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Metabolism Clinical and Experimental 56 (2007) 865–875
Metabolism
Clinical and Experimental
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The cytoprotective effect of α-tocopherol and daidzein against D-galactosamine–induced oxidative damage in the rat liver

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Received 29 August 2006; accepted 17 January 2007

Abstract

We hypothesized that the hepatotoxicity that develops after the induction of oxidative stress (induced by D-galactosamine [GalN]) can be ameliorated by α-tocopherol (ATC) and the soy isoflavone daidzein. To test this, we ranked and assigned male Wistar rats into 6 groups, which involved pretreatment (ATC or daidzein) for 1 hour followed by treatment (GalN) for 23 hours. Histopathologic analysis showed that GalN administration induced marked necrosis ($P < .001$), steatosis ($P < .001$), both lobular and portal inflammations ($P < .001$), overall histopathologic score ($P < .001$), and activation of caspase-3 in the liver ($P < .001$). Immunohistochemical staining of malondialdehyde-protein adducts, a measure of oxidative stress, was increased in response to GalN ($P < .001$). Paradoxically, there were increases in total ($P < .05$) and cytosolic superoxide dismutase ($P < .001$) activities after GalN administration, indicative of an up-regulation of antioxidant defenses. The concentration of total protein ($P < .001$), albumin ($P < .01$), and globulin fractions ($P < .001$) in the plasma, as well as the activity of aspartate aminotransferase ($P < .001$), was significantly perturbed after GalN treatment, reflective of overall acute hepatic injury. Administration of daidzein showed a significant amelioration of the Ga1N-induced increase in malondialdehyde-protein adducts ($P < .01$) and cytosolic superoxide dismutase activities ($P < .01$) in the liver. However, all other variables were not significantly altered in response to daidzein. In response to ATC pretreatment, the total histopathologic score ($P < .05$), degree of necrosis ($P < .05$), and both lobular ($P < .05$) and portal ($P = .05$) inflammations were significantly ameliorated. To conclude, both daidzein and ATC protect the liver against oxidative damage possibly via different pathways.

1. Introduction

In the rat, D-galactosamine (GalN) administration has been used to induce experimental acute hepatitis [1]. The mechanism of this effect is uncertain but may involve oxidative stress as dietary antioxidant therapy has been shown to reduce these changes [2]. For example, a study has shown that vitamin E supplementation is able to reduce the early fat and collagen accumulation in the liver after GalN administration [3]. It is of related interest to investigate the potential protective action of other food components against oxidative stress–induced liver disease. Soy isoflavones are one subclass of the phytoestrogen family of compounds, and recent studies have reported beneficial effects on human health [4]. In previous studies we demonstrated in healthy subjects that consumption of a diet enriched with soy containing isoflavones increased high-density lipoprotein concentrations [5] and decreased the concentration of plasma F_2-isoprostanes compared with consumption of a

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0026-0495/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.metabol.2007.01.005

diet enriched with soy from which the isoflavones had been extracted [6]. The antioxidant properties of soy have also been demonstrated [7], and particularly daidzein has been shown to ameliorate the malondialdehyde (MDA) concentration in the rat [8]. This suggests that phytoestrogens have therapeutic potential to protect against organ injury. However, there is a paucity of information on the protective role of phytoestrogens in liver injury.

The aim of the present study was to investigate the hypothesis that the properties of soy isoflavones (daidzein was selected in this particular study) would be protective against GalN-induced liver damage. α-Tocopherol (ATC) was used as a well-characterized and potent antioxidant for comparison.

2. Materials and methods

2.1. Animals

Male Wistar rats (100-120 g of body weight [BW]), obtained from Harlan UK (Oxfordshire, UK), were used in this study. Rats were ranked by initial weight and assigned into 6 groups of equal mean BWs. They were housed individually in a temperature-controlled environment with 12-hour light-dark cycle. Rats were allowed access to standard laboratory food pellets (Special Diets Services, Witham, Essex, UK) and water ad libitum. The study was conducted under a project license approved by the home office and followed institutional guidelines.

2.2. Experimental design

Rats were subjected to different treatments according to the experimental design (Fig. 1). Rats were "pretreated" for 1 hour followed by a 23-hour "treatment" as follows: A, carrier + saline; B, carrier + GalN; C, ATC + saline; D, ATC + GalN; E, daidzein + saline; and F, daidzein + GalN.

Saline was composed of 0.15 mol/L NaCl and the carrier was intralipid (20% wt/vol fat emulsion). α-Tocopherol (30 mg/kg of BW) and daidzein (100 mg/kg of BW) were freshly prepared and injected intraperitoneally. D-Galactosamine was administered (intraperitoneally) in a single dose of 1 g/kg of BW (1 mL/100 g). Food was withdrawn after the treatment injection. At the time of termination, rats were killed by decapitation, and livers were dissected out, blotted, and weighed. Part of the liver was immediately immersed in 4% (wt/vol) formaldehyde fixative for subsequent embedding in paraffin, while another section of the liver was frozen in liquid nitrogen and subsequently stored at −80°C. Blood was collected into heparinized tubes. Whole blood was centrifuged at 1500*g* for 10 minutes at 4°C, and plasma was stored at −80°C for blood biochemistry analysis.

