A novel antiulcerogenic stable radical prevents gastric mucosal lesions in rats

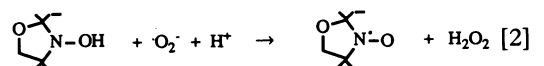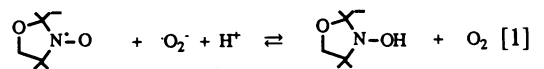D Rachmilewitz, F Karmeli, E Okon, A Samuni

Abstract
The pathogenesis of gastric mucosal injury is still poorly understood. Recent reports implicate redox active metals and reactive oxygen species as mediators of gastric damage induced by ethanol or non-steroidal anti-inflammatory drugs. Attempts were made therefore to prevent gastric injury using chelators and the antioxidant enzymes catalase and superoxide dismutase. These attempts, at best, would only detoxify extracellular reactive species, such as those produced by activated circulating granulocytes and macrophages. This study utilises another strategy by pre-emption of both intra and extracellular reactive species using radical-radical annihilation reactions and by detoxifying redox active transition metals. Nitroxide, stable free radicals were shown to enter mucous cells, protect against the ethanol induced damage, and prevent gastric lesions induced by aspirin, indomethacin, 25% NaCl, or 0·6 N HCl. These findings confirm that gastric mucosal damage from the above agents is mediated by free radicals and, moreover, introduce a prototypical agent within a potential new class of gastric ulcer preventing drugs.
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Oxygen derived radicals and non-radical species have been previously implicated in the pathogenesis of gastric ulceration induced by ethanol.[1-2] Reactive oxygen species have been assumed to be mediators of acute gastric damage induced also by non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and aspirin.[1-6] Previous attempts to attenuate gastric damage, therefore, included pre-treatment with catalase and superoxide dismutase to detoxify presumed reactive oxygen species.[1-2 6-7] In addition, chelating agents have been used to sequester redox active transition metals and prevent them from aggravating the damage. Protective effects of catalase and superoxide dismutase, previously seen, are anticipated if extracellular reactive oxygen species generated by circulating leucocytes predominantly contribute to the damage. The use of reagents, however, which do not enter the cell limits this approach to extracellular reactive species.

If radicals are instrumental in mediating biological damage, their rapid removal should provide protection. Reactions of free radical with diamagnetic molecules, however, give rise to secondary radicals, whereas radical-radical reactions are rapid and often result in stable non-toxic species. Such a strategy has been previously used with nitroxides to protect cells,[8] organs,[9] and whole animals[10] against diverse insults. Nitroxides are cell permeable, comparatively non-toxic[11] stable radicals, which are widely used as biophysical probes for monitoring membrane state, cellular pH, and oxygen concentration,[12-14] intracellular redox reactions,[15] or as contrast agents for nuclear magnetic resonance imaging (MRI).[16] Being capable of undergoing one electron redox reactions, 5-membered ring nitroxides such as 2-ethyl-2,5,5-trimethyl-3-oxazolidinoxyl, have been found to catalytically dismutase superoxide radicals:

$$\text{O}^{\cdot} \text{N-O} + \cdot\text{O}_2^- + \text{H}^+ \rightleftarrows \text{O}^{\cdot} \text{N-OH} + \text{O}_2 \quad [1]$$

$$\text{O}^{\cdot} \text{N-OH} + \cdot\text{O}_2^- + \text{H}^+ \rightarrow \text{O}^{\cdot} \text{N-O} + \text{H}_2\text{O}_2 \quad [2]$$

yielding H_2O_2 and molecular oxygen. Previous studies showed that nitroxides prevent lipid peroxidation[17] and also protect cells by selectively detoxifying other free radicals. Studies using bacterial and cultured mammalian cells showed that nitroxides provide cytoprotection from toxicity induced by H_2O_2,[8 18-19] *t*-butyl hydroperoxide (t-BuOOH), tumour necrosis factor,[20] mitomycin c,[21] and ionising radiation.[22]

This study was undertaken in the light of the clinical challenge to prevent gastric mucosal injury and to gain better understanding of the pathogenesis of mucosal damage, this gastric mucosal damage has been induced in rats by ethanol, indomethacin, aspirin, 25% NaCl, and 0·6 N HCl while the effect of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) on the induction of gastric mucosal injury was investigated. The results show effective protection by TEMPOL against gastric mucosal injury induced by ethanol and non-steroidal anti-inflammatory drugs. This protection also provided direct evidence that free radicals mediate gastric mucosal damage.

Methods

CHEMICALS
Diethylenetriaminopentaacetic acid (DTPA), and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), were purchased from

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Aldrich Chemicals. Fatty acid free bovine serum albumin, indomethacin, superoxide dismutase ferricytochrome c were obtained from Sigma Laboratories, Israel; aspirin (Aspegic, Lab Egic, Amily, France); ranitidine (Zantac, Glaxo, UK), leukotriene B_4 and leukotriene C_4 radioimmunoassay (Amersham, England). Mn(III)-desferrioxamine 1:1 chelate (Mn-DF) has been prepared by dissolving an excess of MnO_2 in DF overnight, followed by centrifugation to remove unreacted MnO_2, as previously described.[23-24] Tri(oxalato) chromiate(III) $K_3[Cr(C_2O_4)_3]\cdot3H_2O$ (CrOx) was prepared as previously described.[25]

ELECTRON PARAMAGNETIC RESONANCE (EPR) MEASUREMENTS

Liquid samples – a volume of 100 ml was drawn into a gas permeable, Teflon capillary of 0·8 mm inner diameter. The capillary was inserted into a quartz tube open at both ends and then placed within the EPR cavity.

