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Ulcer-protecting Effects of Naringenin on Gastric Lesions Induced by Ethanol in Rat: Role of Endogenous Prostaglandins

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Abstract—This study was designed to determine the cytoprotective properties of naringenin and the involvement of endogenous prostaglandins on mucosal injury produced by absolute ethanol. Gastric glands were also histologically analysed. Oral pretreatment with the highest dose of naringenin (200 mg kg^{-1}), 240 min before absolute ethanol, was the most effective in ulcer prevention. Subcutaneous administration of indomethacin (10 mg kg^{-1}) to the animals treated with naringenin (200 mg kg^{-1}) partially inhibited the gastric protection but there was no increase in prostaglandin E_2. All treated groups showed a marked increase in gastric mucus, although this increase was less in animals pretreated with indomethacin. Total proteins and hexosamine content decreased in the groups receiving indomethacin. Histomorphometric evaluation of the gastric damage, with the highest dose of naringenin (200 mg kg^{-1}), confirmed a significant increase of mucus production accompanied by a parallel reduction of gastric lesion.

Several flavonoids prevent gastric mucosal lesions produced by various methods of experimental ulcer and protect the gastric mucosa against various necrotic agents, and have been shown to increase the mucosal content of prostaglandin (Parmar & Ghosh 1981; Konturek et al 1986a, b; Alcaraz & Tordera 1988).

Naringenin, a naturally-occurring flavone, has also been reported to prevent gastric mucosal ulceration in animal models including restraint stress, pyloric occlusion or acetic acid-induced chronic ulcer (Parmar 1983; Motilva et al 1992; Martín et al 1993).

This study was designed to determine the protective properties of naringenin in mucosal injury produced by absolute ethanol. The morphology of the lesions and their modifications were evaluated and the role of endogenous prostaglandins was determined.

Materials and Methods

Animal groups and drug preparation

Male Wistar rats, 180–200 g, were placed in single cages which had wire-net floors to prevent coprophagy. They were fed a normal laboratory diet and given tap water to drink. The animals were deprived of food for 48 h before the experiments but had free access to water. The temperature was maintained at 22–24°C and humidity at 70–75% in a controlled room.

Naringenin (Sigma Chemical Co., USA) was dissolved in distilled water. Drug was prepared freshly each time and administered orally. Indomethacin (Sigma Chemical Co., USA), a cyclo-oxygenase inhibitor, was given subcutaneously. Control rats received distilled water orally in a comparable volume (1 mL/100 g).

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Protection against absolute ethanol. Biochemical study of gastric mucus

Ulceration was induced as described by Robert (1979) by instillation of 1 mL absolute ethanol. The drugs were administered in different doses to different groups of animals and at various intervals before the oral administration of absolute ethanol. One hour after the experimental period, the animals were killed by a sharp blow on the head and their stomachs removed and opened along the greater curvature. Any lesions were examined macroscopically. The number of erosions per stomach was assessed for severity according to our scoring system: 0—no lesions; 1—one or more haemorrhagic ulcers length < 2 mm; 2—one haemorrhagic ulcer length < 5 mm and thin; 3—more than one ulcer grade 2; 4—one ulcer length < 5 mm and wide; 5—two or three ulcers of grade 4; 6— from four to five ulcers of grade 4; 7—more than six ulcers of grade 4; 8—complete lesion of the mucosa. Mean scores for each group were calculated and expressed as the ulcer index.

The gastric mucus was obtained by scraping the mucosa with a glass slide and was immediately homogenized in 4 mL of weighed distilled water. The weight of mucus (mg) was the difference between the weight of homogenate and that of the original 4 mL water. Colorimetry was used to determine total proteins (Lowry et al 1951) and hexosamines (Boas 1953).

The duration of the protective effect against ethanol-induced mucosal damage was examined by giving naringenin (200 mg kg^{-1}) at various intervals (30, 120, 240 and 360 min) before the oral administration of absolute ethanol. At the optimum treatment time found (240 min), the effects of new, lower doses of naringenin (50 and 100 mg kg^{-1}) were investigated.

To study the effect of inhibition of endogenous prostaglandin synthesis by indomethacin, independent groups of rats were employed. Indomethacin was administered subcutaneously (10 mg kg^{-1}), followed 75 min later by water or naringenin (200 mg kg^{-1}) and after a further 30, 120, 240 and 360 min, by ethanol.

