Superoxide anions produced by inflammatory cells play an important part in the pathogenesis of acid and pepsin induced oesophagitis in rabbits

M J Naya, D Pereboom, J Ortego, J O Alda, A Lanas

Abstract

Background—Reactive oxygen metabolites have been associated with gastrointestinal injury.

Objective—To investigate whether mucosal reactive oxygen metabolites are involved in acid and pepsin induced oesophagitis, and if so, which specific metabolites.

Methods—The effects of free radical scavengers and the anti-inflammatory drug ketotifen on rabbit oesophagitis induced by acidified pepsin were studied. Isolated oesophageal cells were obtained before and after oesophageal injury and the generation of superoxide anion and hydrogen peroxide was analysed by flow cytometry. The presence of inflammatory cells was determined by indirect immunofluorescence with a mouse antirabbit CD11b antibody.

Results—Of the free radical scavengers tested, superoxide dismutase, which reacts with the superoxide anion, significantly reduced oesophagitis, whereas catalase, which reacts with hydrogen peroxide, had only a mild effect and dimethylsulphoxide had no effect. Ketotifen significantly reduced the inflammation and also prevented the induction of oesophagitis. Isolated cells obtained from the oesophageal mucosa after acidified pepsin exposure generated increased amounts of superoxide anions, which were mainly produced by CD11b positive cells.

Conclusion—Reactive oxygen metabolites, especially superoxide anion, produced by inflammatory cells play a significant part in the genesis of oesophagitis induced by acid and pepsin in rabbits and might be a target for future medical therapy.

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Keywords: oesophagitis, superoxide anion, free radical, inflammation, acid, pepsin.

Reflux oesophagitis results from contact of the oesophageal epithelium with gastric juice, which contains hydrochloric acid and pepsin. The intrinsic mechanisms of acid and pepsin induced oesophageal mucosal damage are not well known. Recent studies have shown that acid damages oesophageal epithelial cells by inducing cell oedema, cell acidification, and necrosis after affecting the lipid bilayers of the apical cell membranes, their intercellular junction structures, and overwhelming the ability of the acid extruding membrane exchangers and the activity of the Na⁺/K⁺ ATPase. Other potential mechanisms of mucosal damage have not yet been explored.

There is substantial evidence that oxygen derived free radicals play an important part in the pathogenesis of the injury of various tissues including the digestive system. The involvement of oxygen derived free radicals, such as the superoxide anion, hydrogen peroxide, and hydroxyl radical, have been well established in the pathogenesis of ischaemic injury of the gastrointestinal mucosa and in other models of mucosal damage induced by non-steroidal anti-inflammatory drugs, ethanol, haemorrhagic shock, feeding restriction stress, platelet activating factor and Helicobacter pylori. In all these models the presence of acid and pepsin increased and potentiated the mucosal damage. However, it is not clear whether oxygen free radicals are involved in the pathogenesis of oesophagitis induced by acid and pepsin. Potential sources of free radicals include the activated inflammatory cells, the hypoxanthine-xanthine oxidase system, the disrupted mitochondrial electron transport system, the metabolism of arachidonate via the lipoygenase pathway, and vascular endothelial cells. From a theoretical point of view, several of them could be involved in the pathogenesis of mucosal damage, including the lipoygenase pathway and the activated inflammatory cells. Oesophageal mucosal cells metabolise arachidonic acid via both the cyclo-oxygenase and lipoygenase pathways and the presence of inflammatory cell infiltrates in the oesophageal mucosa, lamina propria, and muscularis mucosae, usually accompanies epithelial erosions and ulceration in reflux oesophagitis.

The objective of this investigation was, therefore, to examine the role of inflammatory cells and the superoxide anion and hydrogen peroxide generation in the mechanisms of oesophageal injury induced by acid and pepsin and to determine whether scavenging radicals and the anti-inflammatory drug ketotifen protects against this damage. Finally we sought to determine the cell source of the free radical release.

Methods

This investigation comprised both in vivo and in vitro studies.

In vivo studies

All animal studies were carried out at the Service of Biomedicine and Biomaterials of the
University of Zaragoza, officially inscribed as a “Research Establishment” for the adequate husbandry and use of all research animals under the Good laboratory practices norms. New Zealand white rabbits weighing 2.5–3.5 kg each were studied. The animals were anaesthetised with intramuscular ketamine KCl (75 mg/kg) and intraperitoneal 20% urethane (40 mg/kg) before all studies.