2.3. Histopathologic examination

Sections (4 μm thick) of the liver were stained with hematoxylin-eosin and examined by light microscopy. The sections were graded by an experienced histopathologist

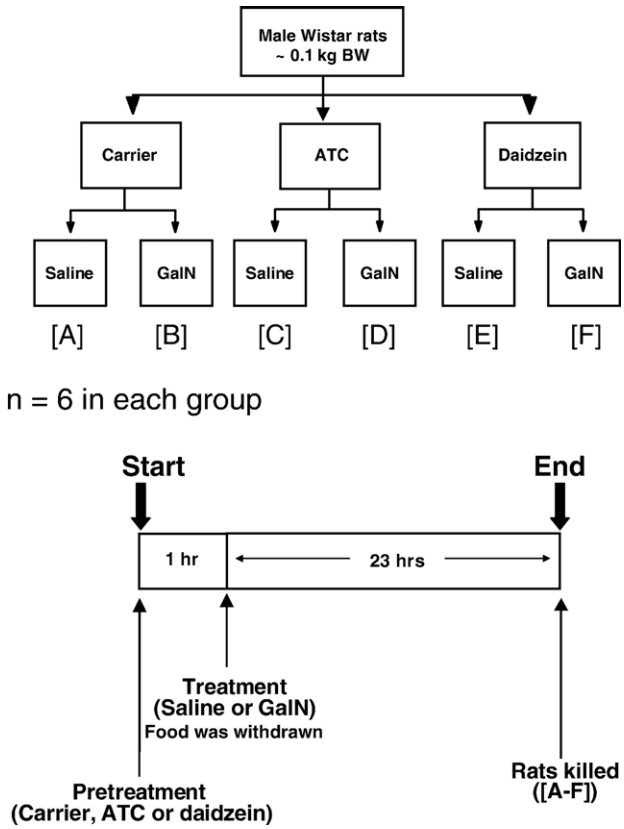

Fig. 1. The schematic diagram shows the experimental design and the treatment timeline. Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein dose is 100 mg/kg BW.

(BP) with a specialist interest in hepatic pathology. For micro-steatosis, macro-steatosis, lobular inflammation, and portal inflammation, the samples were graded from 0 (rarely seen) to 3 (widespread). For necrosis (acidophilic bodies) samples were graded 0 (absent) to 5 (confluent necrosis). Total histopathologic scores were a summation of individual parameters (ie, micro-steatosis + macro-steatosis + lobular inflammation + portal inflammation + necrosis). The histopathologist (BP) was unaware of the groupings when the analysis was carried out, and the codes were only revealed after statistical analysis of the data.

2.4. Immunohistochemistry

Immunohistochemical staining was performed using an automated staining system (Techmate 500+, DakoCytomation) to determine the formation of MDA-protein adducts. Tissue sections were deparaffinized in xylene and rehydratated in decreasing concentrations of ethanol. Endogenous peroxidase was inactivated using peroxidase blocking solution (DakoCytomation, Glostrup, Denmark). A rabbit antibody, specific to MDA adducts, with a 1:4000 dilution was used as a primary antibody, and labeling was detected using PowerVision Homo-Mouse IHC Detection System

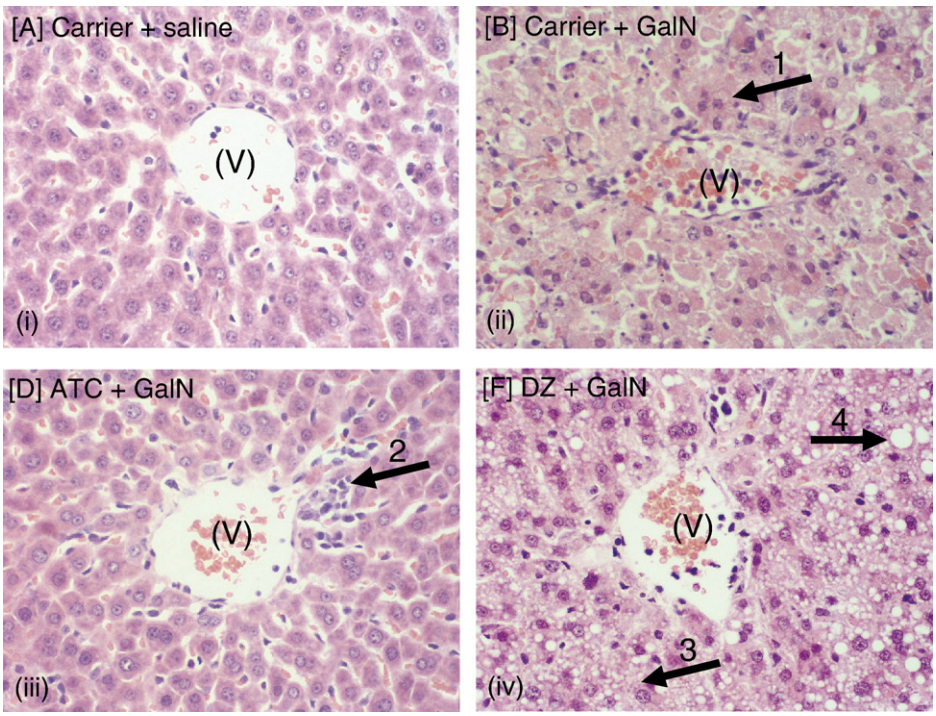

Fig. 2. Liver histology of central venule (V) regions in rats pretreated with either ATC or daidzein followed by GalN. Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein (DZ) dose is 100 mg/kg of BW. Panel (i), normal histology of control liver; (ii), severe necrosis (arrow 1) after treatment with GalN; (iii), mild inflammation (arrow 2) after ATC pretreatment; (iv), widespread microvesicular (arrow 3), and macrovesicular steatosis (arrow 4) after daidzein pretreatment (hematoxylin-eosin, original magnification × 400).

