrieval was routinely performed by immersing the slides in a thermostated bath containing 10 mM citrate buffer (pH 6.0) for 15 min at 97 °C followed by cooling for 20 min at room temperature. Endogenous peroxidase activity was blocked with hydrogen peroxide at 3% in distilled water for 10 min. After blocking with normal horse serum (UltraVision, LabVision, Fremont, CA, U.S.A.), sections were incubated with rabbit polyclonal anti HNE antisera (4-hydroxy-2-nonalen, Alpha Diagnostic, San Antonio, TX, U.S.A.) at 1:500 dilution for 30 min. Staining was achieved using Avidin–Biotin–Peroxidase (ABC) (LabVision, Fremont, CA, U.S.A.). Signal was detected using 3,3-diaminobenzidine (LabVision, Fremont, CA, U.S.A.) as chromogen. Nuclei were counterstained with Mayer’s haematoxylin. Negative controls were performed by substituting the primary antibody with a nonimmune serum. Specimens were examined for both macroscopic evaluation and histopathology in a blinded fashion.

**Measurement of HNE-Histidine Protein Adducts** The gastric mucosa of treated mice was collected, weighted and transferred to an ice-cooled test tube containing 10 ml homogenization buffer (0.1 M phosphate buffer pH 7.4 plus 1 mM EDTA and 10 µM indomethacin) for 1 g of tissue. After the homogenization, samples were centrifuged at 15000×g for 10 min at 4 °C and the supernatants were collected for

### Table 1. Chemico-Physical Properties of Uliveto® Water and Reference Water Used in the Present Study

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Uliveto® water</th>
<th>Reference water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li⁺</td>
<td>0.2 mg/l</td>
<td>—</td>
</tr>
<tr>
<td>Na⁺</td>
<td>113.7 mg/l</td>
<td>1.0 mg/l</td>
</tr>
<tr>
<td>K⁺</td>
<td>11.6 mg/l</td>
<td>0.4 mg/l</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>29.8 mg/l</td>
<td>1.2 mg/l</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>202.2 mg/l</td>
<td>1.2 mg/l</td>
</tr>
<tr>
<td>Sr²⁺</td>
<td>0.7 mg/l</td>
<td>—</td>
</tr>
<tr>
<td>F⁻</td>
<td>1.4 mg/l</td>
<td>—</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>121.4 mg/l</td>
<td>0.54 mg/l</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>683.2 mg/l</td>
<td>17.0 mg/l</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>5.9 mg/l</td>
<td>1.2 mg/l</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>151 mg/l</td>
<td>10 mg/l</td>
</tr>
<tr>
<td>SiO₂⁻</td>
<td>7.6 mg/l</td>
<td>5.6 mg/l</td>
</tr>
<tr>
<td>CO₃⁻</td>
<td>820 mg/l</td>
<td>5.0 mg/l</td>
</tr>
<tr>
<td>pH (20 °C)</td>
<td>6.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Dry residue (180 °C)</td>
<td>986 mg/ml</td>
<td>36.3 mg/ml</td>
</tr>
</tbody>
</table>

Results

**Uliveto® Water Protects against Ethanol-Induced Hemorrhagic Gastric Lesions** Histological examination indicated that a high dose of ethanol caused severe damage of the oxyntic mucosa of the mouse stomach (Fig. 1). In the first series of experiments, where mice were assigned to three groups, the mice of the first group were allowed to drink normal laboratory tap water, the second group Uliveto® water and the third group reference water until 3 h prior to the ethanol challenge. Gastric lesions produced by ethanol, identified as marked and diffused areas of hemorrhage in the stomach of mice drinking laboratory tap water (Fig. 1, upper panels), were similarly observed in the two other groups of
mice (Fig. 1, upper panels) which were allowed to drink reference or Uliveto® water.

In the second series of experiments, mice were allowed to freely drink tap water until 3 h prior to ethanol challenge. Thirty minutes prior to ethanol they received by gavage (35 ml/kg, i.g.) either laboratory tap water, Uliveto® water or reference water. Under these circumstances remarkable areas of lesion were observed in mice receiving laboratory tap water. In contrast, in mice that received Uliveto® water, but not in those that received the reference water, the area of lesions was markedly and significantly reduced (Fig. 1, lower panels).

At the microscopic level ethanol-induced damage consisted of acute erosive hemorrhagic lesions, with diffuse coagulative cell necrosis, multiple superficial erosions, marked vascular congestion and extravasation of erythrocytes (Fig. 2A, upper panel). Scattered inflammatory cells, including neutrophils, were present within the deep mucosal and submucosal layers. Administration of Uliveto® water, but not reference water, strikingly reduced all these signs of inflammation and tissue damage (Fig. 2A, upper panel).

Oxidative stress results in the formation of the reactive aldehydes, HNE, by peroxidation of plasma membrane phospholipids. HNE is, therefore, considered as a suitable marker of oxidative stress. Indeed, exposure to ethanol produced a more intense staining for HNE (Fig. 2A lower panel) and significantly increased HNE-His protein adduct levels (Fig. 2B) in the gastric mucosa. As observed for other signs of inflammation and tissue damage, administration of Uliveto® water, but not reference water, inhibited the HNE staining and significantly reduced the HNE-His levels produced by ethanol challenge (Fig. 2B).