Experimental model
The oesophagitis model is a modification of one described by Lillemoe et al., which has been described elsewhere. In brief, after sedation, the rabbits underwent laparotomy and neck dissection. The oesophagus was cannulated in the neck at the pharyngo-oesophageal junction and in the abdomen at the gastro-oesophageal junction with plastic tubing (internal diameter 2 mm) that was secured in place with umbilical ligatures. The oesophagus was then perfused with 50 ml of the various damaging solutions at a flow rate of 10 ml/min via a recirculating system using a peristaltic pump (Microtube Pump, MP-3, Eyela, Tokyo Rikakikai, Japan). The temperature of the perfusate was maintained at 37°C by a thermoregulator (P-Selecta, Barcelona, Spain). To induce oesophageal damage, acidified pepsin (normal saline acidified to pH 2.0 with 1-0 N HCl+2000 units/ml pepsin; Sigma Chemical Co, St Louis, MO, USA) was perfused for 80 minutes in each animal (exposed period) via a recirculating system. To evaluate ion flux rates after damage, the exposure period was followed by another 40 minute period in which acidified saline (normal saline acidified to pH 2.0 with 1-0 N HCl) was perfused (flux period). In each experiment a five minute washout period with normal saline was performed between the two different solutions perfused.

Aliquots of the flux solution were taken from the reservoir (50 ml) at the beginning and at the end of the perfusion period for the later analysis of pH, K+, and haemoglobin (see below). After the completion of the flux period, each animal was killed with an intracardiac bolus of pentobarbital and the oesophagus removed and opened longitudinally for pathological examination.

Experimental protocol
Six different groups of six to eight animals each were included in this part of the study. In all of them the same protocol of induction of mucosal damage (described earlier) was performed. However, different substances were given 10 minutes before and during the exposure period, when an intraluminal acidified pepsin solution was perfused. Unless otherwise specified, all drugs were given intravenously. These substances were 1 mg/kg dimethylsulphoxide (DMSO); 1 mg/kg intraperitoneal dimethylsulphoxide (one bolus); 90 000 units/kg catalase; 6 mg/kg superoxide dismutase (SOD); 0.05 mg/kg ketotifen; and saline as placebo. All substances were finally dissolved and injected in 50 ml saline. Catalase and DMSO were obtained from Sigma Chemical Co (St Louis, MO, USA) and SOD (Orgoteina-Ontosein) from Tedec-Zambelatti (Madrid, Spain). The SOD used is a liver bovine metaloprotein which has been widely used to evaluate the role of superoxide anion in different experimental models. Keto-
tifen (Zasten) was obtained form Sandoz-Pharma (Barcelona, Spain).

Indicators of damage
Macroscopic and microscopical changes – Immediately after removal, the oesophagus was examined for gross changes, fixed in 10% formalin, and photographed. Histological examination was performed on sections taken from the proximal, central, and distal portions of the oesophagus and stained with haematoxylin and eosin. The extent of both macroscopic and microscopical mucosal damage was graded by two independent observers unaware of the treatments given. The macroscopic and microscopical oesophagitis indices were determined following previously described criteria.

Assessment of mucosal barrier function – As previously described, mucosal permeability was assessed by the calculation of fluxes of H+ (µeq/h), K+ (µeq/h). H+ concentrations were determined by measuring the pH (Crisom, micropH 2001, Barcelona, Spain) and K+ concentrations were assayed by a flame photometer (Eppendorf Genätube, Netherier Hinz GMBH, Hamburg, Germany). Mucosal bleeding was quantified as the total amount of haemoglobin shed into the perfusate in both the exposure and flux periods by a colorimetric method (Quimica Clinica Aplicada SA Terragona, Spain).