Tissue samples – a gastric mucosa sample of 10–40 mg wet weight was excised from the rat stomach immediately after death and placed in a special groove of a standard quartz made tissue cuvette. During the experiment, the sample within the spectrometer cavity was flushed with either air or N_2, without disturbing the sample, and the EPR spectra were recorded on a Varian E9 X-band spectrometer, operating at 9·45 GHz, 100 kHz modulation frequency, 1G modulation amplitude, and 10–20 mW microwave power. The concentrations of the nitroxide in the samples studied were quantitated using a TEMPOL solution of a known concentration as a calibration standard. For the determination of the total concentration of nitroxide + reduced nitroxide, ferricyanide was used to oxidise the hydroxylamine.

ENZYME PREPARATIONS

Catalase (EC 1.11.1.6) was purchased from Sigma (10 U/μg) and from Boehringer Biochemicals (65 U/μg). Superoxide dismutase (EC. 1.15.1.1) was obtained from Sigma. The superoxide dismutase activity was assayed using hypoxanthine/xanthine oxidase as a generator of superoxide in the presence of 100 μM ferricytochrome c and after its superoxide dismutase inhibitable reduction, using a Uvikon dual beam spectrophotometer.[26] Both sample and reference cuvettes contained 130 U/ml catalase to remove H_2O_2. The reference cuvette contained an excess of superoxide dismutase, the reaction was started by adding 0·01 U/ml XO to both cuvettes. The initial rates of superoxide dismutase increase, in the absence (V) and the presence (v) of various amounts of superoxide dismutase were spectrophotometrically followed at 550 nm (ϵ^{550}=21 mM^{-1}cm^{-1}). The superoxide dismutase activity was evaluated from the plots of V/v dependence on the amount of superoxide dismutase added. Boiling the enzyme was not enough for the preparation of inactive-superoxide dismutase. Instead, the enzyme was incubated overnight with 10 mM H_2O_2 at pH 10, followed with dialysis against phosphate buffer pH 7 containing catalase. Subsequently the residual activity was determined as described above and was found to be less then 3%.

ANIMALS AND EXPERIMENT DESIGN

Male rats (Sprague-Dawley strain), weighing 150–200 g were fasted overnight and permitted free access to water. To study the effect of TEMPOL, rats were treated intragastrically with 1–100 mg of TEMPOL/kg bw. Unless otherwise stated, the standard protocol included intragastric treatment with TEMPOL 0·1 g/kg bw five minutes before induction of damage by intragastric administration of 1 ml 96% ethanol. Control animals received equal volumes of saline before intragastric administration of ethanol. Another control group was treated intragastrically with only 0·9% NaCl. To assess the damage 10 minutes after ethanol administration, rats were killed by cervical dislocation; the stomach was removed and washed with ice cold 0·15 M NaCl, and the extent of haemorrhagic erosions in the glandular mucosa was assessed blindly by measuring the area of the gastric lesions. Sections were obtained for morphological studies, the mucosa was then scraped, and samples taken for determination of leukotrienes.

To assess the effect of scavengers, chelating agents, and proteins on the ethanol induced injury, rats were treated intragastrically with either catalase, bovine serum albumin, superoxide dismutase, Mn-DF, or 2,2-dipyridyl 5-15 minutes before intragastric administration of 1 ml 96% ethanol.

To attenuate gastric acid secretion and maintain neutral pH in the stomach, in certain experiments, rats were treated intragastrically with ranitidine (75 mg/kg bw) 30 minutes before intragastric administration of TEMPOL followed five minutes later by intragastric administration of 1 ml 96% ethanol. Control animals in this experiment received ranitidine and ethanol.

When gastric mucosal damage was induced by NSAIDs, rats received intragastric 0·1 g nitroxide/kb bw, five minutes before or one hour and two hours after subcutaneous injections of indomethacin (30 mg/kb bw) or intragastric administration of aspirin (0·1 g/kg bw). In these experiments, rats were killed three hours after NSAID administration.

To assess the nitroxide protection from gastric damage induced by acid or 25% NaCl, TEMPOL was given five minutes before intragastric administration of either 1 ml 0·6 N HCl or 25% NaCl. Control rats were treated with the irritants only; all rats were killed one hour after damage induction.

DETERMINATION OF LEUKOTRIENES

Samples (150 mg) of mucosa were placed in pre-weighed tubes containing 1 ml of 50 mM

phosphate buffer, pH 7·4 minced with scissors, and centrifuged in an Eppendorf centrifuge for 10 seconds. The pellet was resuspended in 1 ml of the above buffer, incubated for one minute in a vortex mixer, 10 μg indomethacin was added, and the tubes were centrifuged for 60 seconds. The supernatants were kept at $-20°C$ until radioimmunoassay(s) were performed. The capability of the mucosa to generate leukotriene C_4 and leukotriene B_4 was expressed as ng/g wet tissue weight.

