Br. J. exp. Path (1986) 67, 149–155

A molecular basis of peptic ulceration due to diet

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Received for publication 1 April 1985
Accepted for publication 4 September 1985

Summary. Fresh rice oil protects against gastric ulceration in rats maintained on an impoverished diet, whereas stored oil is ulcerogenic. Rice oil contains ketoaldehydes which are ulcerogenic but their activity is prevented by the presence of antioxidants such as α-tocopherol, which is lost on storage. Protection may also be restored by the addition of cysteine. These results in rats *in vivo* can be duplicated in a rat liver microsomal system *in vitro*, in which malondialdehyde production is a measure of toxicity. It is proposed that the ulcerogenic activity of rice oil is the direct consequence of the stimulation of endogenous lipid peroxidation due to the lowering of the GSH content in the endoplasmic reticulum by the ketoaldehydes in stored rice oil. A similar mechanism is suggested for the ulcerogenic activity of an impoverished diet which directly lowers the tissue levels of GSH.

Keywords: peptic ulceration, antioxidant, lipid peroxidation, diet

Epidemiological evidence implicates dietary factors in the geographical distribution of duodenal ulceration among people on impoverished diets (Cleave 1962; Cleave 1974; Tovey & Tunstall 1975; Tovey 1979), particularly when polished rice forms the main component. In the pyloric-ligated rat model (Shay *et al.* 1948) an association was demonstrated between gastric ulceration in rats pre-fed with diets corresponding to those areas of India where high and low incidence of human duodenal ulceration are observed (Jayaraj *et al.* 1980). In a study in India, people suffering from duodenal ulceration derived symptom relief by supplementation of their diet with fresh rice bran (Tovey 1972; Malhotra 1978). This observation was confirmed in the rat model in which fresh rice bran and rice bran oil were found to be protective against ulceration. The protective properties of rice bran and rice oil were lost on storage, when they became actively ulcerogenic. It was considered possible that on storage lipid peroxidation of unsaturated fatty acids in the oil had given rise to the production of cytotoxic ketoaldehydes. Support for this proposal was obtained by the demonstration that cysteine suppressed the ulceration induced by stored rice oil. The effect of diet in inducing, or protecing against, ulceration in both humans and the animal model, implies the presence of protective factors in some diets and ulcerogenic compounds in others. This led to the present

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study into the nature of the ulcerogenic and protective substances in rice oil.

Materials and methods

Chemicals. 4-Hydroxy-2,3-transnonenal (HNE), dissolved in chloroform, was a gift from Professor H. Esterbauer.

L-cysteine, α-tocopherol acetate and thiobarbituric acid were bought from Sigma. Malondialdehyde bisdiethylacetal was a gift from Professor T.F. Slater.

Rice oil was prepared as required by the following procedure: 200 g brown rice was boiled with 1 200 ml of distilled water for 40 min. When the mixture was cool, an equal volume of absolute alcohol was added and the mixture was stored for 30 min with occasional stirring. The supernatant was poured off and retained. The slurry was extracted with an equal volume of 30/40 petroleum ether and the petroleum ether retained. The original alcohol extract was extracted with an equal volume of 30/40 petroleum ether and this extract was combined with the first ether extract. The pooled extracts were then allowed to evaporate at room temperature.

The lipid fraction from 'horse gram' (Dolichos biflorus) was prepared as previously described (Jayaraj *et al.* 1980).

Pylorus-ligated rat model. A female Wistar rat (180–200 g) was starved overnight with water *ad libitum*, and the pylorus ligated under ether anaesthesia. The stomach was washed out with 4 ml or more saline until clean and 2 ml of saline was left in the stomach. The rat was allowed water and at 6 h was killed and the stomach, with its contents, removed. The stomach was opened and the number of macroscopic ulcers in the rumen and mucosa were recorded. For purposes of comparison an 'ulcer score' was obtained by dividing the total number of ulcers by the number of rats in a group.