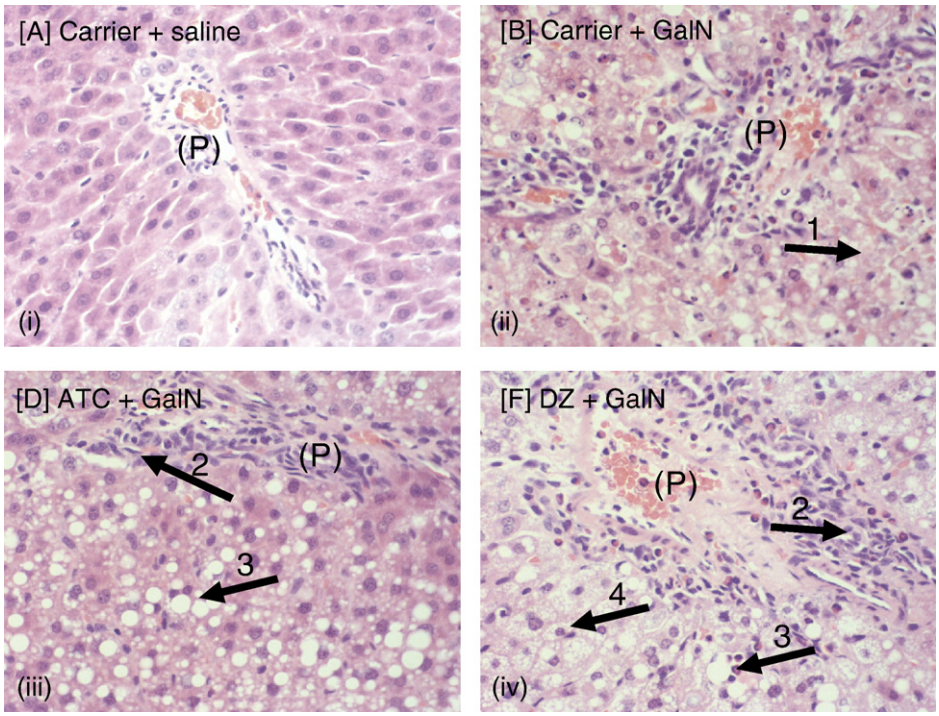

Fig. 3. Liver histology of portal tract region (P) in rats pretreated with either ATC or daidzein followed by GalN. Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein (DZ) dose is 100 mg/kg of BW. Panel (i), normal histology of the control liver; (ii), severe necrosis (arrow 1) treated with GalN; (iii), mild inflammation (arrow 2) with macrovesicular steatosis (arrow 3) after ATC pretreatment; (iv), mild inflammation (arrow 2), microvesicular (arrow 4), and macrovesicular steatosis (arrow 3) after daidzein pretreatment (hematoxylin-eosin, original magnification × 400).

Table 1
The histopathologic scores of the rat liver after different treatments

Treatment	Histopathologic scores		
	Micro-steatosis**	Macro-steatosis**	Necrosis (acidophilic bodies)**
(A) Carrier + saline	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
(B) Carrier + GalN	2.33 ± 0.54	2.17 ± 0.52	2.67 ± 0.78
(C) ATC + saline	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
(D) ATC + GalN	1.83 ± 0.66	1.50 ± 0.47	0.83 ± 0.34*
(E) DZ + saline	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
(F) DZ + GalN	2.83 ± 0.18	2.00 ± 0.28	1.83 ± 0.52

Data are expressed as mean ± SEM (n = 6). The raw scores were ranked, and 2-way analysis of variance (ANOVA) was used to determine whether any of the effects of treatments were significant. Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein (DZ) dose is 100 mg/kg of BW.

* $P < .05$ when compared with group B; 1-way ANOVA between the groups.

** $P < .001$; 2-way ANOVA showed a significant effect due to GalN for all 3 histopathologic indices.

(ImmunoVision Technologies, Brisbane, CA). Sections were counterstained with hematoxylin (DakoCytomation). Substitution of the primary antibody with antibody diluent (DakoCytomation) served as a negative control. The intensity of the staining was scored on a scale of 0 (no reaction) to 5 (strong reaction). The stained sections were photographed with Nikon Eclipse E600 microscope (Tokyo, Japan).

2.5. Measurement of enzymatic activities

The SOD enzyme activity assay was modified from the method of L'Abbe and Fischer [9] for a microplate reader. In brief, livers were homogenized in approximately 40 times their weight as volume of ice-cold 0.2% Trition X-100 solution and centrifuged (16 250*g* for 5 minutes at 4°C). Total SOD activity in the supernatant was measured by the cytochrome *c* reduction assay, which follows absorbance at 550 nm. Reactions were carried out in triplicate in a 96-well plate, and absorbance change was determined using a SPECTRAmax PLUS microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Activity was determined from a standard curve of SOD (1.5-16 U SOD/mL). One unit of SOD activity is defined as that amount of activity that will decrease the rate of cytochrome *c* reduction by 50% under a standardized reaction rate. Protein was determined on a microplate reader using a bicinchoninic acid protein assay kit (Sigma, Oakville, Canada). The glutathione peroxidase (GSHPx) enzyme activity was determined according to the previously described method [10].

2.6. Blood biochemistry

The plasma analytes, including the total protein, albumin, and globulin fractions, as well as the activities of aspartate aminotransferase (AST) were measured by routine laboratory diagnostic procedures [11].