IN VITRO STUDIES
Cell isolation
Isolated cells from the oesophageal mucosa were obtained according to a procedure previously described with small modifications. In brief, the oesophagus from overnight fasted New Zealand white rabbits (3–3.5 kg) was removed after a lethal intravenous injection of 3 ml sodium pentobarbitone (50 mg/kg). The muscle layer was stripped from the submucosa while tissues were bathed in a 4°C Ringer's
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solution previously gassed with 5% CO₂ and 95% O₂. The standard Ringer solution contained 82.2 mM NaCl, 4.0 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 0.8 mM NaSO₄, 17.8 mM NaHCO₃, 0.8 mM NaH₂PO₄, 11.5 mM glucose, and 0.2% bovine serum albumin. The medium was equilibrated with 95% O₂–5% CO₂ and pH was adjusted to 7.4. The oesophageal mucosa was then transferred to a low calcium Ringer solution (Ca²⁺ low medium contained 0.1 mM CaCl₂ and no MgCl₂), containing 1 mg/ml trypsin (Sigma Chemical Co) and 0.1% collagenase (Worthington, New Jersey, NJ, USA; digestion medium). After 30 minutes, the mucosa was cut into small pieces and transferred to a new digestion medium in a shaking water bath at 37°C for another 30 minute period. The digestion medium was continuously oxygenated with 95% O₂ and 5% CO₂. During this period the tissue was subjected to repetitive pipetting for several minutes. Finally, the tissue fragments and cells were collected by centrifugation and re-suspended in Ringer’s solution allowing the tissue fragments to settle and leaving isolated mucosal cells suspended in solution. This suspension was collected and then filtered through 100 μm Teflon mesh. A small aliquot of cells was removed for a cell count. Cells were >90% viable as determined by flow cytometry using the fluorochromes rhodamine²³ and propidium iodide to measure live and dead cells respectively.²³

**Cytometric determinations**

The cellular content of superoxide anion and hydrogen peroxide, cell mortality, and population studies were carried out by flow cytometry with an EPICS Elite (Coulter, Hialeah, FL, USA) equipped with an Argon Laser (ILT 550, Salt Lake City, UT, USA). All determinations were carried out with over 10,000 cells.

**Intracellular superoxide anion** – ‘O₂’ was measured by intracellular ethidium bromide formation from hydroethidine as previously described.²⁴ Briefly, the separated cells were resuspended at 1×10⁶ cell/ml in Hank’s balanced salt solution medium with 20 μM hydroethidine and incubated for 20 minutes; the transformation of ethidium bromide by ‘O₂’ was measured with a DL filter of 625 nm.

**Intracellular hydrogen peroxide** – Hydrogen peroxide was determined as rhodamine²³ formation from the oxidation of dihydroethidium.²³,²⁵ Oesophageal isolated mucosal cells were resuspended at 1×10⁶ cell/ml in Hank’s balanced salt solution and incubated for 20 minutes with 2 μM dihydroethidium²³ after which the rhodamine²³ fluorescence was recorded at 510–540 nm with a band pass filter.

**Determination of the cell populations of the oesophageal mucosa** – The oesophageal isolated mucosal cells were cytometrically studied by size and granularity and two populations were clearly separated. The histological analysis of these populations obtained by cytometric sorting showed that in one of them there was a predominance of squamous cells and that in the other area of greater granularity the cell population was composed of smaller cells from the oesophageal basal layers. Moreover, no mast cells could be identified either in isolated cells or in histological specimens (toluidine blue stain).

The presence of monocytes, macrophages, and neutrophils in isolated oesophageal mucosal cells was determined by indirect immunofluorescence. The cells were firstly labelled with a mouse antirabbit CD11b (Labgen, Barcelona, Spain) antibody for 45 minutes at 4°C and then with a goat antimouse Ig G1-FITC (Southern Biotechnology Associates Inc, Birmingham, AL, USA) for 45 minutes at 4°C. FITC fluorescence was determined at 525±15 nm with a band pass filter.

**In vitro experimental protocol**

Superoxide anion, hydrogen peroxide, and cell mortality were measured in isolated cells obtained from the oesophageal mucosa of rabbits with and without previous 80 minutes of oesophageal acidified pepsin perfusion. In both types of isolates simultaneous measurements of superoxide anion and CD11b positive cells were performed. Simultaneous measurements of hydrogen peroxide in CD11b positive cells could not be undertaken because the fluorochromes used to determine the presence of hydrogen peroxide and CD11b receptors have the same wavelength. The cytometric measurements were performed in isolated mucosal cells as a group and individually in each population identified by flow cytometry.

