

## The effects of acute and chronic administration of *n*-6 and *n*-3 polyunsaturated fatty acids on ethanol-induced gastric haemorrhage in rats

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Female weanling rats in three equal groups (*n* 12) were given orally by intubation 1 ml micellar solution of taurocholic acid (10 mM) and either arachidonic acid (20:4 *n*-6), linoleic acid (18:2 *n*-6) or eicosapentaenoic acid (20:5 *n*-3) at a concentration of 120 mM. After 1 h the rats were given intragastrically 2 ml absolute ethanol and were killed 1 h later. Rats given oral 20:4 *n*-6 showed a significant reduction ( $P < 0.05$ ) in the extent (%) of gastric mucosal haemorrhage compared with either the rats given 20:5 *n*-3 or 18:2 *n*-6 (8.3 (SD 7.3), 23.2 (SD 10.4) and 21.4 (SD 10.4) respectively). In a second experiment, four equal groups (*n* 12) of female Wistar rats were fed for 5 weeks on either a control diet of standard laboratory rat food, or the same diet enriched with either maize oil or fish oil or butterfat at a level of 100 g/kg. Following a 24 h fast the rats received an intragastric dose of 2 ml ethanol and were killed 1 h later. Examination of the extent (%) of gastric lesion showed a significant reduction ( $P < 0.05$ ) with the feeding of either maize oil or fish oil compared with the controls (12.2 (SD 8.2), 15.3 (SD 13.2) and 29.3 (SD 14.0) respectively). The butterfat diet was not significantly different from the control diet (23.8 (SD 8.1)).

**Arachidonic acid: Fish oil: Gastric haemorrhage**

There is as yet no readily available animal model for human gastric or duodenal ulcer. In the absence of such, a number of noxious agents have been used to induce gastric mucosal injury in rats including absolute ethanol, 0.6 M-hydrochloric acid, 0.2 M-sodium hydroxide, sodium chloride (250 g/l) and aspirin (Robert *et al.* 1979). The potential of exogenously administered prostaglandins to exert a cytoprotective effect against these and other agents and procedures has also been widely studied (Robert *et al.* 1979; Hollander *et al.* 1982; Tarnawski *et al.* 1988). This cytoprotective effect is not mediated by inhibition of gastric HCl secretion since it operates at levels below the anti-secretory effect of prostaglandins and since neither antacids nor anti-secretory compounds are effective as cytoprotectors (Miller & Jacobson, 1979). These authors have also shown that even those prostaglandins without an action on acid secretion have cytoprotective properties. The most popular mechanisms proposed for these cytoprotective prostaglandins centre on the increased secretion of both mucus and bicarbonate, on the maintenance of blood flow and on the stimulation of the sodium pump in gastric mucosal cells (Robert 1979; Tarnawski *et al.* 1988).

Since the prostaglandins are capable of mediating cytoprotection, several workers have examined the potential of the precursor fatty acid, arachidonic acid (20:4 *n*-6), in this respect. Hollander *et al.* (1982) showed that exogenous administration of 20:4 *n*-6 to rats

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stimulated the synthesis of prostaglandins, PGE<sub>2</sub> in particular, and significantly reduced ethanol-induced gastric haemorrhage in rats. Exogenously administered linoleic acid (18:2 *n*-6) has also been reported to stimulate gastric prostaglandin PGE<sub>2</sub> production (Grant *et al.* 1988) and to protect against ethanol injury of rat gastric mucosa (Tarnawski *et al.* 1985).

Surprisingly little attention has been directed towards the longer-term effects of dietary oils rich in the *n*-6 polyunsaturated fatty acids (PUFA) on rat gastric cytoprotection. Given the opposing effects of oils rich in either the *n*-6 or *n*-3 PUFA on the development of circulatory disease (Bolton-Smith *et al.* 1988), the potential of the latter (fish oil) to exert an effect, via blood flow changes in mesenteric microvessels, requires attention. The present study set out to examine the relative cytoprotective effects of the *n*-6 PUFA 20:4 *n*-6 and 18:2 *n*-6 and the main *n*-3 PUFA eicosapentaenoic acid (20:5 *n*-3), acutely administered, in protecting against ethanol-induced gastric injury in rats. The study also compared the effects of chronic feeding of the *n*-6-rich PUFA maize oil and the *n*-3 PUFA fish oil with both a low-fat control diet and with a high-fat diet rich in saturated fatty acids (butterfat) in this model.