MEASUREMENT OF LEUKOTRIENE B_4

Immunoreactivity of leukotriene B_4 was determined by radioimmunoassay kit (Amersham, TRK 940). The assay combines the use of a high specific activity leukotriene B_4 tracer, an antiserum specific for leukotriene B_4 (cross reactivity 100%), and leukotriene standard (range 1·6–200 pg/tube). The specific binding of tracer is 42·5%, non-specific binding 2·4%. Fifty per cent B/B_0 displacement is obtained with 15 pg/tube and 90% B/B_0 displacement with 2·2 pg/tube of leukotriene B_4.

MEASUREMENT OF LEUKOTRIENE C_4

Immunoreactivity of leukotriene C_4 was determined by a radioimmunoassay kit (Amersham, TRK 905). The assay combined the use of a high specific activity leukotriene C_4 tritiated tracer with a monoclonal antibody specific for leukotriene C_4 and leukotriene C_4 standard. The standard curve covers the range 8–500 pg/tube and was performed in serial dilution. The assay uses highly specific leukotriene C_4 antiserum (cross reactivity 100%) and has low cross reactivity with leukotriene D_4 (<5%). The specific binding of tracer is 40·2%, non-specific binding 3%. B/B_0 displacement of 50% is obtained with 34 pg/tube and 80% B/B_0 displacement with 9·5 pg/tube of leukotriene C_4.

Radioimmunoassay was performed as follows: 100 μl of either standard or sample

buffer were pipetted to assay tubes and 100 μl antibody was added to all tubes except for total and blank tubes. The tubes were incubated at 4°C for 30 minutes. 100 μl tritiated leukotriene were added to all tubes and incubated at 4°C overnight. 200 μl buffer was added, followed by 200 μl dextran coated charcoal solution, excluding the total tube to which 200 μl buffer was added. The tubes were vortexed, incubated at 4°C for 10 minutes, and centrifuged in a refrigerated Beckman centrifuge at 1600 g for 10 minutes. The supernatant was transferred to scintillation vials to which 7 ml scintillation fluid was added and counted in a Kontron liquid scintillation counter.

MORPHOLOGICAL STUDIES

Sections of the gastric mucosa from five representative rats of each treatment group were fixed in phosphate buffered formaldehyde, embedded in paraffin wax, and routine 5 μm sections were prepared. Tissues were routinely stained with haematoxylin and eosin and blindly evaluated for severity of damage by light microscopy.

STATISTICAL ANALYSIS

The data are expressed as the mean (SEM) of the number of rats in each experimental group. Statistical comparisons for parametric data were made by Student's t test for unpaired data. The Mann-Whitney U test for statistical comparisons of non-parametric data was also used. A probability of $p<0·05$ was considered as statistically significant.

Results

EFFECT ON GASTRIC MUCOSAL LESIONS

Figure 1 shows the effect of 96% ethanol when given by itself or after pre-treatment with TEMPOL, on the gastric mucosa. In ethanol treated rats, lesion area averaged 56 (4) mm^2/rat (n=48), which represent about 10% of the glandular part of the stomach. Figure 1 shows that intragastric pre-administration of TEMPOL dose dependently decreased the extent of damage. Nitroxide at 0·1 g/kg bw totally prevented the ethanol induced mucosal lesions. As cultured mammalian cells tolerated up to 1 mM TEMPOL,[11] only subtoxic concentrations of nitroxide were used and the average TEMPOL concentration used in this study did not exceed 0·5 mM. In control experiments, no adverse effects in TEMPOL treated rats were detected for a week.

HISTOLOGICAL FINDINGS

Histological sections from the gastric wall of rats treated with 96% ethanol showed multiple mucosal lesions. The erosions were sharply demarcated from the surrounding mucosa with considerable congestion, oedema, and acute inflammatory infiltrate underneath (Fig 2 A). In rats pre-treated with 0·1 g/kg bw TEMPOL, the gastric mucosa was normal and could not
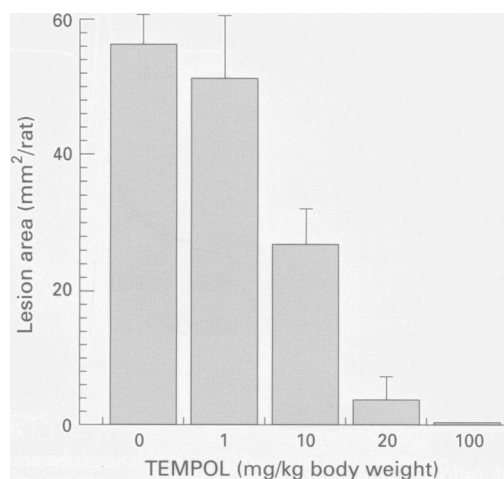