Measurement of prostaglandin E_2 (PGE_2) in gastric mucosa

Three groups of six rats were used: control 1 (distilled water); control 2 (water and absolute ethanol, 240 min later); naringenin (200 mg kg^{-1}) followed 240 min later by absolute ethanol. After the rats were killed their stomachs were removed, opened along the greater curvature and rinsed with 0·9% NaCl (saline). Gastric mucosa was minced with a scalpel and homogenized in 4 mL triethylammonium formate buffer (TEAP) 0·04 M, pH 3·15 with 15% ethanol in cold medium. The homogenate was centrifuged (18 000 *g*, 10 min, 4°C). The supernatant was removed and kept at 4°C until assayed for PGE_2.

The method described by Navarro et al (1988) was used for the analytical determination of prostanoids. Briefly, the supernatant was passed through a reversed-phase octadecylsilica C18 Sep Pak cartridge which was washed with 0·05 M TEAP pH 3·15 (10 mL) and petroleum ether (10 mL). The final elution was accomplished with 5 mL methyl formate. The eluate from the last fraction was collected in polypropylene tubes and maintained at −40°C until analysis. Each fraction was evaporated and dissolved in 100 mM Tris-HCl buffer, pH 7·4. PGE_2 was determined in two 200 μL samples by RIA, using a Kit (Eria, Diagnostic Pasteur, France) and ^3H as tracer.

Histological evaluation of gastric glands

Three groups of five rats were used: control 1 (distilled water); control 2 (water and absolute ethanol later); naringenin (200 mg kg^{-1}) followed 240 min later by absolute ethanol. The method described by Escolar et al (1987) was followed. The rat was killed, the stomach was exposed and an incision made in the duodenum. A polythene cannula was placed into the stomach via the oesophagus and the gastric lumen was washed with 10–15 mL saline. To maintain a similar intragastric pressure in all animals, the same amount of Carnoy fixative (10% acetic acid, 30% chloroform, 60% absolute ethanol) was perfused through a cannula inside the gastric cavity. After the solution had emptied through the duodenal incision, the oesophagus and duodenum were ligated. Fifteen minutes later, the stomach was removed and placed in the same fixative solution. Samples from the glandular portion were dehydrated with absolute ethanol and embedded in paraffin. Two series of sections (5 μm thick, separated by at least 1 mm), were made by cutting the block in a plane perpendicular to the mucosal surface. Sections were stained with haematoxylin/eosin, periodic acid Schiff (PAS) and alcyan blue (pH 0·5). For the PAS and alcyan blue methods, only gastric glands from macroscopically intact mucosa were used.

A Video Imagine Analyzer CCD Camera Mintron coupled to an Olympus microscope was used for the morphometric study.

Gastric lesions were examined in haematoxylin/eosin stained sections (5 μm thick) and quantified according to the scale proposed by Lacy & Ito (1982). For each rat, the mean length of gastric mucosa examined (mm), the percentage of damaged mucosal length, and the percentage of the mucosal length for each degree (depth) of damage were tabulated.

Cross-sectional areas of the portion of the gland stained red by PAS were measured to give the total glycoprotein present in the gastric pits.

Cross-sectional areas of the gastric gland stained blue by the alcyan blue method were measured to give the sulphated macromolecular content in the gastric pits.

In the latter two assays, the mean length of gastric mucosa examined from each stomach (mm) and the areas stained by PAS and alcyan blue were expressed as $\mu m^2/10\ \mu m$ of mucosa length examined.

Statistical analysis

Values are given as arithmetic means ± s.e.m. The significance of differences between means was evaluated by Student's *t*-test for unpaired data.

Results

Table 1 shows the protective effects of naringenin on ethanol-induced lesion. The treated groups showed a marked increase in the concentration of total proteins compared with control.

In this experimental model, oral pretreatment with naringenin at different times, prevented ulceration (Fig. 1). Subcutaneous administration of indomethacin (10 mg kg^{-1}) to the animals treated with naringenin partially inhibited gastric protection. The intensity of mucosal lesions increased and total proteins and hexosamines content were less in these groups at different intervals.

Table 2 shows that ethanol treatment increased the levels of PGE_2 present in the mucus. The amount of PGE_2 in the group of animals treated with naringenin was augmented

Table 1. Inhibition by naringenin of ethanol-induced gastric mucosal damage. Naringenin was administered at the doses shown 240 min before ethanol and animals were killed 60 min after receiving ethanol.