#### MATERIALS AND METHODS

Weanling female Wistar rats (50–100 g) were used for the study. All animals were maintained on a standard laboratory diet before the start of the experiment. For the chronic study four groups of twelve rats each were fed for 5 weeks on a standard laboratory rat diet (Red Mills, Kilkenny) enriched with 100 g/kg of either fish oil (Maxepa; Seven Seas Ltd, Hull), maize oil or butterfat. The oils were dissolved in diethyl ether and dispersed evenly over the feed pellets which were subsequently dried under nitrogen. The fourth group of rats was given the low-fat control diet consisting of the standard laboratory diet similarly treated with fat-free diethyl ether. All diets were offered *ad lib.* for 5 weeks and the diets presented fresh each day from frozen portions to minimize oxidative damage (Bolton-Smith *et al.* 1988). In the acute study the three groups of twelve rats were maintained on the standard laboratory rat diet for 5 weeks. At 1 h before the intubation with ethanol to induce the gastric haemorrhage, the rats in the acute study were given orally 1 ml of a micellar solution containing 10 mM-taurocholic acid and 120 mM-eicosapentaenoic acid (20:5 *n*-3), -linoleic acid (18:2 *n*-6) or -arachidonic acid (20:4 *n*-6). The latter two fatty acids were obtained from Sigma (St Louis, USA) while the former was prepared from an ethyl ester of 20:5 *n*-3 (Mochida, Tokyo, Japan) by hydrolysis under ethanolic potassium hydroxide (50 g/l) for 12 min at 65° under N<sub>2</sub> in the dark. The saponified material was extracted in water followed by chloroform-methanol (2:1, v/v). The material was identified as pure free fatty acid by thin-layer chromatography and the purity of the 20:5 *n*-3 confirmed by gas-liquid chromatography. The ethanol-induced gastric haemorrhage was achieved in both the chronic and acute study by an oral dose of 2 ml absolute ethanol. The gastric haemorrhage was induced following a 24 h fast in both the chronic and the acute study and 1 h post intubation with fatty acids in the chronic study. Three control groups (*n* 4) were orally dosed with the fatty acids and 1 h later with 2 ml normal saline (9 g NaCl/l) to assess possible uptake of fatty acids by the gastric mucosal cells and to serve as an ethanol control.

At 1 h after the ethanol or saline dose the animals were anaesthetized, killed by cardiac puncture and their stomach removed for gross examination of the area for haemorrhagic lesions and for microscopic evaluation of histological changes. The stomach was opened along the line of greater curvature and thoroughly rinsed with normal saline. The total area of the stomach and the total area lesioned were marked on a transparent grid with 1 mm<sup>2</sup> markings which was overlaid on the stomach. The stomachs were then placed in formalin

Table 1. *Chronic effects of dietary fat source on the phospholipid fatty acid composition (mol/100 mol) of rat gastric epithelium\**

(Mean values with their pooled standard errors)

