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Protective Effect of Daidzein against Acute Ethanol-induced Lipid Peroxidation in Rat Jejunum

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Ethanol causes extensive damage to the intestinal tract from the oropharynx to the rectum. The jejunum has also been shown to be particularly vulnerable to the deleterious effects of ethanol. We hypothesized that (I) the pathogenesis of acute alcohol-mediated injury in the small intestine involves generation of reactive oxygen species, and consequentially, enhanced lipid peroxidation; (II) the pathogenic changes due to alcohol can be ameliorated with daidzein pretreatment. To test these hypotheses male Wistar rats (n=24) were divided into four groups as follows (*pretreatment* followed by *treatment*): [A] carrier+saline (control); [B] daidzein+saline; [C] carrier+ethanol; [D] daidzein+ethanol. Daidzein (100 mg/kg) or carrier (Intralipid) pretreatment was twice administered as a single dose, whereas ethanol (75 mmol/kg) or saline (0.15 mol/l NaCl) treatment was administered once only. At 24 h after ethanol or saline was administered, rats were sacrificed. The analytes 7α-and 7β-hydroperoxycholest-5-en-3β-ol (7α-OOH and 7β-OOH), 7α-and 7β-hydroxycholesterol (7α-OH and 7β-OH), and 7-ketocholesterol (7-keto) in jejunum were analyzed by HPLC.

The data showed that daidzein *per se* did not affect levels of cholesterol hydroperoxides nor oxysterols. However, there were significant increases in 7α- and 7β-OOHs, 7α- and 7β-OHs, and 7-keto after ethanol dosage compared to controls. Daidzein ameliorated these effects, i.e., values in the daidzein+ethanol group were similar to those in the carrier+saline (control) group. This is the first report showing that (1) cholesterol-derived markers of oxidative stress are increased in the rat jejunum in response to ethanol, indicative of metabolic damage; (2) daidzein pretreatment has protective effects against ethanol-induced injury.

Acute and chronic exposure of the small intestine to alcohol causes a variety of structural and functional abnormalities. These range from induction of blebs to perturbations of active transport mechanisms. For example, ethanol inhibits the absorption of numerous nutrients including monosaccharides, several L-amino acids, lipid, and vitamins (10,11). Moreover, acute administration of alcoholic solutions leads to mucosal damage in the small intestine which increases gut permeability and translocation of endotoxins. The intermittent endotoxaemia stimulates Kupffer cells in the liver, thereby enhancing the production of reactive oxygen species and proinflammatory mediators (10). The aforementioned structural and functional lesions due to alcohol may be related to oxidative stress in the small intestine, though hitherto this has received comparatively little attention.

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Oxysterols and cholesterol hydroperoxides are derived from cholesterol via exposure to free radicals or oxidative modification (Figure 1). Previously, we have developed an analytical procedure for these cholesterol oxidation products (1,5). We found elevated plasma phosphatidylcholine hydroperoxide (6) and accumulation of cholesterol hydroperoxide in erythrocyte membrane (2) of alcoholic patients. Moreover, we also demonstrated accumulation of oxysterols and/or cholesterol hydroperoxides in heart (5), skeletal muscles (14), and liver (8) of rats subjected to ethanol dosage. However, hitherto, neither oxysterols nor cholesterol hydroperoxides have been measured in mammalian small intestine.

We hypothesized that the pathogenesis of ethanol-induced injury in the small intestine involves generation of reactive oxygen, resulting in enhanced lipid peroxidation with specific increases in cholesterol hydroperoxides or oxysterols. We also hypothesized that such changes can be ameliorated with agents known to prevent oxidative stress. Soy isoflavones are one sub-class of the phytoestrogen family, and recent studies have reported beneficial effects of these compounds on human health (25). The antioxidant properties of soy have also been demonstrated in isolated cells (i.e., lymphocytes (13) and Caco-2 intestinal cells (22)), and in the whole rat (7). However, there is a paucity of information on the protective role of phytoestrogens in ethanol-induced injury.