**DATA ANALYSIS**

Results are expressed as mean (SEM). The differences between means were evaluated for significance (p<0.05). After logarithmic Box-Cox transformation, continuous data were analysed by analysis of variance (ANOVA) with post hoc comparison where appropriate. Two-group comparisons were conducted with Student’s unpaired t test (two tailed). Categorical data were analysed by Fisher’s exact test. Cell mortality and CD11b labelling studies were analysed by Kruskal Wallis test and Mann-Whitney U test.

**Results**

**IN VIVO STUDIES**

**Effects of radical scavengers on acid and pepsin induced oesophageal mucosal damage**

Exposure of the oesophageal mucosa to acidified pepsin for 80 minutes induced severe gross and microscopical mucosal damage (Fig 1). Mucosal barrier function was also severely affected, showing loss of hydrogen ions from the lumen and a positive flux of potassium and haemoglobin into the oesophageal lumen (Figs 2 and 3). The parenteral administration of either intraperitoneal or intravascular DMSO, a scavenger of hydroxyl radicals, during the exposure period failed to modify this mucosal
damage. However, catalase, a scavenger of hydrogen peroxide radicals, induced a weak reduction in mucosal injury induced by acidified pepsin. All the indicators of damage were reduced but the differences were only statistically significant for macroscopic damage (Figs 1–3). By contrast, treatment with SOD, a scavenger of superoxide anion radicals, greatly reduced the mucosal injury induced by acidified pepsin, and all indicators of damage were significantly improved (Figs 1–4).

Effects of ketotifen on acid and pepsin induced oesophageal mucosal damage

The parenteral administration of ketotifen during the exposure period of the oesophageal mucosa to acidified pepsin also induced a dramatic improvement of all indicators of mucosal damage. The effect was similar to that observed with SOD treatments (Figs 1–3). The effects of ketotifen on acid and pepsin induced oesophageal mucosal damage were associated with a significant decrease in the presence of inflammatory cells in the mucosa and lamina propria of the oesophagus (Fig 5).

IN VITRO STUDIES

Isolated cells from the oesophageal mucosa obtained after the in vivo rabbit oesophageal exposure to acidified pepsin for 80 minutes induced a significant increase (300%) in generation of superoxide anions compared with control experiments (isolated cells without previous in vivo acidified pepsin exposure; Table I). The cell populations identified by flow cytometry showed similar increases in the production of superoxide anions, but the high correlation obtained between the superoxide anion content and the immune CD11b staining suggest that most superoxide anions were produced by the inflammatory cells that were equally distributed in the epithelial populations identified by flow cytometry (Fig 6). The percentage of CD11b positive cells isolated from the oesophageal mucosa increased after the rabbit oesophageal exposure in vivo to acidified pepsin from 2·97 (1·08) to 6·06 (0·97)% (p<0·01). Hydrogen peroxide production by isolated cells from this preparation did not differ from production by control cells (Table II).

Discussion

The data obtained in this study show that generation of superoxide anions is involved in the pathogenesis of acid and pepsin induced oesophageal damage in rabbits. The administration of SOD, a scavenger of this radical, significantly reduced the oesophageal damage induced by acidified pepsin. Other free radical scavengers were either weaker or did not offer any significant mucosal protection. Involvement of oxygen free radicals in mucosal injury has usually been assessed indirectly, but we also directly evaluated and confirmed the presence of superoxide anion in cells isolated from the oesophageal mucosa after acid exposure by measuring ethidium bromide, which is intracellularly formed by the oxidation of hydroethidine in the presence of the superoxide anion. This study also suggests that the presence of inflammation is associated with oesophageal damage as the anti-inflammatory drug ketotifen, which is not a free radical scavenger, was as effective as SOD in the prevention of oesophageal damage induced by acid and pepsin, and that activated anti-inflammatory cells (neutrophils, or monocytes, or macrophages) are one of the main sources of this free radical, although other potential, but probably minor, sources were not excluded.