The administration of rice oil, HNE, α-tocopherol and cysteine in the rat model was carried out as follows: 0.1 ml of rice oil, with or without the addition of cysteine or α-tocopherol, was injected by needle directly into the lumen of the pylorus-ligated stomach. Chloroform was evaporated from an appropriate quantity of HNE solution. The residue was dissolved in liquid paraffin and 0.1 ml of the solution was administered by direct injection into the lumen. In some cases, rats were treated orally with α-tocopherol dissolved in liquid paraffin 72 h before ligation of the pylorus. The quantities of agents used are indicated in Table 2.

Lipid peroxidation assay. Incubation mixtures containing rat liver microsomes were prepared as previously described (Slater & Sawyer 1971). Briefly, for each experiment a 10% homogenate in 0.25 M sucrose was prepared from the liver of one rat. 6.0 ml post-mitochondrial supernatant was mixed with 32.4 ml standard stock solution containing 83.5 mM KCl 5 mM glucose 6-phosphate, 8.4 iu glucose 6-phosphatase 0.245 mM $NADP^+$ 10 mM acetamide, 37.2 mM Tris-HCl pH 8.0. This mixture was pipetted in 2.5 ml aliquots into 20 ml screw-cap glass vessels to which various agents had been added. All incubations were performed in triplicate. The quantities of agents used are indicated in Table 3. Lipid peroxidation was determined by measuring malondialdehyde production in the incubation mixture. After incubating the sample for 90 min an equal volume (2.5 ml) 10% trichloroacetic acid was added. The precipitate was removed by centrifugation and 2.0 ml of the supernatant was mixed with 2.0 ml 0.67% w/v thiobarbituric acid (Sigma). The assay sample was boiled for 10 min, cooled, and read at 532 nm in a Unicam SP8–400 dual beam spectrophotometer. The blank consisted of the same microsomal mixture precipitated with trichloroacetic acid before the start of the incubation. The malondialdehyde concentration was determined from a standard curve of malonaldehyde bisdiethylacetal in non-incubated microsomal mixture.

HPLC analysis of rice oil. Rice oil 0.1 ml was mixed with 5 ml of a 0.048% 2,4-dinitrophenyl hydrazine in $CHCl_3$:CH_3COOH 9:1 v/v and left at room temperature for 3.5 h. The sample was evaporated to dryness in a rotary evaporator and redissolved in 10 ml methanol (HPLC grade). 20 μl of this solution was injected onto a partisil ODS reverse phase HPLC column. The sample was eluted with methanol/H_2O mobile phase 80/20. Peaks of absorbance were monitored at 378 nm.

Results

HPLC analysis of fresh and stored rice oil

Preliminary observations suggested the possibility that ketoaldehydes may be the ulcerogens in rice oil. To determine whether such compounds were actually present in stored rice oil, 2,4-dinitrophenyl hydrazine was added to the oil and the coloured hydrozones separated by HPLC, as described in the materials and methods section. This analysis revealed that the derivatized oil contained 10 distinct compounds including one major component with a shorter retention time than that of the hydrozone of HNE. A similar analysis performed on fresh oil showed, surprisingly, no obvious difference between the HPLC profiles. Thus, storage had not significantly increased the level or range of ketoaldehydes but, as they are present in both fresh and stored oil, it is possible that a protective factor may have been inactivated on storage.

The protective effects of α-tocopherol and cysteine against rice oil-induced ulceration

A lipid-soluble natural product with cytoprotective properties is α-tocopherol which, in addition, is destroyed on storage in the light. The effect of α-tocopherol on the ulcerogenic activity of stored rice oil was investigated and it proved protective both when administered simultaneously with, or 72 h before, the rice oil in doses as low as 40 μg per stomach. The results of these experiments, together with previously published data for comparison, are shown in Table 1.