Naringenin (mg kg^{-1})	Inhibition (%)	Protein (%) (mg (mg mucus)$^{-1}$)	Hexasamine (%) (μg (mg mucus)$^{-1}$)
50	15·4 ± 1·2	39·5 ± 4·2	51·9 ± 4·8
100	17·0 ± 1·0	52·1 ± 6·6	100·4 ± 13·7
200	82·2 ± 7·5	113·9 ± 12·4	116·1 ± 20·8

Table 2. PGE_2 in gastric mucosa following induction of ulcers by absolute ethanol. Naringenin (200 mg kg^{-1}) was administered 240 min before ethanol.

Treatment	PGE_2 (pg)	Mucus (mg)	PGE_2 (pg (mg mucus)$^{-1}$)
Control 1 (distilled water)	1062 ± 252	85 ± 2·4	9·55 ± 0·9
Control 2 (100% ethanol alone)	2271 ± 150**	363 ± 58**	7·06 ± 0·7
Naringenin (200 mg kg^{-1})	1675 ± 293**	410 ± 54**	4·83 ± 0·5**

** $P < 0·01$ compared with control 1.

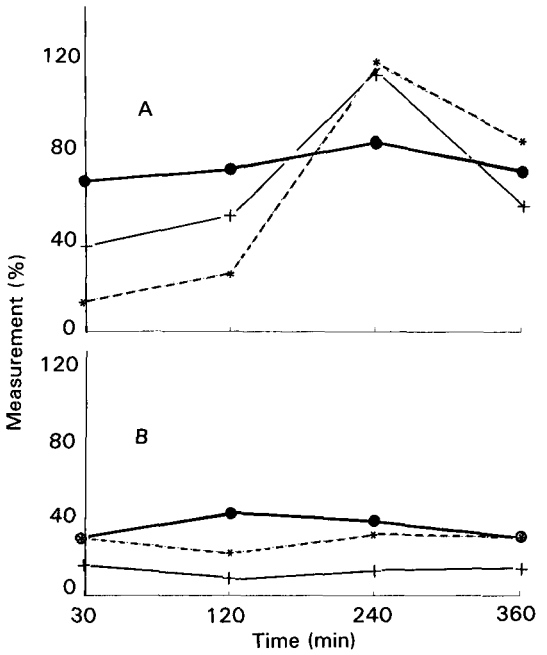

FIG. 1. Inhibition by naringenin (%) of gastric mucosa damage induced by ethanol (●——●). Also shown are the percentage protein (* - - *) and hexosamine (+——+) content of the mucosa. A. Naringenin (200 mg kg^{-1}). B. Indomethacin (10 mg kg^{-1}, s.c.) 75 min before naringenin (200 mg kg^{-1}).

compared with control 1; however, there was no increase compared with the ethanol treatment group. The increase observed in the total amount of PGE_2 between control 2 or the naringenin groups and control 1 disappeared when the values of PGE_2 were expressed as wet weight of mucous material (pg (mg mucus)$^{-1}$).

Table 3 shows the histomorphometric evaluation of the mucosal damage induced by absolute ethanol. The treated groups showed a significant reduction in the percentage of damaged mucosa ($P<0.01$) compared with the animals receiving only absolute ethanol. In the latter there was obvious damage in all the mucosal surfaces. Histologically, there was extensive cell damage in the upper third of the gastric mucosa (degree 3, 77·9±7·6). This damage was considerably less following administration of naringenin (19·6±2·5). The percentages of mucosa with grade 1 damage were high in the treated group (37·7±1·5) and notably higher than those for grade 2 (19·7±0·4).

Treatment-induced modifications to the morphometric parameters of glandular glycoprotein content are shown in Table 4. The administration of absolute ethanol increased both the PAS area (neutral glycoproteins) and the alcyan blue area (sulphated glycoproteins). Naringenin (200 mg kg^{-1}) 240 min before absolute ethanol, increased both parameters significantly with respect to control group 1 (distilled water) and control group 2 (water and ethanol).