Oxygen free radicals are detrimental to the integrity of biological tissues and mediate their injury. The mechanisms of damage involve lipid peroxidation, which destroys cell membranes with the release of intracellular...
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Figure 4: Microphotographs of oesophageal specimens in control experiments (A and B) and animals treated with superoxide dismutase (C). Superoxide dismutase reduced the microscopical mucosal damage and inflammation induced by acidified pepsin. (A) Oesophageal epithelial nacerlation and oedema, vascular congestion, haemorrhage, and polymorphonuclear cell infiltration; (B) Detail of the polymorphonuclear cell infiltration; (C) Small superficial epithelial loss with oedema and vascular congestion. No polymorphonuclear cell infiltration was seen in this specimen.

![Image of microphotographs](image)

Figure 5: Effects of different free radical scavengers and ketotifen on the presence of inflammatory cells in the mucosa and lamina propria of the oesophagus after the intraluminal perfusion of acidified pepsin in rabbits (DMSO=dimethylsulfoxide; SOD=superoxide dismutase).

![Image of graph](image)

**TABLE I** Measurement of superoxide anion production by cell flow cytometry in isolated cells obtained from the oesophageal mucosa before and after the perfusion of acidified pepsin in vivo

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Without damage</th>
<th>With damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>45:48 (25:43)</td>
<td>152:50 (20:37) *</td>
</tr>
<tr>
<td>Population A</td>
<td>84:46 (40:70)</td>
<td>255:70 (8:75). *</td>
</tr>
<tr>
<td>Population B</td>
<td>44:27 (20:04)</td>
<td>121:13 (4:83)</td>
</tr>
</tbody>
</table>

The generation of superoxide anion is expressed as the mean (SEM) of arbitrary units of fluorescence of rhodamine=HE) in four experiments. In population A there was a predominance of squamous cells and in population B a predominance of basal cells. *p<0.05.

**TABLE II** Measurement of hydrogen peroxide production by cell flow cytometry in isolated cells obtained from the oesophageal mucosa before and after the perfusion of acidified pepsin in vivo

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Without damage</th>
<th>With damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>5:59 (1:74)</td>
<td>6:36 (3:03)</td>
</tr>
<tr>
<td>Population A</td>
<td>7:31 (2:70)</td>
<td>6:95 (4:15)</td>
</tr>
<tr>
<td>Population B</td>
<td>4:25 (1:87)</td>
<td>4:54 (1:99)</td>
</tr>
</tbody>
</table>

The generation of hydrogen peroxide is expressed as the mean (SEM) of arbitrary units of fluorescence of rhodamine=HE) in four experiments. In population A there was a predominance of squamous cells and in population B a predominance of basal cells.

generation of the superoxide anion as a mechanism of damage is well established in different models of acute and chronic injury in the stomach and intestine but it is not well established whether this radical is involved in the pathogenesis of acid and peptic induced oesophageal damage. In a recent report by Wetscher et al., oesophagitis induced by duodenogastro-oesophageal reflux after duodenoejunal ligation in rats was prevented or significantly reduced by the blockade of the superoxide anion with SOD, but it was not reduced with catalase, dimethylthiourea, allopurinol, or vitamin E succinate. Treatment with catalase reduced lipid peroxidation by 56% but was not sufficient to inhibit oesophagitis on gross appearance. In a different model of oesophagitis, induced by perfusing components, such as lysosomal enzymes, leading to further tissue damage. The radicals also promote mucosal damage by causing degradation of the epithelial basement membrane components, complete alteration of the cell metabolism, and DNA damage. The...
controlled amounts of acid and pepsin for fixed periods, we have shown both directly and indirectly that superoxide anions were directly involved in the oesophageal damage induced by acid and pepsin in rabbits. In our model, catalase was much less effective than SOD in preventing or reducing the mucosal damage, and generation of hydrogen peroxide was not detected in mucosal cells isolated after damage, but we cannot exclude that small amounts of this radical might be produced in vivo. The involvement of these two radicals seems plausible because SOD efficiently and specifically scavenger the superoxide radical by catalysing its dismutation to hydrogen peroxide and oxygen.27 Hydrogen peroxide, which is a relatively non-toxic substance can be scavenged by catalase to form water and molecular oxygen.27