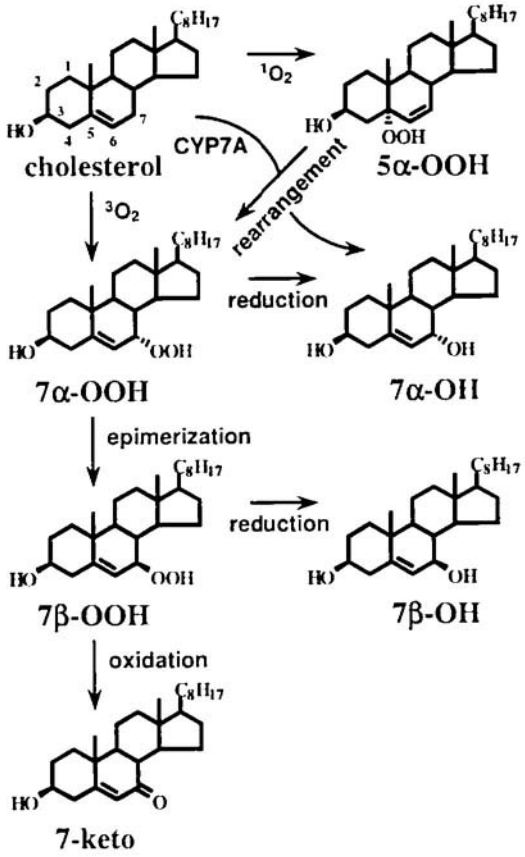

Figure 1:
Putative pathway from cholesterol to oxysterols.

5α-OOH, 5α-hydroperoxycholest-6-en-3β-ol;
7α-OOH, 7α-hydroperoxycholest -5-en-3β-ol;
7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol;
7α-OH, 7α-hydroxy- 5-en-3β-ol;
7β-OH, 7β-hydroxy-5-en-3β-ol;
7-keto, 3β-hydroxycholest-5-en- 7-one;

To address these hypotheses we measured the levels of 7α-and 7β-hydroperoxycholest-5-en-3β-ol (7α-OOH and 7β-OOH), 7α-and 7β-hydroxycholesterol (7α-OH and 7β-OH), and 7-ketocholesterol (7-keto) in the intestine of rats subjected to ethanol dosage with or without daidzein pretreatment. These aforementioned products of cholesterol were measured because the metabolism of the oxysterols and hydroperoxides have been characterized more precisely than malondialdehyde (MDA) and thiobarbituric acid reacting substances (TBARS) which are also thought to be unspecific.

MATERIALS AND METHODS

Materials

3, 5-Di-tert-butyl-4-hydroxytoluene (BHT), luminol (3-aminophthaloylhydrazine) and cytochrome c (from horse, type VI) were purchased from Wako Pure Chemical Co.(Osaka, Japan). β-Sitosterol (as an internal standard (IS)), 7-keto, 7α-OH, and 7β-OH were purchased from Stelaroids (Wilton, NH). 5α-Hydroperoxycholest-6-en-3β-ol (5α-OOH), 7α-OOH and 7β-OOH, and β-sitosterol-5α-hydroperoxide (as IS) were prepared as described previously (1).

Animals

Twenty-four male Wistar rats (100-120 g body weight), obtained from Harlan UK (Oxfordshire, UK) were used in this study. Rats were ranked by initial weight and assigned into 4 groups of equal mean body weights. They were housed individually in a temperature-controlled environment with 12 h light-dark cycle. Rats were allowed to access to standard laboratory food pellets and water *ad libitum*. The study was conducted under a Project License approved by the Home Office and followed institutional guidelines.

Experimental design

Rats were subjected to different treatments according to the experimental design outlined in Figure 2. Rats were "pre-treated" for 2 days which was followed by a single "treatment". Rats were killed 24 h after the treatment (Figure 2) and the groups were:

[A], Carrier+saline
[B], Daidzein+saline
[C], Carrier+ethanol
[D], Daidzein+ethanol