Figure 1: Effect of TEMPOL on ethanol induced gastric mucosal damage. Rats were treated with intragastric TEMPOL 5 minutes before intragastric administration of 1 ml 96% ethanol. The rats were killed 10 minutes after ethanol administration, and the area of macroscopically visible gastric lesions was measured. Results are mean (SEM).
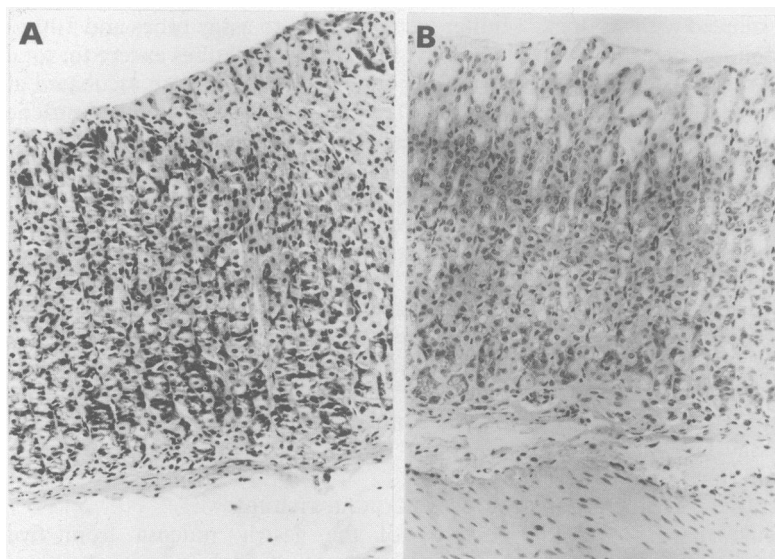
Figure 2: Histological sections (haematoxylin and eosin, original magnification ×173) of gastric mucosa of rats treated with 1 ml 96% ethanol only (A); or after pre-treatment with TEMPOL 0·1 g/kg bw (B). (A) shows (on top right side) an erosion that is sharply demarcated from the surrounding mucosa with considerable congestion, oedema, and acute inflammatory infiltrate underneath. (B) a section obtained from rats pre-treated with TEMPOL 0·1 g/kg bw, shows normal gastric mucosa.

be distinguished from that in control rats. The surface epithelium of the entire mucosa was examined and found to be totally protected and normal looking (Fig 2B).

TIME DEPENDENCE OF NITROXIDE EFFECT

To examine the duration of the nitroxide effect, rats were treated with intragastric nitroxide at various periods before induction of damage. TEMPOL 0·1 g kg, when given 30 or 60 minutes before administration of ethanol, prevented lesion formation (Fig 3). With a lower dose of 20 mg nitroxide/kg bw, given 30 minutes and 60 minutes before ethanol, the protection was partial and lesion area decreased by 76% and 45% respectively. When TEMPOL (0·1 g/kg bw) was given 5 minutes after ethanol administration, no

protection was seen (61 (9) and 56 (4) mm^2/rat respectively).

PARTITION OF NITROXIDE INTO CELLS

To ascertain that TEMPOL does indeed enter the mucous cells, the effect of an extracellular paramagnetic line broadening agent on the EPR signal of the nitroxide was examined. In principle, a distinction between the EPR signals arising from intra and extracellular species can be achieved with selective line broadening agents that cannot readily enter the cells. CrOx, which was previously found to be superior to ferricyanide or Ni(II),[27] was used in our study. After mixing 40 μl of 300 mM CrOx with 40 μl of 10 mM TEMPOL, the EPR signal of the nitroxide disappeared (data not shown). Later, immediately after the addition of a tissue sample of gastric mucosa to the solution, the EPR signal of TEMPOL has been instantaneously restored. Figure 4 shows the results of a typical experiment that compares the EPR signal seen upon scanning a sample containing 38 mg wet weight gastric mucosa soaked with 40 μl of 5 mM TEMPOL in phosphate buffered saline, before (upper spectrum) and immediately after the addition of 40 μl of 300 mM CrOx (lower spectrum). As Fig 4 shows, the intensity of the restored signal, because of the intracellular TEMPOL species inaccessible to the line broadening agent, represented only a small fraction of the total signal, reflecting the volume fraction occupied by the cells. The results showed that TEMPOL readily enters the mucous cells, as previously found for isolated cells in tissue culture.[28] The TEMPOL distribution between the intra and extracellular compartments is readily evaluated from the respective spectra. The volume fraction occupied by the cells and inaccessible for CrOx is less than 15% of the total sample volume (38 mg of gastric mucosa containing about 15 mg of mucous cells immersed in a
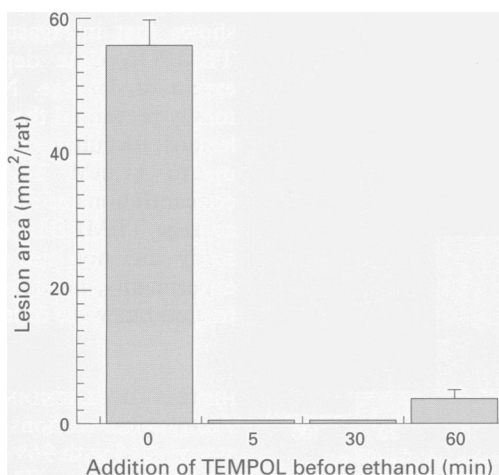