| Dietary fat ...  | Control            | Fish oil           | Maize oil         | Butterfat         | Pooled SEM | Statistical significance $P =$ |
|------------------|--------------------|--------------------|-------------------|-------------------|------------|--------------------------------|
| 16:0             | 28.3 <sup>ab</sup> | 30.5 <sup>a</sup>  | 24.2 <sup>b</sup> | 25.0 <sup>b</sup> | 1.06       | 0.004                          |
| 18:0             | 10.6 <sup>a</sup>  | 11.8 <sup>ab</sup> | 14.2 <sup>b</sup> | 10.3 <sup>a</sup> | 1.24       | 0.073                          |
| 16:1             | 1.0 <sup>a</sup>   | 1.9 <sup>b</sup>   | 0.9 <sup>a</sup>  | 1.2 <sup>a</sup>  | 0.16       | 0.003                          |
| 18:1             | 23.2 <sup>a</sup>  | 21.9 <sup>a</sup>  | 19.6 <sup>b</sup> | 29.9 <sup>c</sup> | 0.66       | 0.003                          |
| 18:2 <i>n</i> -6 | 18.0 <sup>a</sup>  | 14.9 <sup>b</sup>  | 23.1 <sup>c</sup> | 15.9 <sup>b</sup> | 0.77       | 0.000                          |
| 18:3 <i>n</i> -3 | 1.0 <sup>ab</sup>  | 1.2 <sup>a</sup>   | 0.8 <sup>b</sup>  | 0.8 <sup>b</sup>  | 1.11       | 0.021                          |
| 20:4 <i>n</i> -6 | 15.5 <sup>a</sup>  | 7.1 <sup>b</sup>   | 14.6 <sup>a</sup> | 13.1 <sup>a</sup> | 0.44       | 0.000                          |
| 20:5 <i>n</i> -3 | 0.7 <sup>a</sup>   | 6.6 <sup>b</sup>   | 0.5 <sup>a</sup>  | 1.1 <sup>a</sup>  | 0.27       | 0.000                          |
| 22:6 <i>n</i> -3 | 0.9 <sup>a</sup>   | 2.2 <sup>b</sup>   | 1.1 <sup>a</sup>  | 1.1 <sup>a</sup>  | 0.16       | 0.000                          |

<sup>a, b, c</sup> Mean values for a given fatty acid with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* For details of diets and procedures, see pp. 502–503.

for histological examination. Stomachs from the control animals which had received the saline dose were similarly treated with the exception that the epithelial cells were removed by gentle scraping for subsequent lipid analysis.

The cells were washed in saline and the lipids extracted in 2 ml chloroform–methanol (2:1; v/v) followed by 2 ml chloroform alone. All organic solvents used in the study contained butylated hydroxytoluene (0.1 g/l) as an antioxidant. Phospholipids were isolated by thin-layer chromatography using light petroleum (b.p. 40–60°)–diethyl ether–formic acid (80:20:2, by vol.). Fatty acid methyl esters were synthesized using sodium methoxide and the fatty acid composition determined by gas–liquid chromatography as previously described (Kearney *et al.* 1989).

The data were analysed by analysis of variance and, where indicated by the *F* test, significant differences between means were determined using the least significance difference test.

## RESULTS

The effects of a 5-week intake of diets containing 100 g fish oil, maize oil or butterfat/kg on the phospholipid fatty acid composition of rat gastric mucosal cells is given in Table 1 and the mean values compared with those of a low-fat control diet. The fish-oil diet significantly increased the levels of 16:1 *n*-9, 20:5 *n*-3 and 22:6 *n*-3 and significantly decreased the levels of 18:1 *n*-9, 18:2 *n*-6 and 20:4 *n*-6 ( $P < 0.05$ ). The maize-oil diet significantly increased the levels of 18:0 and 18:2 *n*-6 ( $P < 0.05$ ) and significantly reduced the levels of 18:1 *n*-9 ( $P < 0.05$ ). The butterfat diet led to a significant increase in 18:1 *n*-9 and a significant decrease in 18:2 *n*-6 ( $P < 0.05$ ). Thus, the chronic administration of a dietary fat tended to influence fatty acid composition along the lines predictable from the fatty acid composition of the fat fed.

The effects of the acute administration of 20:5 *n*-3, 20:4 *n*-6 and 18:2 *n*-6 on rat gastric mucosal cell phospholipid fatty acids are given in Table 2. There were no significant treatment effects insofar as the acute administration of a particular fatty acid did not lead to an elevation of that fatty acid in mucosal phospholipids. The significant differences otherwise noted were small, particularly in relation to those fatty acids involved in eicosanoid metabolism (20:4 *n*-6 and 20:5 *n*-3).