Hydroxyl radicals are formed by the reaction of superoxide anions with hydrogen peroxide via the Haber-Weiss reaction. This reaction proceeds at too slow a rate to be of physiological relevance. However, the reaction rate is accelerated by the presence of transition metals such as iron. In these conditions the generation of hydroxyl radicals, which are highly reactive and cytotoxic, is particularly harmful. In our model, parenteral DMSO, a scavenger of hydroxyl radicals, did not have a significant effect on oesophageal damage, suggesting that this radical is not involved in the pathogenesis of acid and pepsin induced oesophagitis. Similar results were reported by Westcher et al,18 who did not find any significant effect with dimethylthiourea alone, another hydroxyl radical scavenger, on rat oesophagitis. However, the hydroxyl radical is extremely reactive and it is unlikely that DMSO in vivo experiments will reach the site of generation of this radical in sufficient concentration to prevent hydroxyl radical induced local damage and therefore, despite our findings and Westcher’s data, a role for hydroxyl radicals in the pathogenesis of acid and pepsin induced oesophagitis cannot be definitely excluded.

An important question related to this investigation concerns the source of the oxygen radicals produced during oesophageal injury induced by acid and pepsin. Our in vitro studies suggest that the inflammatory infiltrate is the main source of generation of superoxide anions. As measured by flow cytometry, isolated oesophageal cells obtained after the perfusion of acidified pepsin showed increased generation of superoxide anions. Pretreatment with a monoclonal antibody directed against subunits of the granulocyte adherence glycoprotein CD11b showed that most of the superoxide anion was generated in CD11b positive cells, which include neutrophils, monocytes, and macrophages in rabbits. Eosinophils are not labelled by the CD11b antibody in rabbits and, therefore, we cannot exclude the possibility that this type of cell, usually present as a small percentage of the inflammatory infiltrate, may contribute to the generation of superoxide anions. In a recent study, the indirect measurement of reactive oxygen species by luminol chemiluminescence from human oeso-

phageal biopsy specimens showed an increased production of these compounds in patients with oesophagitis that was partially reduced by the in vitro treatment with the myeloperoxidase inhibitor azide and catalase, suggesting that neutrophils were not the only source of mucosal luminol chemiluminescence.28

In our model, ketotifen also significantly and dramatically reduced the oesophageal damage induced by acidified pepsin. The effect was similar to that obtained with the perfusion of the superoxide anion scavenger SOD. Ketotifen is an anti-inflammatory drug, usually known as a “mast cell stabiliser” with other important effects that include the inhibition of neutrophilic migration,29 30 the reduction of eosinophil viability,31 and inhibition of cell NADPH oxidase,32 and the inhibition of leukotrienes, platelet activating factor, prostaglandin E2, and tromboxane B2 concentrations in inflamed tissues.33 35 Previous studies have also shown that treatment with ketotifen either reduced or totally prevented mucosal damage in different experimental conditions.33 35 Clostridium difficile toxin A induced enteritis in rat ileum and ethanol and gastric mucosal damage induced by non-steroidal anti-inflammatory drugs in rats and humans.29 33 35

The presence of increased amounts of neutrophils and macrophages in the oesophageal mucosa after damage suggests the involvement of soluble chemotactic mediators causing migration of inflammatory cells into the mucosa. In other models of damage, mast cells are involved in both the induction and amplification of the inflammatory process by releasing soluble mediators. Therefore, the effects of ketotifen on this model of acid and pepsin induced oesophagitis could be due to an effect on resident oesophageal mast cells. However, mast cells, which are present in the adult North American opossum and humans, are very rare in rabbit oesophageal mucosa,37 38 suggesting that most effects of ketotifen in this model of oesophagitis might be mast cell independent and probably related to other drug actions (for example, inhibition of neutrophilic migration). Our histological studies have shown a significant reduction in the presence of inflammatory cells only in those experiments in which rabbits were treated with ketotifen.

The results of this study demonstrate that oxygen derived free radicals, especially the superoxide anion, are involved in the pathogenesis of acid and pepsin induced oesophagitis in rabbits, and that the inflammatory infiltrate – namely, neutrophils, macrophages, and monocytes – are the main source of this radical and the drugs that modulate this inflammatory response or radical scavengers dramatically reduce the acid and pepsin induced injury. Because oesophagitis in humans is often characterised by epithelial erosions, ulcers and accompanying inflammatory infiltrate,1 these results may be useful in the development of future treatment of reflux oesophagitis.

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Investigaciones Sanitarias de la Seguridad Social (FIS). Part of this work was presented at the 1995 meeting of the American Gastroenterological Association.


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