Table 2
The histopathologic scores of the liver under different treatments

Treatment	Histopathologic scores	
	Lobular inflammation*	Portal inflammation*
(A) Carrier + saline	0.00 ± 0.00	0.00 ± 0.00
(B) Carrier + GalN	1.83 ± 0.52	1.83 ± 0.52
(C) ATC + saline	0.00 ± 0.00	0.00 ± 0.00
(D) ATC + GalN	0.83 ± 0.34**	1.00 ± 0.40**
(E) DZ + saline	0.00 ± 0.00	0.00 ± 0.00
(F) DZ + GalN	1.50 ± 0.24	2.17 ± 0.18

Data are expressed as mean ± SEM (n = 6). The raw score of the data was ranked, and 2-way ANOVA was used to analyze any significant effect on the treatments. Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein (DZ) dose is 100 mg/kg of BW.

* $P < .05$ when compared with group B; 1-way ANOVA between the groups.

** $P < .001$; 2-way ANOVA showed a significant effect due to GalN on both histopathologic indices.

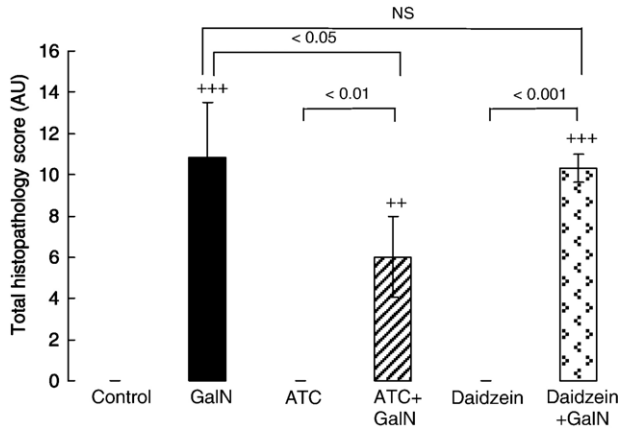

Fig. 4. The total histopathologic scores of the liver with different pretreatments (ATC or daidzein) and drug treatment (GalN). Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein dose is 100 mg/kg BW. Data are expressed as mean ± SEM (n = 6) of the histopathologic score. The data were ranked, and the values were analyzed using 2-way ANOVA ($F_{5,30} = 21.170$, $P < .001$; GalN treatment, $P < .001$). Symbols above the histograms refer to differences from the control ($^{++}P < .01$, $^{+++}P < .001$) using 1-way ANOVA followed by LSD post hoc test. NS indicates not significant.

2.7. Immunohistochemical detection of active caspase-3

Briefly, formalin-fixed tissue sections were rehydrated, and subjected to microwave/citric acid buffer antigen retrieval, before incubation with rabbit antihuman active caspase-3 immunoglobulin G (IgG) (0.8 ng/mL: AF835, R&D Systems, Abingdon, UK) in Tris-buffered saline (TBS) with 10% (vol/vol) goat serum/0.1% (vol/vol) Tween 20. Immunodetection was carried out using biotin-labeled goat antirabbit IgG, and horseradish peroxidase Vector ABC elite reagents (Vector Labs, Peterborough, UK), with 3′,3′-diaminobenzidine as a substrate (Sigma, Poole, UK). A 2% (vol/vol) hydrogen peroxide/methanol incubation during the rehydration procedure was used to block endogenous peroxidase activity in all sections. Normal rabbit IgG fraction (X0903, Dako Labs, Ely, UK) was used as a negative control.

2.8. Statistical analysis

Data are expressed as means ± SEM and analyzed by using SPSS (ver 11.0; SPSS, Chicago, IL). The histopathologic scores, MDA-protein adduct scores, and immunostaining score were analyzed after ranking. The differences between groups were compared using 2-way analysis of variance, with pretreatment (carrier, ATC, daidzein) and treatment (GalN, no GalN) as independent factors. For relevant comparisons, the data were further analyzed by the post hoc least significant differences (LSD) test to determine any statistically significant main effects or higher-order interactions. A P value of less than .05 was considered to be significant. Posterior LSD analysis was conducted only after a careful visual scrutiny of graphically represented data to reduce the number of post hoc comparisons to minimum and to protect the data analysis from an elevated risk of type I statistical error [12].

2.9. Methodological considerations

D-Galactosamine is used as an effective agent to induce experimental hepatitis, and also oxidative stress in animal models [1,2]. In the present study, GalN was administered, and either ATC or daidzein pretreatment was used as cytoprotective agents to try to reduce the ensuing damage. Food was withdrawn from the rats after the injection of either saline or GalN to control for the anorexic effect