Table 2. *Acute effects of intragastric intubation with free eicosapentaenoic acid, arachidonic acid and linoleic acid on the fatty acid composition (mol/100 mol) of rat gastric epithelial cell phospholipids\**

(Mean values with their pooled standard errors)

| Fatty acid administered | Eicosapentaenoic acid<br>(20:5 <i>n</i> -3) | Arachidonic acid<br>(20:4 <i>n</i> -6) | Linoleic acid<br>(18:2 <i>n</i> -6) | Pooled SEM | Statistical significance:<br><i>P</i> = |
|-------------------------|---|--|-------------------------------------|------------|---|
| 16:0                    | 25.1 <sup>a</sup>                           | 27.2 <sup>b</sup>                      | 27.9 <sup>b</sup>                   | 0.64       | 0.043                                   |
| 18:0                    | 10.0 <sup>a</sup>                           | 12.5 <sup>b</sup>                      | 11.1 <sup>a</sup>                   | 0.50       | 0.023                                   |
| 16:1                    | 1.2   | 1.7                                    | 1.2                                 | 0.23       | 0.398                                   |
| 18:1                    | 23.3  | 25.7                                   | 25.1                                | 0.80       | 0.147                                   |
| 18:2 <i>n</i> -6        | 21.3 <sup>a</sup>                           | 15.2 <sup>b</sup>                      | 17.5 <sup>b</sup>                   | 0.30       | 0.000                                   |
| 18:3 <i>n</i> -3        | 0.9   | 0.8                                    | 0.7                                 | 0.97       | 0.071                                   |
| 20:4 <i>n</i> -6        | 15.7 <sup>a</sup>                           | 14.3 <sup>a</sup>                      | 12.9 <sup>b</sup>                   | 0.51       | 0.011                                   |
| 20:5 <i>n</i> -3        | 0.6 <sup>a</sup>                            | 0.6 <sup>a</sup>                       | 0.8 <sup>b</sup>                    | 0.05       | 0.034                                   |
| 22:6 <i>n</i> -3        | 1.0   | 1.3                                    | 1.1                                 | 0.11       | 0.163                                   |

<sup>a, b</sup> Mean values for a given fatty acid with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* For details of diets and procedures, see pp. 502–503.

The effects of chronic and acute fat intake on ethanol-induced gastric haemorrhage in the rat are given in Table 3. In the chronic study the maize-oil and fish-oil diets led to a significant fall in the extent of the stomach lesion ( $P < 0.05$ ). In the acute study the intubation with 20:4 *n*-6 before administration of ethanol led to a significant decline in the area of stomach lesion compared with rats given oral 20:5 *n*-3 or 18:2 *n*-6 ( $P < 0.05$ ).

Histologically the haemorrhagic mucosal lesions showed loss of surface epithelium and of crypt epithelium, the latter extending for varying degrees of depth. There was separation and disarray of surviving crypt epithelial cells. Mucosal congestion and haemorrhage were present. In some sections there was submucosal oedema and occasional focal haemorrhage in the vicinity of blood vessels. A very slight infiltrate of inflammatory cells was present immediately beneath the mucosa in a few sections.

#### DISCUSSION

In the last few years a series of papers have been published on the effect of *n*-3 and *n*-6 PUFA on rat gastric ulceration. The prostaglandin precursor 20:4 *n*-6, acutely administered to rats, is clearly protective against ethanol-induced gastric injury. This is evident from the present study and from the findings of Hollander *et al.* (1982). The acute effects of 18:2 *n*-6, similarly administered, did not show comparable effects in the present study which contrasts with the findings of Tarnawski *et al.* (1985). These authors, in a preliminary communication, did show that acutely administered 18:2 *n*-6 reduced the severity of ethanol injury in rats. However, the details are insufficient to discern when exactly the cytoprotective effect emerged or, indeed, the degree of cytoprotection. The interpretation of the present study is that, whereas exogenous 20:4 *n*-6 provides an immediate substrate for eicosanoid synthesis which might account for the observed cytoprotective effect, the exogenous 18:2 *n*-6 would require elongation and desaturation by mucosal cells to achieve the same effect which would not be evident within the 1 h of study.