Induction of ulceration by HNE

Previous studies in the rat (Benedetti *et al.* 1980) have shown that 4-hydroxy-2, 3-transnonenal is the most cytotoxic aldehydic product of lipid peroxidation. It was therefore

Table 1. Gastric ulceration induced by stored rice oil and protection from ulceration by α-tocopherol and cysteine

Experiment No.	Treatment	No. of Rats	Dose	No. of ulcers–group		Ulcer Score
				Rumenal	Mucosal	
1	Nil	11	—	22	8	2.7
	α-tocopherol	2	10 mg/kg	0	0	0
	Stored rice oil	2	0.1 ml	175	5	90.0
	Stored rice oil +α-tocopherol*	4	0.1 ml 10 mg/kg	0	0	0
	Stored rice oil +α-tocopherol†	4	0.1 ml 10 mg/kg	0	0	0
2	Stored rice oil	2	0.1 ml	118	3	60.5
	Stored rice oil +cysteine	2	0.1 ml 1 mg	2	0	0.7

* Simultaneous addition.
† Administered 72 hours before stored rice oil.

considered of interest to employ this chemically-defined compound in the rat model. The results given in Table 2 show that HNE is extremely ulcerogenic, producing widespread ulceration of the gastric rumen and mucosa. As with stored rice oil, protection was achieved with cysteine and α-tocopherol. Since the lipid fraction of horse gram has been shown to give protection in animal experiments (Jayaraj *et al.* 1985) the results of its action against HNE are included in Table 2.

Does stored rice oil induce lipid peroxidation?

Antioxidants such as α-tocopherol are free radical scavengers, which help to prevent or terminate chain reactions such as lipid peroxidation. They would not, therefore, be expected to protect against the cytotoxic

Table 2. The effect of various treatments on HNE-induced rumenal and mucosal ulceration

Experiment No.	Treatment	No. of Rats	Dose	No. of ulcers–group		Ulcer Score
				Rumenal	Mucosal	
1	HNE	2	0.26 μmoles	37	5	21.0
	HNE +cysteine	2	0.26 μmoles 2.6 μmoles	1	2	1.5
	HNE +cysteine	2	0.26 μmoles 5.2 μmoles	0	1	0.5
2	HNE	2	0.26 μmoles	20	6	13.0
	HNE+ α-tocopherol	2	0.26 μmoles 10 mg/kg BW	0	0	0
	HNE+ horse gram lipid	2	0.26 μmoles 0.1 ml	0	0	0

Table 3. The effect of various additions upon lipid peroxidation in rat liver microsomes *in vitro*

Experiment No.	Addition	Dose	Malondialdehyde Production nmoles/ml
1	Nil	—	0.25
	CCl_4	5 μl	6.33
	HNE	500 μg	3.90
	Fresh rice oil	5 μl	0.33
	*Stored rice oil	5 μl	3.98
	*Stored rice oil + α-tocopherol	5 μl 664 μg	0.86
2	Nil	—	0.62
	CCl_4	5 μl	5.45
	Stored rice oil	5 μl	5.58
	Stored rice oil +cysteine	5 μl 500 μg	1.72

* Ulcerogenic in pylorus-ligated rats.

action of those products of lipid peroxidation which are already present in rice oil. The protective action of α-tocopherol in the rat stomach model nevertheless suggests that lipid peroxidation is an essential step in the ulcerogenic action of ketoaldehydes. It has been shown that HNE stimulates lipid peroxidation and that this stimulation is blocked by α-tocopherol in a rat liver microsomal system, (White & Rees 1984). Using the same system, we examined the effects of ulcerogenic rice oil and α-tocopherol on lipid peroxidation and found that stored rice oil stimulated lipid peroxidation, and this effect is reversed by the addition of α-tocopherol or cysteine (Table 3).

Discussion

The results of the present investigation reveal that ulcerogenic agents are present in both fresh and stored rice oil. The ability of ulcerogenic oil and HNE to stimulate lipid peroxidation *in vitro* and its reversal by cysteine and α-tocopherol, suggest that lipid peroxidation is implicated in the events leading to gastric ulceration in the pylorus-ligated rat model.

It is accepted that peptic ulcers occur only in the presence of acid and pepsin (Wormsley 1979), but it might be expected that erosion would proceed more rapidly should cells be destabilized by lipid peroxidation.