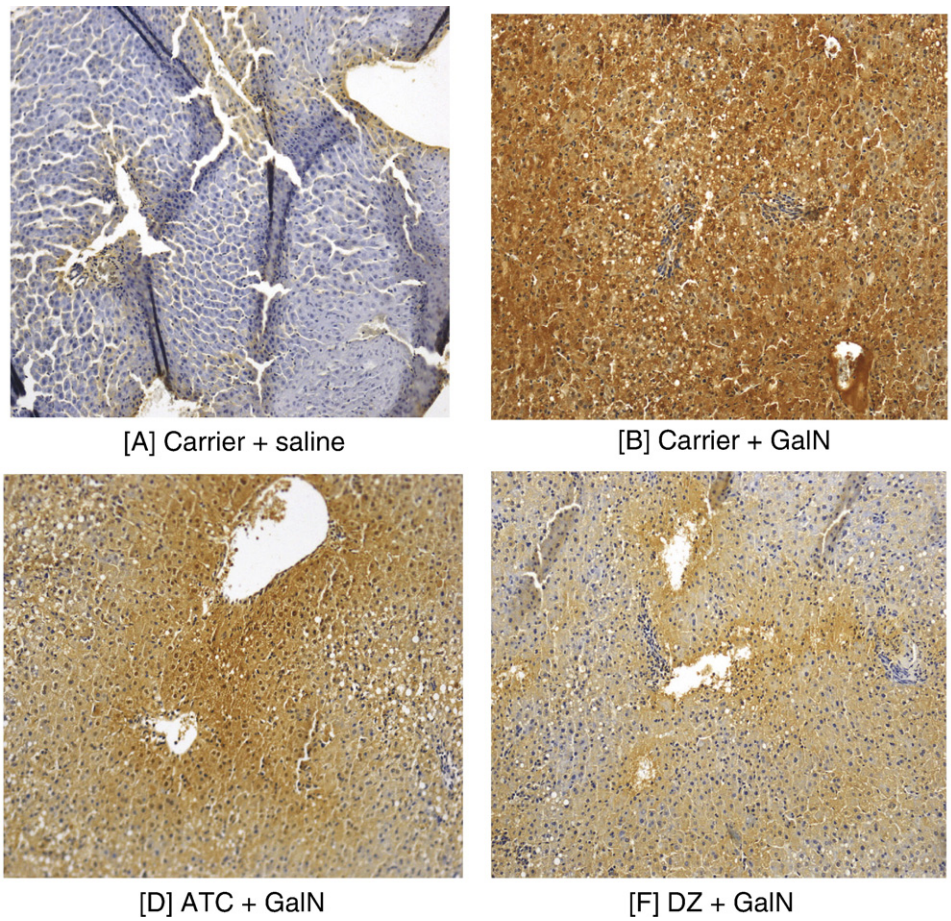

Fig. 5. The immunohistochemical observation of the liver section specifically stained with MDA-protein adducts. Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein (DZ) dose is 100 mg/kg of BW. Panels show the representative photomicrographs of the liver sections. The GalN-treated rats showed a distinct staining for the adducts. The ATC + GalN–treated group and the DZ + GalN–treated group showed a reduced immunostaining. For the semiquantitative scoring and results, refer to Fig. 6.

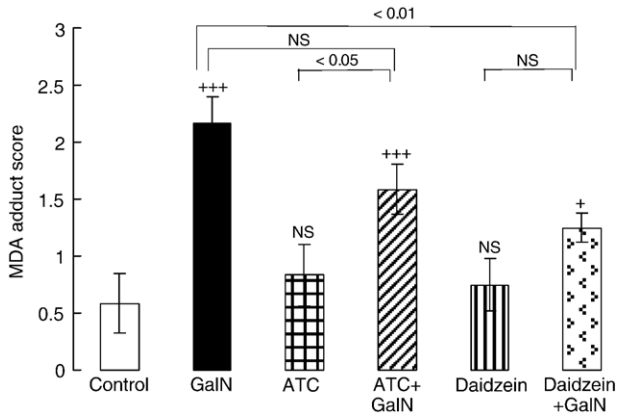

Fig. 6. Immunohistochemically stained MDA-protein adduct scores of the liver. Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein dose is 100 mg/kg of BW. Data are expressed as mean ± SEM (n = 6). The data were ranked, and the values were analyzed using 2-way ANOVA ($F_{5,30}$ = 8.040, P < .001; GalN treatment, P < .001). Symbols above the histograms refer to differences from the control (^{+}P < .05, ^{+++}P < .001) using 1-way ANOVA followed by LSD post hoc test.

caused by GalN [13]. Thus, none of the subsequent effects were due to differences in nutritional status.

α-Tocopherol at a dose of 30 mg/kg of BW was chosen as an effective regimen to enhance antioxidant capacity and protect against GalN-induced hepatic injury. This regimen was based on a previous animal study, which showed an increase in tissue ATC concentration after administration of this dose [14]. Isoflavones, such as daidzein, have also been studied extensively, and we have shown that consumption of isoflavones increase the resistance against oxidative damage in human (eg, see reference [6]). In laboratory animals, administration of isoflavones in doses from 10 to 230 mg/kg per day are effective in producing therapeutic responses in various models (eg, see references [15-17]). We therefore used a comparable dose of daidzein (100 mg/kg of BW per day). Although the metabolite of daidzein, equol, has been shown to have a higher antioxidant property in vitro [18-20], daidzein per se has also been shown to have an antioxidant effect in hepatic cells [21,22]. Moreover, both ATC and daidzein were injected intraperitoneally rather than orally. This was to ensure greater bioavailability, an approach used in other studies [14,16].

3. Results

3.1. Effects of galactosamine

Histopathologic examination of the liver in GalN-treated rats showed widespread necrosis (acidophilic bodies) affecting both the perivenular ("V") and periportal ("P") areas (Figs. 2 and 3). Other histopathologic parameters including macro- and micro-steatosis, and portal and lobular inflammations showed a significant increase after treatment with GalN (Tables 1 and 2). Total histopathologic scores (a summation of individual parameters) were also increased by GalN (Fig. 4).

The immunohistochemistical location of MDA adducts is shown Fig. 5. In the control rats, the immunostaining for MDA adducts was generally weak and restricted to the periportal region. D-Galactosamine markedly induced MDA adduct staining in the hepatocytes and the immunoreactivity was present in all zones of the hepatic lobule. Quantitative analysis of the immunohistochemistry data also showed a significant increase in the formation of MDA adducts in the liver from the rats treated with GalN (Fig. 6). D-Galactosamine administration also induced widespread activation of caspase-3 hepatic cells, as measured by immunohistochemistry (Fig. 7).

Neither GSHPx nor selenium-dependent glutathione peroxidase (SeGSHPx) activities were changed because of GalN (data not shown). Administration of GalN significantly increased the total SOD (combined manganese and copper/zinc [Cu/Zn] isoforms) and Cu/Zn-SOD (ie, cytosolic) activities (P < .01 and P < .001 when compared to control; Fig. 8).