Figure 3: Protection by TEMPOL given at various time intervals before ethanol administration. Rats were treated, intragastrically with TEMPOL 0·1 g/kg bw at various time intervals before intragastric administration of 1 ml 96% ethanol. Rats were killed after 10 minutes and the area of macroscopically visible gastric lesions was measured. Results are mean (SEM).
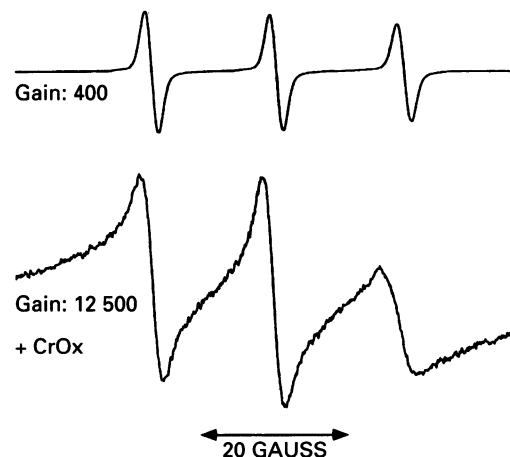

Figure 4: Nitroxide partition into mucous cells. Electron paramagnetic resonance spectra recorded for tissue sample of gastric mucosal soaked with 5 mM TEMPOL in 0·1 M pH7, phosphate buffered saline before (upper spectrum, gain=400) and immediately after the addiiton of CrOx (lower spectrum, gain 5=12 500). Experimental conditions: room temperature, aerobic conditions, 9·45 GHz, 3360 Gauss centre field, 10 mW incident microwave power, and 1G modulation amplitude.

TABLE I *Effect of catalase, superoxide dismutase, bovine serum albumin (BSA), superoxide dismutase-mimic and chelating agents on gastric mucosal lesions induced by ethanol*

Additive	Rats (n)	Time before administration of ethanol (min)	Lesion area mm^2/rat
Control ethanol only	48		56 (4)
Catalase (Sigma), 50 000 U/kg bw	5	5	8·4 (2·6)*
Catalase (Sigma), 50 000 U/kg bw	10	15	40 (14)
Catalase (Boeheringer), 50 000 U/kg bw	5	5	42 (8)
BSA, 15 mg/kg bw	5	5	18 (7·6)*
BSA, 15 mg/kg bw	5	10	33 (10·7)
BSA, 15 mg/kg bw	5	15	90 (13)
Superoxide dismutase 12 000 U/kg bw	10	5	56 (12)
Superoxide dismutase 60 000 U/kg bw	10	5	19 (6·3)
Superoxide dismutase 60 000 U/kg bw	9	15	24 (7·8)*
Inactive superoxide dismutase 60 000 U/kg bw	5	5	37 (7·8)
Mn-DF, 0·1 g/kg bw	8	5	37 (7·8)
DF, 0·1 g/kg bw	10	5	38 (9·2)
2,2dipyridyl, 0·1 g/kg bw	5	5	63 (19)

The additives were given intragastrically before treatment of the rats with 1 ml 96% ethanol. The rats were killed 10 minutes later and mucosal lesions measured. Results are mean (SEM) for rats in each group. *Significantly different from rats treated with ethanol alone.

total volume of 118 µl). Taking into account the respective 2:1 ratio in signal heights shown in Fig 4 and a 1:31 ratio of spectrometer gain yields a ratio of 1:2 for nitroxide concentration inside and outside the cells.

PERSISTENCE OF NITROXIDE IN THE MUCOSAL COMPARTMENT

To evaluate the persistence of the nitroxide in the mucosal tissue, rats were treated intragastrically with 0·1 g/kg bw TEMPOL, either five minutes or 60 minutes before administration of 1 ml saline or ethanol 96% (n=4). Five minutes later, the rats were killed 20 mg wet weight samples were excised from the gastric mucosal tissue, blended in 1 ml phosphate buffered saline, and frozen. To estimate the residual nitroxide concentration in the tissue, the frozen suspension were thawed, samples of 100 µl were drawn into a Teflon capillary, placed in the EPR cavity, and the EPR spectrum of the TEMPOL was recorded (see methods). Because TEMPOL predominantly decays within cells by reduction to the hydroxy-
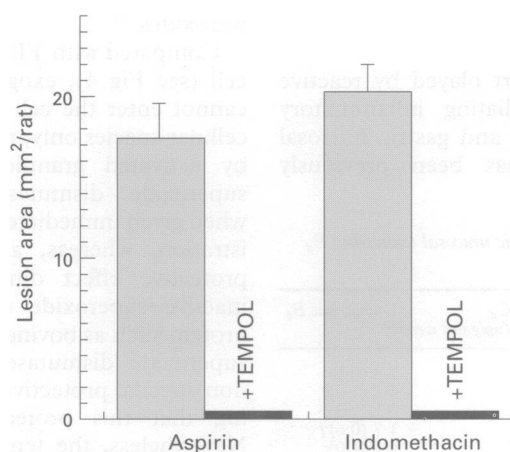