The Bicarbonate Content of Uliveto® Water Protects against Ethanol-Induced Hemorrhagic Gastric Lesions

Prostaglandins are considered major protective agents in the gastric mucosa. To explore the mechanisms by which Uliveto® water affords protection against ethanol, we measured PGE₂ levels in the gastric mucosa of mice under various experimental conditions. PGE₂ levels found in the gastric mucosa of mice treated with ethanol and laboratory water were slightly and not significantly different from that observed after the vehicle of ethanol (Table 2). Pretreatment with Uliveto® water produced a moderate increase in PGE₂ levels in the gastric mucosa, but the difference did not reach...
Table 2. Effect of Ethanol and a Bicarbonate-Alkaline Water (Uliveto®) on PGE₂ Levels in the Mouse Gastric Mucosa

<table>
<thead>
<tr>
<th></th>
<th>Gastric mucosal PGE₂ generation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
</tr>
<tr>
<td>PGE₂ (pg/mg of tissue)</td>
<td>35.3±4.1</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. of at least 8 mice. §

Fig. 3. Macroscopic Images and Pooled Data of the Hemorrhagic Lesions Produced in the Mouse Stomach 30 min after the Administration of Intragastric Ethanol (23 ml/kg)

Mice were allowed to freely drink tap water until 3 h prior to ethanol challenge. Then, 30 min prior ethanol, they were treated by gavage with 35 ml/kg of reference water added with NaHCO₃ and Ca(HCO₃)₂ (74.8, 813.8 mg/l, respectively), a combination of salts contained in Uliveto® water (LiCl 1.3 mg/l, Na₂SO₄ 82.4 mg/l, NaCl 227.5 mg/l, KCl 21.7 mg/l, MgSO₄ 282.9 mg/l, SeSO₄ 1.8 mg/l, NaF 3.8 mg/l, CaCl₂ 557.1 mg/l, NaN₂O 6.8 mg/l, SiO₂ 1.2 mg/l) or a combination of salts (LiCl 1.3 mg/l, Na₂SO₄ 82.4 mg/l, NaCl 185.5 mg/l, KCl 21.7 mg/l, MgSO₄ 282.9 mg/l, SeSO₄ 1.8 mg/l, NaF 3.8 mg/l, NaNO₃ 6.8 mg/l, SiO₂ 1.2 mg/l) plus NaHCO₃ and Ca(HCO₃)₂. Columns are mean±S.E.M. of at least 8 mice: §p<0.05 vs. vehicle; *p<0.05 vs. tap water.

DISCUSSION

The present findings show that pre-exposure (30 min before the challenge) to a bicarbonate-alkaline mineral water markedly reduces the area of lesions produced by the administration of a high dose of ethanol in mice. In contrast, no reduction in ethanol-evoked lesions was observed after a few days of drinking the bicarbonate-alkaline mineral water, provided that water ingestion was stopped 3 h before the ethanol challenge. Thus, it is likely that the protection is given by the presence of the water in the stomach shortly before the exposure to the injurious insult. However, results clearly indicate that time constraints are not the sole requirement for protection, because neither tap water nor reference water, even though administered 30 min before ethanol, were able to reduce the gastric injury. This finding also excludes the possibility that protection is due to the relatively high volume of water introduced into the stomach and that might have diluted (and rendered less damaging) the ethanol load. In fact, if this was the case, a similar protective effect would have been equally produced by tap or reference water. Prostaglandins represent one of the defense lines to maintaining cellular integrity of the gastrointestinal mucosa. However, our data do not support the hypothesis that PGE₂ plays a role in the protective effect of Uliveto® water. Rather, protection seems to be due to one or more of the solutes contained in the Uliveto® water and absent in tap or reference water.

Ethanol-induced injury was characterized, as previously reported, by erosive hemorrhagic lesions, multiple superficial erosions, marked vascular congestion and extravasation of erythrocytes. Pre-ingestion of Uliveto® water reduced or abolished all these signs of inflammation and tissue damage. There is evidence that the detrimental effect of ethanol in the gastric mucosa is largely mediated by the generation of reactive oxygen species (ROS) within the mucosal tissue. One major action of ROS is the generation of reactive carbonyl species (RCS) principally through peroxidation of plasma membrane polyunsaturated fatty acids. HNE is a specific and stable RCS and alkylating agent that reacts with proteins, generating various forms of adducts with cysteine, lysine and histidine residues.

For this reason, HNE localization by immunohistochemistry and the evaluation of HNE-His protein adducts levels are used as reliable markers of oxidative stress. The ability of the Uliveto® water to reduce the increase in HNE immunostaining, observed mainly in the superficial part of the gastric mucosa and the levels of HNE-His adducts in gastric mucosa homogenates, indicates that this type of water inhibits several, if not all, the proinflammatory mechanisms, that activated by ethanol, contribute to the gastric tissue damage. HNE has been recently identified as a signaling molecules that selectively targets the transient receptor potential ankirin 1 (TRPA1) channel, expressed on terminals of primary sensory neurons. By this mechanism HNE causes pain and neurogenic inflammation at both cutaneous and visceral level, including the gastrointestinal tract. Thus, inhibition by Uliveto® water of HNE formation may also exert addi-
tional anti-inflammatory action by reducing the neurogenic component of ethanol-evoked inflammation.

The low pH of the gastric juice is considered the major contributing factor to gastric mucosal injury. Therefore, it is not surprising that the mucus-bicarbonate-phospholipid “barrier” constitutes the first line of mucosal defense.\(^1\) In particular, secretion of HCO\(_3^-\) into a stable, adherent mucus gel layer creates a pH gradient at the epithelial surface in the stomach and duodenum and provides the first line of mucosal defense against luminal acid.\(^1\) There is evidence in experimental animals that addition of NaHCO\(_3\) to the water supply reduces the pH of the gastric juice.\(^1\) It is therefore possible that the anti-inflammatory effect of this type of water may protect from gastric mucosal damage in man.

**Acknowledgments**

The study was supported by grants from Acqua e Termi di Uliveto S.p.A. and by Consorzio Ferrara Ricerche.

**REFERENCES**