Galactosamine increased the activity of plasma AST (Fig. 9) and reduced the total, albumin, and globulin protein fractions (Fig. 10).

3.2. Effects of ATC

α-Tocopherol per se had no effect on any variable. However, ATC treatment prevented GalN-induced changes in the liver (Figs. 2 and 3). Necrosis (P < .05; Table 1); lobular (P < .05; Table 2) and portal inflammations

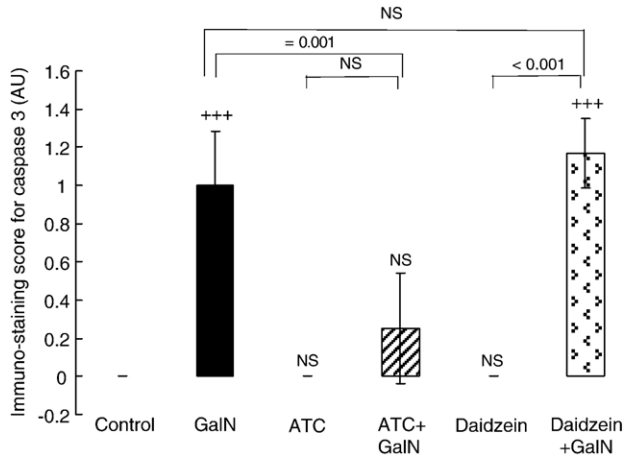

Fig. 7. The immunostaining score of activated caspase-3 activity with different pretreatments (ATC or daidzein) and drug treatment (GalN). Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein dose is 100 mg/kg of BW. Data are expressed as mean ± SEM (n = 6). The mean scores for control, ATC, and daidzein are zero. The data were ranked, and the values were analyzed using 2-way ANOVA ($F_{5,30}$ = 20.343, P < .001; GalN treatment, P < .001). Symbols above the histograms refer to differences from the control (^{+++}P < .001) using 1-way ANOVA followed by LSD post hoc test.

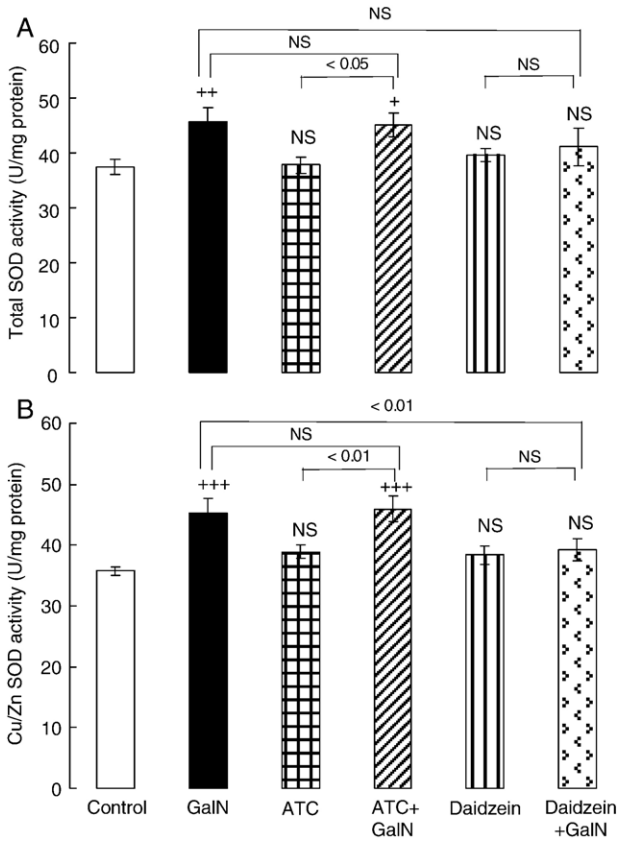

Fig. 8. The effects of different pretreatments (ATC or daidzein) and drug treatment (GalN) on the total SOD and Cu/Zn-SOD activities in the liver. Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein dose is 100 mg/kg of BW. Data are expressed as mean ± SEM (n = 6). The total SOD data and the Cu/Zn-SOD data were analyzed using 2-way ANOVA ($F_{5,30}$ = 3.187, P < .05 [GalN treatment, P < .01] and $F_{5,30}$ = 7.014, P < .001 [GalN treatment, P < .001]; interaction, P < .05). Symbols above the histograms refer to differences from the control (^{+++}P < .001) using 1-way ANOVA followed by LSD post hoc test.

(P < .05; Table 2); and total histopathologic scores (P < .05; Fig. 4) were significantly reduced by ATC compared with those of GalN-treated animals.

Although the immunohistochemical findings showed a trend that ATC treatment decreases the intensity of MDA adduct staining especially in the periportal and midzonal areas (Fig. 5), there was no statistically significant effect of ATC on mean MDA adduct scores (Fig. 6). However, caspase-3 activation was reduced by ATC compared with that of GalN-treated animals (P = .001; Fig. 7).

Despite these beneficial changes in histologically derived parameters, ATC treatment did not ameliorate the changes in SOD activities or plasma analytes that arose as a consequence of GalN treatment (Figs. 8-10).

3.3. Effects of daidzein

There was no significant effect of daidzein per se on any variable. However, daidzein ameliorated GalN-induced MDA adduct formation (P < .01; Fig. 6). The intensity of the positive staining was reduced in the rats that received daidzein treatment in addition to GalN (Fig. 5). In these rats, the most prominent reactions were localized to the periportal region. Daidzein + GalN also reduced the cytosolic Cu/Zn-SOD activity when compared with the GalN treatment group (P < .01; Fig. 8). All other analytes and measurements were unaffected by daidzein + GalN treatment compared with GalN treatments (P > .05, not significant).