Figure 5: Effect of nitroxides on NSAID induced gastric mucosal lesion. Rats, fasted overnight, were treated intragastrically with 0·1 g TEMPOL/kg bw, five minutes before as well as one hour and two hours after subcutaneous administration of indomethacin (30 mg/kg bw) or intragastric administration of aspirin (0·1 g/kg bw). The rats were killed three hours after NSAID administration and the area of macroscopically visible gastric lesions was measured.

lamine 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1oxyl (TEMPOL-H), the total concentration of TEMPOL+TEMPOL-H was also evaluated. To oxidise the TEMPOL-H that was accumulated 2 µl of 0·1 M $K_3Fe(CN)_6$ was added to each sample and the EPR spectrum was recorded again. From the intensity of the EPR signal, the total concentration of TEMPOL+TEMPOL-H was calculated, taking into account the respective dilutions. When TEMPOL was given five minutes before treatment with ethanol, the residual (TEMPOL) was 1·3 (0·2) mM while (TEMPOL)+(TEMPOL-H) was 3·65 (1·1) mM. When TEMPOL was given 60 minutes before treatment with ethanol the respective values were: 0·32 (0·24) mM and 1·7 (0·65) mM. This shows that after one hour, the total concentration of TEMPOL decreased by half, from which, over 80% was reduced to TEMPOL-H. This explains the decrease with time in the protective effect of nitroxide (see Fig 3).

EFFECT OF PH

To find out if acidic pH was required for the protective activity of TEMPOL, its effect on gastric mucosal injury in ranitidine treated rats was studied. Under such conditions, gastric acid secretion was inhibited, the pH remained neutral, and no free gastric acid was detectable. Administration of ethanol, in the absence of TEMPOL, 30 minutes after treatment with ranitidine resulted in lesion areas of 55 (10) mm^2/rat (n=7). Conversely, in ranitidine treated rats, a pre-treatment with TEMPOL totally prevented ethanol induced gastric damage, showing that neither the extent of damage nor the protective effect of TEMPOL differ from these obtained under normal gastric pH1·7.

EFFECT OF EXOGENOUS CATALASE, SUPEROXIDE DISMUTASE, AND BOVINE SERUM ALBUMIN

As the deleterious effect of ethanol is exerted in fasted, but not fed, animals, the intragastric administration of enzymic protein might have some non-specific effect resembling that of rat feeding. To distinguish between specific and non-specific protective effects, the effects of intragastric administration of superoxide dismutase, inactive superoxide dismutase, catalase, and bovine serum albumin was studied. In addition, the time interval between the administration of protein and ethanol was varied. When given five minutes before ethanol, even a foreign protein such as bovine serum albumin or inactive superoxide dismutase, diminished the extent of gastric mucosal lesions. Similarly, the protection afforded by catalase of Boheringer was smaller than that exerted by catalase of Sigma, which contained sixfold more protein (Table I). When bovine serum albumin was given 10–15 minutes before ethanol, however, no protection against ulceration was evident.

TABLE II *Effect of acid, 25% NaCl, and TEMPOL on gastric mucosal lesions and eicosanoids*

Treatment	Rats (n)	Leukotriene C_4 (ng/g wet weight)	Leukotriene B_4 (ng/g wet weight)	Lesions (mm²/rat)
0·6 N HCl	9	8·2 (0·9)	6·4 (1·0)*	106 (18)
0·6 N HCl + TEMPOL	10	6·8 (0·6)	3·6 (0·5)*	28 (7)*
25% NaCl	14	6·1 (1·4)	1·0 (0·3)	34 (10)
TEMPOL + 25% NaCl	9	8·9 (1·0)	0·2 (0·03)	0*

TEMPOL (0·1 g/kg/bw) was given intragastrically 5 minutes before intragastric administration of saline or irritant. Rats were killed 60 minutes later, mucosal lesions were measured and eicosanoids were determined as described in methods. Results are mean (SEM) for rats in each group. *Significantly different from rats treated with 0·6 N HCl or 25% NaCl alone.

EFFECT OF MN-DF SUPEROXIDE DISMUTASE-MIMIC

Mn-DF, a low molecular weight, cell permeable, superoxide dismutase-mimic, is reportedly effective in protecting various cells against superoxide induced damage.[23] Pre-treatment with Mn-DF before ethanol administration, however, did not decrease the extent of gastric mucosal injury (Table I) to any statistically significant extent.

PROTECTION BY TEMPOL AGAINST DAMAGE INDUCED BY NSAIDS AND OTHER IRRITANTS

The protection provided by nitroxide was also evaluated with respect to damage induced by NSAIDs and other irritants. TEMPOL (three doses, each of 0·1 g/kg bw) prevented gastric ulceration induced by either indomethacin or aspirin (Fig 5). Similarly, pre-treatment with TEMPOL (0·1 g/kg bw) prevented gastric lesions induced by 25% NaCl and partially protected from 0·6NN HCl (Table II).