The effects of the *n*-3 PUFA on eicosanoid metabolism have generally been studied following chronic ingestion, allowing phospholipid 20:5 *n*-3 levels to achieve a steady-state (Croft *et al.* 1984). The lack of effect of acutely administered 20:5 *n*-3 observed in the

Table 3. *Effects of chronic intakes of dietary fats and acute intakes of fatty acids on ethanol-induced haemorrhage\* (% with stomach lesions)*

(Mean values with their pooled standard errors)

|  |                   |
|--|-------------------|
| Chronic study ( <i>n</i> 12)             |                   |
| Control                                  | 29.3 <sup>a</sup> |
| Fish oil                                 | 15.3 <sup>b</sup> |
| Maize oil                                | 12.2 <sup>b</sup> |
| Butterfat                                | 23.8 <sup>a</sup> |
| SEM                                      | 3.24              |
| Statistical significance: <i>P</i> =     | 0.002             |
| Acute study ( <i>n</i> 12)               |                   |
| Eicosapentaenoic acid (20:5 <i>n</i> -3) | 23.2 <sup>a</sup> |
| Arachidonic acid (20:4 <i>n</i> -6)      | 8.3 <sup>b</sup>  |
| Linoleic acid (18:2 <i>n</i> -6)         | 21.4 <sup>a</sup> |
| SEM                                      | 2.74              |
| Statistical significance: <i>P</i> =     | 0.000             |

<sup>a, b</sup> Mean values with unlike superscript letters, in either the chronic or acute study, were significantly different (*P* < 0.05).

\* For details of diets and procedures, see pp. 502–503.

present study and the mucosal phospholipid fatty acid data would indicate that this uptake of 20:5 *n*-3 into mucosal cell phospholipids did not occur in the hour between fatty acid and ethanol administration. However, this contrasts directly with the findings of Leung (1988) who noted a significant effect of fish oil (as opposed to free 20:5 *n*-3) in reducing ethanol-induced gastric haemorrhage in rats. The higher the dose of fish oil the greater was the protection, and the longer the time-lapse between fish-oil dosing and ethanol injury the smaller was the degree of protection. Because this author did not include a control oil, it remains probable that the protective effect was a physical one. Thus, the balance of evidence supports the view that of the three fatty acids, 20:4 *n*-6, 18:2 *n*-6 and 20:5 *n*-3, only the first is cytoprotective when acutely administered.

Several groups have examined the chronic effects of dietary fats on ethanol-induced gastric haemorrhage. Faust *et al.* (1989) fed rats for 4 weeks on either a low-fat control diet or the same diet enriched with 100 g/kg of either maize oil or fish oil. The latter exerted a significant cytoprotective effect against ethanol injury compared with either of the other two diets. The mode of injury was, however, quite different from that used originally by Hollander *et al.* (1982) on which the design of the present study was based. Faust *et al.* (1989) used a preliminary dose with a mild ethanol irritant (150 ml/l) followed 15 min later by the more substantive irritant of absolute ethanol. The authors provide findings to show that this mechanism induces an 'adaptive protection'. Direct comparison with the present study is, therefore, difficult. However, both studies show that the chronic administration of fish oil reduces ethanol-induced gastric injury in rats, albeit to different degrees. The studies differ in their findings with respect to maize oil which was not cytoprotective in the study of Faust *et al.* (1989) and clearly cytoprotective in the present study. This latter finding tends to be supported by the findings of Mosnier *et al.* (1990). These authors fed rats for 3 weeks on diets containing either no added fat or 50 g supplements of maize oil or fish oil/kg. They induced gastric lesions using ethanol (400 ml/l). Thus, their study differed from the present one in having a slightly shorter time-period (3 *v.* 4 weeks), a lower level of dietary oil (50 *v.* 100 g/kg) and a milder ethanol irritant (400 *v.* 1000 ml/l). Nonetheless, their findings showed that both fish oil and maize oil led to a significant reduction in the number of injured rats (12.5 and 45% respectively) compared with rats given the low-fat