In some circumstances, agents considered as "protective" may act as prooxidants, particularly in high doses. For example, ATC [23] and daidzein [21] have both been reported to be cytotoxic in high levels. However, neither daidzein nor ATC per se induced hepatic damage in terms of the histopathologic score, MDA-protein adduct score, caspase-3 activation, or blood analytes. This confirms that the dosage regimen used in this study was acceptable from an experimental view at the levels used, that is, neither daidzein nor ATC was toxic or injurious to the animals.

4. Discussion

4.1. Effects of GalN

The current data show that GalN caused hepatic injury as indicated by severe necrosis and widespread micro- and macro-steatosis (fatty degeneration) and both lobular and portal inflammations. Raised plasma AST activities and reductions in total protein, albumin, and globulin fractions were also observed supporting the contention that metabolic functions of the liver were altered by GalN treatment.

The impairment of liver metabolism and gross architecture, leading to liver cell death (reflected by increased

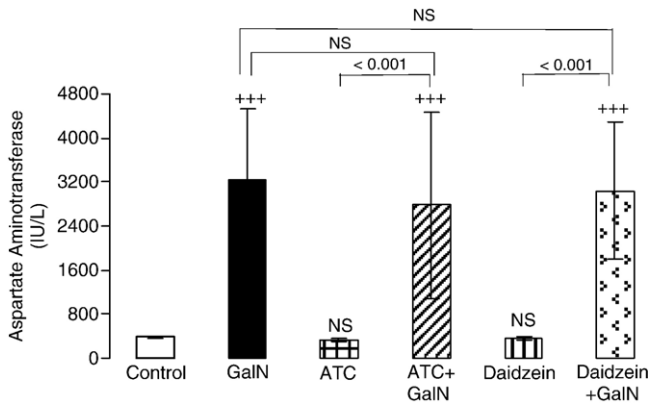

Fig. 9. The effect of different pretreatments (ATC or daidzein) and drug treatment (GalN) on plasma AST activity. Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein dose is 100 mg/kg of BW. All data are expressed as mean ± SEM (n = 6). The data were log transformed, and the values were analyzed using 2-way ANOVA ($F_{5,30}$ = 19.142, P < .001; GalN treatment, P < .001). Symbols above the histograms refer to differences from the control (^{+++}P < .001) using 1-way ANOVA followed by LSD post hoc test.

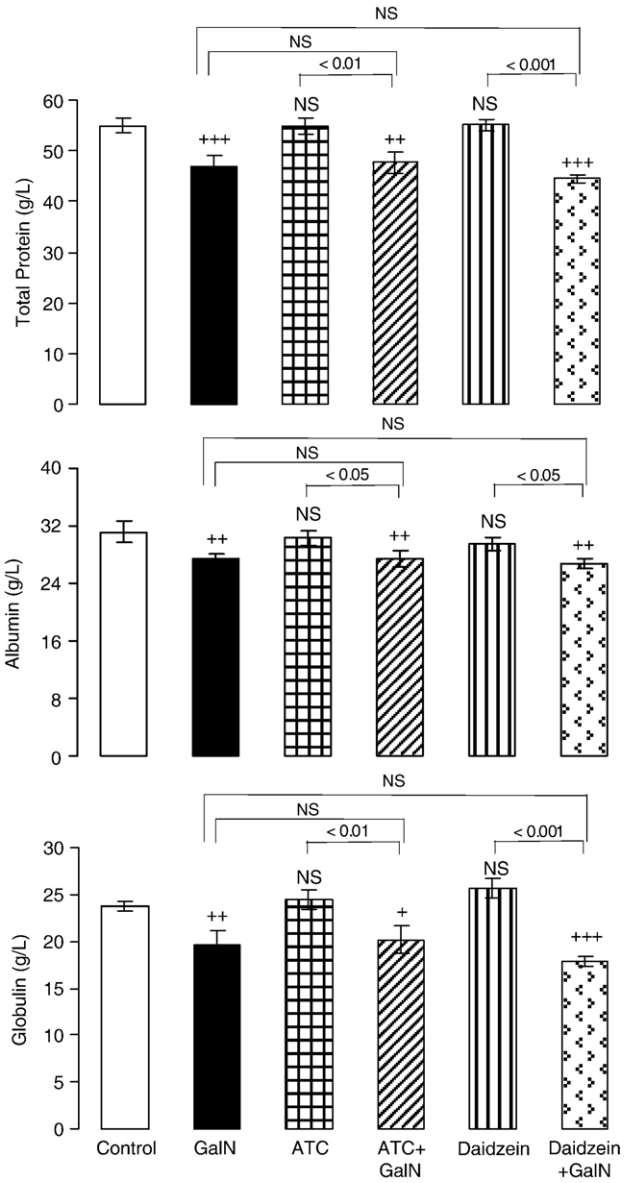

Fig. 10. The change of blood total protein concentration (top), and albumin (middle) and globulin fractions (bottom) in response to different pretreatments (ATC or daidzein) and treatment (GalN). Rats were pretreated with either ATC or daidzein then followed by either saline or GalN treatment 1 hour after the previous injection. Rats were killed 23 hours after the second injection. ATC dose is 30 mg/kg of BW, GalN dose is 1 g/kg of BW, and daidzein dose is 100 mg/kg of BW. All data are expressed as mean ± SEM (n = 6). The total protein data were analyzed using 2-way ANOVA ($F_{5,30}$ = 10.610, $P < .001$; GalN treatment, $P < .001$). The albumin data were analyzed using 2-way ANOVA ($F_{5,30}$ = 3.788, $P < .01$; GalN treatment, $P < .001$). The globulin fraction data were analyzed using 2-way ANOVA ($F_{5,30}$ = 9.914, $P < .001$; GalN treatment, $P < .001$). Symbols above the histograms refer to differences from the control ($^{+}P < .05$, $^{++}P < .01$, $^{+++}P < .001$) using 1-way ANOVA followed by LSD post hoc test.

caspase-3 activation), has been reported to involve the activation of Kupffer cells in the liver that in turn leads to uridine triphosphate depletion and release of tumor necrosis factor α [24,25]. These data also suggest that GalN-induced liver injury involves multiple pathways including both direct oxidative damage and indirect apoptosis induction.