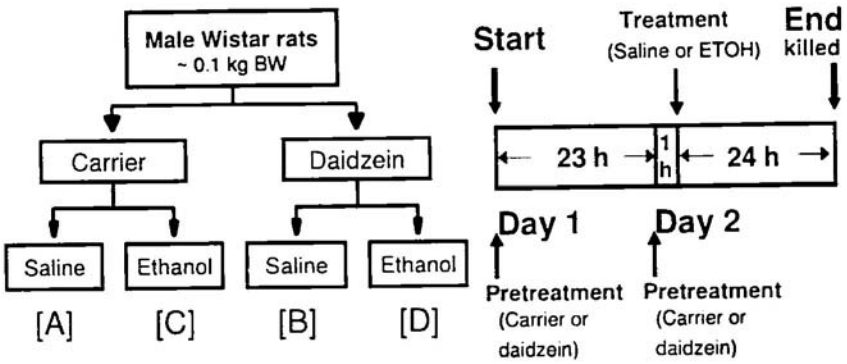

Figure 2: Experimental design and protocol

Saline was 0.15 mol/l NaCl and the carrier was Intralipid (20% w/v fat emulsion). Daidzein (100 mg/kg body weight) was obtained from Hangzhou FST, Republic of China, and freshly suspended in Intralipid via homogenization prior to injection intraperitoneally (i.p.). At 1 h after the second pretreatment injection, rats were treated with an intraperitoneal injection (10 ml/kg body weight) of solution containing either saline (0.15mol/l NaCl) or ethanol (75 mmol/kg body weight). In this experimental design, we selected the i.p. route for administration to ensure greater bioavailability of the compounds. At 24 h after the last injection, rats were killed by decapitation. The jejunum was rapidly dissected and flushed with saline.

Extraction

Total lipids were extracted and the cholesterol fraction was isolated by solid phase extraction using a silica column (Sep-Pak-NH_2) as previously described (3).

HPLC-CL analysis

Cholesterol hydroperoxides were measured by HPLC with post-column chemiluminescence (HPLC-CL) as previously described (3). A TSK gel Octyl-80Ts column (Tosoh, Tokyo, Japan, 150 × 4.6 mm internal diameter) and methanol/water/acetonitrile (89:9:2) as the mobile phase were used.

HPLC-UV analysis of oxysterol

7α-OH, 7β-OH, and 7-keto were determined by HPLC with a UV detector set at 210 nm and 245 nm as previously described (5). An Inertsil ODS-2 column (GL Sciences, Osaka, Japan, 5 μm, 150 × 4.6 mm internal diameter) and acetonitrile/methanol/water (46:45:9) as the mobile phase were used. The calibration curves were linear in the range of 25-200 ng of 7α-OH, 50-200 ng of 7β-OH and 7-keto using 250 ng of IS. The recoveries from the tissue extracts were determined by comparison of the peak area with a known concentration. The recoveries were about 70%.

Statistical analysis

All data are expressed as means ± SD. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Fisher's PLSD post hoc test (Stat View 5.0). Values of $p<0.05$ were considered statistically significant.

RESULTS

Cholesterol hydroperoxides

There was clear separation of cholesterol hydroperoxides (5α-OOH, 7α-OOH, and 7β-OOH) together with the internal standard β-sitosterol-5α-hydroperoxide in standard solutions as well as in jejunum samples. Lipid extracts from jejunum contained 7α-OOH and 7β-OOH, but not 5α-OOH, as previously described (4).

The mean concentrations of 7α-OOH and 7β-OOH in jejunum of control rat are shown in Table 1. In jejunum from control rats, the 7α-OOH concentration was lower than the 7β-OOH, which may reflect the fact that 7α-OOH is easily epimerized to 7β-OOH (4).

Following treatment with daidzein, concentrations of 7α-OOH and 7β-OOH in jejunum were unaffected (Table 1).

There were significant increases in 7α-OOH (90%) and 7β-OOH (32%) in jejunum following acute ethanol administration (Table 1).

Cholesterol hydroperoxides were significantly affected by combined daidzein+ethanol treatment compared to carrier+ethanol administration. The decreases of 7α-OOH and 7β-OOH in group [D] were 69% and 75%, respectively, compared to group [C] (Table 1). Thus, the effects of daidzein+ethanol (i.e., Group [A] versus [D]) were quite different to

treatments with carrier+ethanol (i.e., Group [A] versus [C]) implicating a potentially therapeutic role for daidzein in ameliorating ethanol-induced damage.