control diet (87%). Whilst the results are expressed differently in the two studies (% rats injured *v.* % area lesioned), the findings confirm those of Faust *et al.* (1989) that fish-oil feeding is cytoprotective in this model and support the finding that maize oil is also cytoprotective. Further supportive evidence of a cytoprotective effect of maize oil in gastric lesions is available from the findings of Schepp *et al.* (1988) that chronic ingestion of 18:2 *n*-6, the main fatty acid in maize oil, is cytoprotective in a different model for inducing ulcers in rats, *i.e.* the cold-restraint model. The present study, which included a high-saturated-fat diet which did not differ from controls in the degree of injury, shows that whatever effect is ascribed to maize oil is not due merely to the physical presence of lipid in the diet.

The present study showed a marked elevation in mucosal phospholipid 20:5 *n*-3 and a marked depression in 20:4 *n*-6 in fish-oil-fed animals. Such effects are widely reported for other tissues in fish-oil-fed animals, and the consequent effects on eicosanoid synthesis can be predicted from the extensive literature on the subject. These would include a reduced synthesis of the 2-series of eicosanoids and an increased synthesis of the 3-series. The increased levels of 18:2 *n*-6 in the mucosal phospholipids of maize-oil-fed rats might provide the basis for an increased rate of endogenous synthesis of C<sub>20:4</sub> and an increased endogenous production rate of the 2-series of prostaglandins in these animals. The findings of Grant *et al.* (1988) in humans show that chronic ingestion of maize oil augments gastric PGE<sub>2</sub> production. The level of increase in eicosanoid production observed by these workers was, however, only three-fold, whereas Hollander *et al.* (1982) found that in rats the increase in PGE<sub>2</sub> synthesis following acute loading with 20:4 *n*-6 was 5000-13000 fold. In light of these effects two hypotheses are proposed to explain the cytoprotective effects of the *n*-6 and *n*-3 oils. In the case of the *n*-6 oils it is proposed that an increased endogenous production of PGE<sub>2</sub> exerts its cytoprotection in the same way that the acute administration of 20:4 *n*-6 does. A variety of mechanisms, previously discussed, could be involved. In the case of the *n*-3 oils it is proposed that maintenance of mucosal blood flow and reduced inflammatory response are the protective mechanisms. *n*-3 oils give rise to increased synthesis of thromboxane A<sub>2</sub>, a weakly thrombotic eicosanoid, and to increased synthesis of PGI<sub>2</sub>, a strong anti-thrombotic and vasodilatory agent (Knapp *et al.* 1986). Mesenteric venule thrombosis lies at the heart of the mucosal injury induced by ethanol (Guth *et al.* 1984; Hawkey & Rampton, 1985; Szabo *et al.* 1985; Gronbeck *et al.* 1988; Oates & Hakkinen, 1988). Concentrated ethanol rapidly causes necrosis of superficial mucosal cells and the release of vasoactive mediators by mast cells. This triggers a series of strong constrictions that rapidly progress from small mucosal venules to larger venules. This coincides with a strong arteriolar and arterial vasodilation and leads to the engorgement of mucosal capillaries, creating visible hyperaemia and ultimately mucosal haemorrhage. The histological findings of the present study are consistent with this pattern. The histological findings of haemorrhagic necrosis of mucosa with varying degrees of epithelial cell loss are similar to those described by others in acute alcohol-induced mucosal injury. No vascular injury was seen but the haemorrhage and occasional submucosal oedema and perivascular haemorrhage suggest that this was occurring at a submicroscopic level. The established capacity of 20:5 *n*-3 to reduce the inflammatory response (Endres *et al.* 1989) and to sustain mucosal blood flow would seem an attractive basis on which to explain their cytoprotective effect in ethanol-induced gastric injury.

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