EFFECT ON GASTRIC MUCOSAL MEDIATORS

The generation of mucosal leukotriene B_4 and C_4, was increased two to threefold 10 minutes after damage induction by ethanol compared with their generation in saline treated rats (Table III). TEMPOL at 0·1 g/kg bw prevented the ethanol induced increase in leukotriene C_4 and B_4 generation (p<0·05, Mann-Whitney test, Table III).
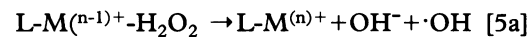
Discussion

Evidence for the key part played by reactive oxygen species in mediating inflammatory bowel disease in general and gastric mucosal injury in particular, has been previously

TABLE III *Effect of ethanol, NSAIDs, and TEMPOL on gastric mucosal leukotrine B_4 and leukotriene C_4*

Treatment	Rats (n)	Leukotriene C_4 (ng/g wet weight)	Leukotriene B_4 (ng/g wet weight)
Saline	30–31	3·6 (0·7)	2·4 (0·5)
Ethanol	14–32	11·3 (1·6)*	6·2 (1·0)*
TEMPOL	10	2·4 (0·7)	0·7 (0·3)*
TEMPOL+ethanol	9–10	5·3 (0·6)†	1·7 (0·6)†
Indomethacin	10	2·1 (0·4)	4·1 (1·0)
TEMPOL+Indomethacin	10	2·1 (0·3)	2·6 (0·5)
Aspirin	5	1·2 (0·06)	1·5 (0·3)
Aspirin+TEMPOL	10	2·3 (0·5)	1·8 (0·2)

TEMPOL (0·1 g/kg bw) was given intragastrically five minutes before intragastric administration of saline or 96% ethanol. Rats were killed 10 minutes later and leukotriene B_4 and leukotriene C_4 were determined as described in methods. The control values of leukotriene B_4 and leukotriene C_4 were the same with and without saline treatment. Results are mean (SEM) for rats in each group. *Significantly different from saline treated rats; †significantly different from ethanol treated or NSAIDs treated rats.

reported for diverse types of stress.[1-7 29-36] The central finding of this study is that TEMPOL provided total protection from gastric mucosal injury induced by alcohol and NSAIDs. Moreover, this protection was prolonged even when the nitroxide was given one hour before initiating damage. Nitroxide stable radicals selectively react with free radicals and with other paramagnetic species. As such, nitroxides serve not only as radical chain reaction terminators and effective cytoprotective agents, but also as sensitive probes for free radical mediated processes. Therefore, the complete protection provided by TEMPOL confirms the role of free radicals and redox active metals in ethanol induced cellular damage in general[37] and in the pathogenesis of gastric mucosal injury.

ROLE OF EXTRACELLULAR O_2^-

Previous reports of protective effects of chelating agents, catalase, and superoxide dismutase, implicated reactive oxygen species in either triggering gastric mucosal lesions or contributing to the development of necrosis once the process has started.[1 6] According to this hypothesis O_2^-, H_2O_2, and ·OH or hypervalent metal are instrumental in the pathogenesis of gastric mucosal injury by a metal catalysed Haber-Weiss reaction (L denotes some cellular ligand that binds the metal):

$$L-M^{n+}+O_2^- \rightarrow L-M^{(n-1)+}+O_2 \quad [3]$$

$$L-M^{(n-1)+}+H_2O_2 \rightarrow L-M^{(n-1)+}-H_2O_2 \quad [4]$$

$$L-M^{(n-1)+}-H_2O_2 \rightarrow L-M^{(n)+}+OH^-+·OH \quad [5a]$$

$$L-M^{(n-1)+}-H_2O_2 \rightarrow L-M^{(n+1)+}+2OH^- \quad [5b]$$

Practically, however, under normal gastric pH of 1·7 the superoxide radical anion does not exist. The hydroperoxyl radical HO_2, the protonated form of superoxide, is much less oxidisable than O_2^- and has been previously shown to react faster than superoxide with nitroxides.[38]

Compared with TEMPOL, which enters the cell (see Fig 4), exogenously added enzymes cannot enter the cell and can detoxify extracellular species only, probably those generated by activated granulocytes.[6] In this study, superoxide dismutase protected primarily when given immediately before ethanol administration, whereas, a few minutes later its protective effect diminished. Moreover, as inactive superoxide dismutase or a foreign protein such as bovine serum albumin, lacking superoxide dismutase activity showed some non-specific protective effect, it is not surprising that this protection rapidly subsided. Nevertheless, the temporal relation, between the protecting agent and the initiation of gastric mucosal damage does not exclude a contribution of exogenous superoxide. A previous study[39] showed that addition of superoxide dismutase, with or without catalase, only slightly (twofold) decreased the indomethacin induced lesion area. Conversely,

nitroxides, which act both intra and extra-cellulary are fully protected. The dramatic difference between the effects of superoxide dismutase and nitroxide clearly shows the important part played by intracellular radicals.
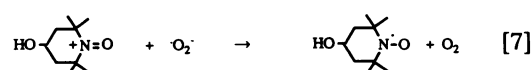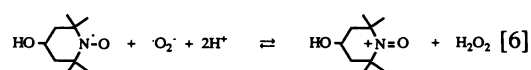
In this study, Mn-DF did not show any protection. This synthetic superoxide dismutase-mimic, which reportedly[23] can provide cytoprotection by removing O_2^- is prone to dissociate in biological systems and lose its catalytic activity.[24] Therefore, the failure of Mn-DF to prevent gastric mucosal injury does not necessarily exclude the role of intracellular O_2^- toward mucosal damage.