Table 1. 7-Hydroperoxycholesterol concentrations in jejunum of rats from four groups 24 h after acute ethanol or saline administration

	nmol/g	
	7 α -OOH	7 β -OOH
[A] Control	2.10 ± 0.3 [a]	9.95 ± 1.3 [a]
[B] Daidzein	2.55 ± 0.7 [a b]	9.55 ± 2.1 [a b]
[C] EtOH	4.01 ± 0.5 [b]	13.12 ± 4.3 [b]
[D] Daidzein+EtOH	2.77 ± 0.8 [a]	9.91 ± 3.2 [a]

Values are mean ± SD. n=6 Within a column, means with different superscripts are different ($p<0.05$). 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol and 7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol. Control, Carrier+saline; Daidzein, Daidzein+saline; EtOH, Carrier+ethanol; Daidzein+EtOH, Daidzein+ethanol; Carrier, 20% w/v fat emulsion; Saline, 0.15mol NaCl; Daidzein, Daidzein at 100 mg/kg body weight ; ethanol, ethanol at 75 mmol/kg body weight. The study design included a pre-treatment and treatment stage to ensure all rats were treated identically. Other details are contained in the Materials and Methods section.

Oxysterols and cholesterol

There was successful separation of oxysterols and cholesterol. The mean concentrations of 7α-OH and 7β-OH, and 7-keto of control rats are shown in Table 2. In the control rats, 7α- and 7β-OHs levels were higher than 7α- and 7β-OOH levels. The

Table 2. Oxysterol concentrations in jejunum of rats from four groups 24 h after acute ethanol or saline administration

	nmol/g		
	7 α -OH	7 β -OH	7-keto
[A] Control	32 ± 8 [a c]	195 ± 10 [a]	226 ± 44 [a]
[B] Daidzein	25 ± 8 [a b]	184 ± 18 [a b]	175 ± 48 [a b]
[C] EtOH	51 ± 14 [b]	270 ± 66 [b]	327 ± 88 [b]
[D] Daidzein+EtOH	36 ± 10 [c]	195 ± 40 [a]	222 ± 45 [a]

Values are mean ± SD. n=6 Within a column, means with different superscripts are different ($p<0.05$). 7 α -OH, cholest-5-ene-3 β ,7 α -diol; 7 β -OH, cholest-5-ene-3 β ,7 β -diol; 7-keto, 3 β -hydroxycholest-5-en-7-one. Control, Carrier+saline; Daidzein, Daidzein+saline; EtOH, Carrier+ethanol; Daidzein+EtOH, Daidzein+ethanol; Carrier, 20% w/v fat emulsion; Saline, 0.15mol NaCl; Daidzein, Daidzein at 100 mg/kg body weight; ethanol, ethanol at 75 mmol/kg body weight.

concentration of 7-keto was 18 times as large as the sum of 7α-OOH and 7β-OOH. The cholesterol concentration in jejunum was 6785±530 nmol/g.

Daidzein administration did not affect jejunal cholesterol or oxysterols. At 24 h after acute ethanol dosage, jejunal 7α-OH and 7β-OH, and 7-keto were significantly elevated. The increases were 59%, 38%, and 45%, respectively.

Oxysterols were also significantly affected by daidzein pretreatment prior to ethanol. These levels in group [D] were approximately 70% of group [C] (Table 2). Thus, the effects of daidzein+ethanol (i.e., Group [A] versus [D]) were quite different to treatments with carrier+ethanol (i.e., Group [A] versus [C]).

DISCUSSION

Methodological considerations

The aims of this study were to (I) assess oxidative stress by measuring cholesterol hydroperoxide and oxysterol levels in small intestine of rats acutely dosed with alcohol and (II) investigate the effect of pretreatment with daidzein as an antioxidant. In laboratory animals, administration of isoflavones at doses ranging from 10 mg/kg to 230 mg/kg per day has been shown to have various metabolic effects and responses in different tissues such as the prevention of NF-kappa B activation (15). We used a comparable dose of daidzein (100 mg/kg body weight per day) in the present study. However, it could be argued that dosage via the intraperitoneal route was suboptimal. This criticism can be discounted because, as mentioned in the Methods section, the i.p. route ensures greater bioavailability of daidzein and ethanol. Furthermore, our studies were intentionally focused on acute responses, rather than the long-term effects of either daidzein or ethanol. In this regard, it is important to emphasize that in most pathological or therapeutically orientated studies, distinct responses are obtained in acute and chronic situations. For example, the acute and chronic effects of ethanol dosage on the small intestine are quite distinct, with largely biochemical changes in the former and compositional perturbations in the latter (20, 21).