Discussion

The present study confirms the protective effect of naringenin, a naturally occurring flavone. It significantly reduced the ulcer index and increased both the amount of mucus and its glycoprotein content. Enhanced total proteins, hexosamines, neutral glycoproteins (PAS area) and sulphated macromolecules (alcyan blue area) were found. The possible role of prostaglandin in naringenin-induced gastroprotection was shown by our finding that inhibition of prostaglandin biosynthesis by pretreatment with indomethacin reversed, in part, the protection afforded by naringenin. This study confirms that this flavonoid, in this experimental model and at the dose of 200 mg kg^{-1}, does not enhance PGE_2 levels but does prevent the gastric necrosis induced by absolute ethanol. There is increasing evidence that the beneficial effects of many mucosal-strengthening drugs occur through mechanisms other than the prostaglandin-mediated (Glavin & Szabo 1992) including endogenous glutathione production (Szabo et al 1981), polyamines (Mozsik & Javor 1988), and the more recently characterized gastroprotectants including endothelium-derived relaxing factor (nitric oxide) (McNaughton et al 1989) and dopamine (Glavin & Szabo

Table 3. Morphometric evaluation of gastric lesions in haematoxylin/eosin sections. Naringenin (200 mg kg^{-1}) was given 240 min before absolute ethanol.

Treatment	Length gastric mucosa examined (mm)	Total length mucosa damage (%)	Length damage scale (%)		
			1	2	3
Control 1 (distilled water)	5·0±0·4	0	—	—	—
Control 2 (100% ethanol)	4·9±0·5	100±0·0	6·3±0·9	11·4±0·3	77·9±7·6
Naringenin (200 mg kg^{-1})	5·0±0·4	76·8±9·2**	37·7±1·5	19·7±0·4	19·6±2·5

** $P<0.01$ compared with control 2.

Table 4. Morphometric evaluation of gastric glands in periodic acid Schiff (PAS)- and alcyan blue-stained sections. Naringenin (200 mg kg^{-1}) was given 240 min before absolute ethanol.

Treatment	Total length (mm)	PAS (μm^2)	Alcyan blue (μm^2)
Control 1 (distilled water)	1·93±0·4	262·2±30·7	23·5±4·1
Control (100% ethanol alone)	1·81±0·3	322·9±14·3	42·2±5·0*
Naringenin (200 mg kg^{-1})	1·84±0·8	528·3±39·3**‡	56·3±5·4**†

* $P<0.05$, ** $P<0.01$ compared with control 1. † $P<0.05$, ‡ $P<0.01$ compared with control 2. The stained area is per 10 μm mucosal length examined.

1990). Furthermore, since vascular changes in gastric mucosa appear to be the most pronounced feature of absolute ethanol injury, it is possible that maintenance of mucosal vasculature and normal blood flow may be the major mechanism of cytoprotection. Prostaglandins appear to preserve microvascular integrity and to reduce the underlying vasocongestion. However, not all eicosanoids exert a protective effect on the gastric mucosa. Leukotrienes, potent vasoconstrictors, are generated by the gastric mucosa, and have been identified in the gastric mucosa, particularly after exposure to ethanol damage (Peskar et al 1986). Leukotrienes exert various biological actions, beside the vasoconstrictor effect, that could contribute to their role as mediators of ischaemic and tissue damage. Recent reports showed that inhibition of leukotriene synthesis was accompanied by a decrease in gastric mucosal damage in different experimental models including ethanol-induced gastric ulcers (Konturek et al 1988; Osada et al 1990). Flavonoids can interfere with the production of arachidonic acid metabolites, through lipoxygenase enzyme inhibition, and reduce the concentration of leukotrienes in different biological systems (Landolfi et al 1984; Robak & Gryglewski 1988; Ferrandiz et al 1990). The gastroprotection exerted by naringenin could be through regulation of release of vasoactive substances such as leukotrienes.

In addition, free radicals may be involved in the pathogenesis of acute gastric mucosal injury including lesion formation induced by ethanol (Salim 1990; Glavin & Szabo 1992). Naringenin has been found to be a free radical scavenger (Baumann et al 1980) and showed a dose-dependent inhibition of lipid peroxidation (Younes & Sregers 1981). Thus, scavenging these radicals could stimulate the healing process for this injury.

In conclusion, data presented here confirm that naringenin exerts protective mucosal activity in this experimental model. The impression that this could be partly explained through a complex non-prostaglandin-dependent mechanism, involving free radical scavenging properties and inhibition of lipid peroxidation and enhancement of physicochemical properties of mucus gel and its constituents, is reinforced.

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