A similar conclusion can be drawn from the failure of DF to provide protection. DF is a chelating agent, which avidly binds transition metal ions[40] particularly iron (III); however, because it enters the cell very slowly[8] the cytoprotective effect of DF requires about one hour of preincubation. As Table I shows, DF did not provide protection against gastric mucosal injury.

MECHANISM OF NITROXIDE ACTION

Reactions of free radicals with diamagnetic molecules yield secondary radicals, which might propagate injurious process. Conversely, nitroxides, which are free radicals themselves, terminate radical chain reactions. Several mechanisms might underly the nitroxides effect on the biological damage, all of which entail reaction with paramagnetic species. Firstly, five membered ring nitroxides have been found capable of catalytically dismutating O_2^-. Both the nitroxide and its respective hydroxylamine can react with O_2^- yielding O_2 and H_2O_2, whereas the concentration ratio of the redox couple [nitroxide]/[hydroxylamine] remains time invariant and independent of O_2^- flux.[38] Secondly, more recently, 6 membered ring nitroxide derivatives were found to remove O_2^- by a different, yet catalytic, mode. Both the nitroxide and its respective oxoammonium cation, acting as an superoxide dismutase mimic, rapidly react with O_2^-, yielding H_2O_2 and O_2[41]:

Note that generally the concentration of cellular enzymes is dwarfed by that of their respective substrates. The exception for this rule are cellular superoxide dismutase and nitroxide superoxide dismutase-mimics whose concentrations exceeds that of O_2^- by several orders of magnitude. Thirdly, both 5 and 6

$$HO-\underset{}{\bigcirc}N\text{-}O + O_2^- + 2H^+ \rightleftharpoons HO-\underset{}{\bigcirc}{}^+N\text{=}O + H_2O_2 \quad [6]$$

$$HO-\underset{}{\bigcirc}{}^+N\text{=}O + O_2^- \rightarrow HO-\underset{}{\bigcirc}N\text{-}O + O_2 \quad [7]$$

membered ring nitroxides have been found to protect cells through reaction with reduced metal ions. 'Normally', in aerated systems, reduced redox active metal ions react with

H_2O_2 yielding a peroxo complex (reaction 4). As cellular transition metals do not exist as 'free' ions, but rather coordinated to cellular components, deleterious species, such as authentic secondary ·OH (reaction 5a) and hypervalent metal (reaction 5b), are formed 'site specifically' in a close proximity to critical biological targets. This site specific formation is responsible for damage potentiation and accounts for the frequent failures of radical scavengers to provide protection.[42 43] The reactions of nitroxides with transition metals have been previously studied and reported.[8 19] These results suggested that nitroxides can compete with H_2O_2 and oxidise the reduced metal ions[19]:

$$L\text{-}M^{(n-1)+} + H^+ + \text{nitroxide} \rightarrow$$
$$L\text{-}M^{n+} + \text{hydroxylamine} \quad [8]$$
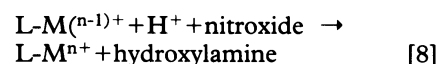
thus inhibiting reaction 4. Finally, nitroxides have been previously shown to protect hypoxic cells also where reactive oxygen species are practically absent. The hypoxic cytotoxicity of either t-BuOOH[8] or mitomycin c[21] to Chinese hamster cells could be totally eliminated by nitroxides. While t-BuOOH induced toxicity is catalysed by transition metals, that of mitomycin c is not. The cytoprotective effect of TEMPOL, in the last case, was attributed to the rapid removal of the deleterious semiquinone radicals of mitomycin c through reaction with nitroxide.[21]

The findings of this study confirmed that in ethanol treated rats, the generation of gastric mucosal leukotriene B_4 and leukotriene C_4 increases, suggesting that they participate in some fashion in the pathogenesis of the damage.[44] TEMPOL effectively prevented gastric mucosal damage (Figs 1 and 2) and attenuated the increase in leukotriene B_4 and leukotriene C_4 generation induced by ethanol (Table III). Yet, gastric mucosal generation of leukotrienes in rats treated with indomethacin or aspirin, did not change in the presence of nitroxide (Table III). These findings suggest that leukotrienes and platelet activating factor do not contribute toward gastric mucosal injury but are formed after damage induction after free radical generation. This may partly explain why specific receptor antagonists or synthesis blockers of leukotrienes do not provide total protection.

In conclusion, our results suggest that TEMPOL prevents gastric mucosal injury by breaking some chain reaction through a termination reaction with free radicals. Thus, the strategy of using selective radical-radical reactions to block biological damage, seems applicable also for the prevention of gastric injury. While the part played by free radicals in the mediation of gastric mucosal injury is well established and the potential of nitroxides for intervention is evidenced, further research is still needed to select better nitroxides and optimise their protective effect.

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