Oxidative stress in the ethanol exposed small intestine

Ethanol administration causes oxidative imbalance via a number of pathways including the generation of reactive oxygen species via xanthine oxidase (12), and an impairment of defense mechanisms such as via decreased glutathione peroxidase activities secondary to selenium deficiency (24). However, to our knowledge, there are no reports showing increases in specific markers of oxidative stress in the small intestine after acute ethanol.

The main finding of this study was the HPLC-detected increases in jejunal cholesterol hydroperoxides (7α- and 7β-OOHs) and oxysterols (7α-OH, 7β-OH, and 7-keto) following acute ethanol dose. This is the first report on the simultaneous measurements of multiple sterols in the small intestine. Of particular note was the observation that these markers of oxidative stress increased after 24 h, reflective of a remarkable sensitivity to ethanol. These observations are similar to studies on skeletal muscle, which also shows an increase in cholesterol hydroperoxides 24 h after ethanol dosing (4).

The protective effects of daidzein

Isoflavones have also been studied extensively, and overall appear to show a beneficial effect, implicating their usage in the diet, as supplements or as pharmacological agents. For example, flavonoids including quercetin, myricetin (flavonol), luteolin (flavone) and (-)-epigallocatechin gallate (flavanol) prevent the formation of MDA due to hydrogen peroxide and Fe^{2+} treatment in Caco-2 intestinal cells (22). Also, supplementation of Jurkat T-cell and primary lymphocytes with daidzein significantly decreases production of MDA and protects DNA from oxidative damage (7).

In the present study daidzein dosage *per se* had no effect on cholesterol hydroperoxides nor oxysterols compared to controls. However, jejunal concentrations of cholesterol hydroperoxides (7α- and 7β-OOHs) and oxysterols (7α- and 7β-OHs and 7-keto) were reduced by daidzein+ethanol treatment, compared to rats in the carrier+ethanol group: reductions were marked and of the order of approximately 70%. Thus, daidzein significantly suppressed the ethanol-induced oxidative stress. To our knowledge, this is the first report showing daidzein has the ability to ameliorate the oxidative stress arising as a consequence of ethanol administration. We do not know the precise mechanism whereby this protective effect of daidzein occurs though some beneficial effects of daidzein on alcohol-related pathology have been reported elsewhere. For example, at a dose of 100 mg/kg body weight/day, daidzein decreases ethanol intake by 75% (19). Moreover, both daidzin and daidzein suppress free-choice ethanol intake by golden Syrian hamsters (16). Daidzin and daidzein are also potent inhibitors of mitochondrial aldehyde dehydrogenase (ALDH-2) (17). Thus, acetaldehyde accumulates in the blood and tissue as a consequence of daidzein or daidzin administration. As a result, the "spontaneous" (i.e., free choice) intake of ethanol is reduced. These effects on ALDH-2 are isoflavonoid-specific since neither puerarin, daidzin nor daidzein administration affects liver aldehyde dehydrogenase activities (18, 19). As both daidzein and daidzin have antidipsotropic effects, and purified puerarin at 50 mg/kg/day abolishes withdrawal symptoms (9), they are reported to be effective therapeutic agents for alcohol abuse. Such an interpretation requires some caution, as paradoxically we would assume that inhibition of ALDH-2 by daidzein would potentiate the damaging effects of ethanol on cholesterol hydroperoxides or oxysterols in the small intestine. However, inhibition of ALDH-2 in ethanol-dosed rats (by cyanamide pre-treatment) does not lead to further elevations in skeletal hydroperoxides, compared to ethanol alone (3). Clearly, further work into the potential of using daidzein in ameliorating ethanol-induced damage is warranted.

Conclusions

In conclusion, this is the first report showing that cholesterol-derived markers of oxidative stress are increased in the rat jejunum in response to ethanol, indicative to metabolic damage; (2) daidzein pretreatment has protective effects against ethanol-induced injury.

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