It has been suggested previously that the cytotoxicity of HNE might be mediated by a peroxidative mechanism (White & Rees 1984). Ketoaldehydes are electrophilic compounds which react readily with intracellular -SH groups. Glutathione (GSH), which is a major cytosolic constituent involved in many metabolic processes, acts as an antioxidant by interaction with lipid hydroperoxides (Lawrence & Burk 1976; Burk *et al.* 1978). Ketoaldehydes, which enter cells readily and lower to GSH concentration, would be expected to increase the level of endogenous lipid peroxidation. Since lipid peroxidation results in further ketoaldehyde production the process accelerates, eventually destroying cell membranes. It is proposed that this mechanism may underly the ulceration seen in the experimental model. This pattern of activity is summarized in Fig. 1. No other aldehyde was tested in the rat model. It would be of interest to observe the effect of an unsaturated aldehyde without the hydroxy group, such as nonenal.

Endogenous lipid peroxidation is not nor-

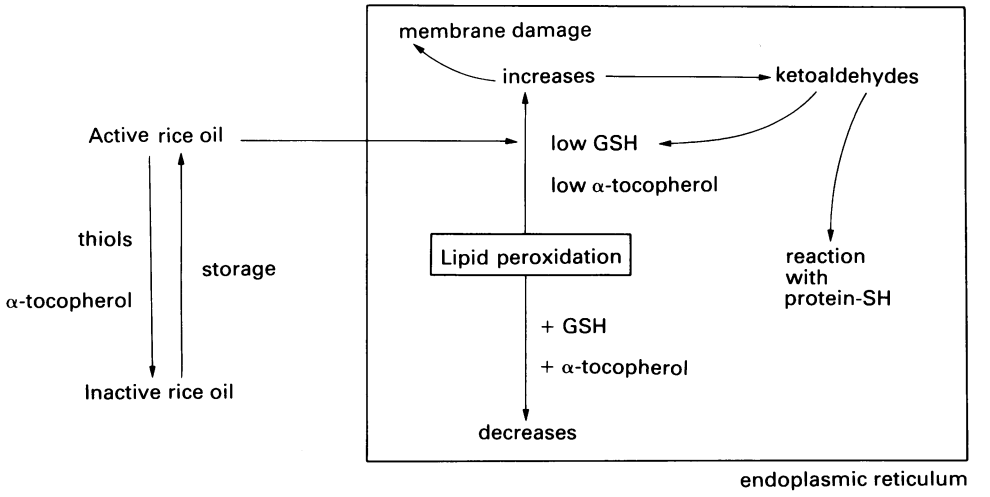

Fig. 1. A molecular basis of peptic ulceration due to diet. Diagrammatic representation of events leading to peroxidative damage in cells exposed to toxic rice oil.

mally damaging to cells because it is controlled by antioxidants such as α-tocopherol and GSH. The levels of antioxidants in tissues are determined by the diet. Protein deprivation produces a substantial depletion of GSH in rats (Edwards & Westerfield 1952), which has been found to be associated with the onset of necrotic lesions (Lindan & Work 1953). It is possible that ulceration in rats fed a low-protein diet results from lowered levels of GSH and antioxidants.

The Shay model of rat gastric ulceration is an accepted model for the study of human peptic ulceration (Lee & Bianchi 1971). Circumstantial evidence, based on the present studies and observations in the field, implicates dietary factors in the aetiology of human duodenal ulceration. The association of a human diet composed almost entirely of polished rice with duodenal ulceration (Tovey 1972; Malhotra 1978) may be explained by the observations reported here. These findings also offer an alternative explanation for the observations of Szabo *et al.* (1981), who reported that alcohol-induced ulceration in the rat stomach model was prevented by -SH compounds and potentiated by compounds interacting with -SH groups which depleted GSH. They concluded that -SH played a role in potentiating prostaglandin-induced gastric cytoprotection. Alcohol is oxidized to acetaldehyde, which would interact with -SH groups thus lowering the GSH concentration with a commensurate increase in lipid peroxidation. This would produce a situation analogous to that of dietary ulceration described in the present study.

Not all plant oils have the high toxicity of rice oil, yet they all contain a substantial proportion of polyunsaturated fatty acids. Other oils may possess higher concentrations of antioxidants, or cytoprotection may be achieved by other mechanisms; these possibilities are currently under investigation.

Acknowledgements

We would like to thank the AICR for the provision of running expenses to Dr J.S. White, and funding from the Surgical Unit, University College Hospital, which permitted this work to be carried out. We would also like to thank Professor C.G. Clark and Professor P.A. Riley for helpful discussions.

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