In the present study, GalN administration induced the formation of hepatic MDA-protein adducts, reflective of lipid peroxidation. As far as we are aware, we are the first to report enhanced MDA-protein adduct formation in a model of nonalcoholic liver injury. Previous studies have suggested that the activation of Kupffer cells are involved in the generation of protein adducts in the liver [26]. This supports, but does not prove, the aforementioned suggestion that Kupffer cells play an important role in the pathogenesis of GalN-induced injury [24,25,27,28].

Previous studies showed that there was a decrease in SOD, GSHPx, and SeGSHPx activities 24 hours a after a single dose of GalN [29,30]. These aforementioned observations contradict our present findings, which show a significant increase in Cu/Zn-SOD activities, although GSHPx and SeGSHPx activities were refractory. We are unable to explain these differences between the aforementioned studies. Whereas the former studies used a dosage regimen of 0.5 g/kg [29,30], we used 1 g/kg of BW, which is of the same order of magnitude. However, an increase in SOD activities after administration of hepatotoxic agents or induction of oxidative stress is not unusual. For example, it has been shown that lipopolysaccharide administration increases SOD activities in the liver of rats 24 hours after dosage [31], comparable to our observations. Indeed, increases in SOD have also been observed in the liver in response to a variety of catabolic or pathologic conditions in vivo such as exposure to alcohol [32]; hepatectomy [33]; metabolism of catecholamines [34]; x-ray and hepatopathy [35]; aging [36]; sepsis [37]; and liver transplantation [38]. Nevertheless, we speculate that the up-regulation of total and cytosolic Cu/Zn-SOD activities may be associated with adaptive mechanisms to counteract increased oxidative stress.

4.2. Protective effects of ATC and daidzein

One of the major findings was that ATC and daidzein pretreatment was protective against liver injury as shown by several variables. Supplemental ATC significantly ameliorated changes in necrosis, lobular and portal inflammations, total histopathologic scores, and caspase-3 activation. Daidzein ameliorated GalN-induced changes in MDA adduct scores and Cu/Zn-SOD activity. Thus, both agents appeared to be protective in distinct ways.

4.2.1. Mechanisms of protection with ATC

α-Tocopherol is a well-known antioxidant inhibiting lipid peroxidation and modulating SOD activities [39-42]. Thus, it was reasonable to have assumed that ATC would have behaved similar in the present study. However, we failed to demonstrate a protective effect of ATC on lipid peroxidation as reflected by MDA-protein adducts. α-Tocopherol was also ineffective in ameliorating the GalN-induced changes in SOD activities. The failure to replicate the aforementioned [40-42] may be because either (1) the dosage period of ATC was insufficient to exert an antioxidant effect or (2) the protective effect of

ATC against histopathologic changes involves some nonoxidative pathways. Regarding the first assumption, various studies have shown that ATC may exert an effect on lipid peroxidation in the liver in periods as short as 17 hours [43] or even 2 hours [44]. This leads to the second assumption that the protective effects of ATC on liver histology may involve nonoxidative pathways. This is not unreasonable because ATC affects a variety of pathways and processes such as protein kinase C, protein phosphatase 2A, cyclooxygenase, nuclear factor κB, cell adhesion proteins, chemokines, and transcriptional regulation [45]. Further work is thus needed to elucidate the mechanisms whereby ATC ameliorates GalN-induced morphological damage by nonoxidative pathways.

4.2.2. Mechanisms of protection with daidzein

In contrast to ATC, daidzein pretreatment before GalN administration ameliorated changes in MDA-protein adducts and Cu/Zn SOD activity, which were all related to the imbalance of oxidative status. The precise mechanism whereby these protective effects of daidzein occurred is presently unknown, but both indirect and direct processes have been proposed. For example, similar findings were reported in a study that showed that daidzein administration significantly reduced the MDA concentration in the liver of control animals, by indirect mechanism [8]. These authors ascribed their findings to a mobilization of ATC from nonhepatic sources because the concentration of ATC in the liver and plasma increased after daidzein dosage [8]. We, however, do not think that a similar indirect mechanism occurs in response to GalN; otherwise, we would have seen an ATC-like response in the daidzein-treated animals.

Daidzein pretreatment before GalN administration leads to changes in MDA-protein adducts and Cu/Zn SOD activity that are likely to be protective. However, in contrast to ATC pretreatment, because there was a lack of improvement in the histopathology and a lack of decreased necrosis, it is possible that changes in MDA-protein adduct formation and Cu/Zn SOD activity may not play a direct role in the mechanisms leading to hepatic damage after GalN administration. Indeed, is possible that the data (Tables 1 and 2 and Figs. 4 and 6) suggest a dissociation between MDA-protein adducts and liver histopathology after daidzein pretreatment, and this could be because of a lack of a daidzein-mediated reduction in capase-3 activity, in contrast to ATC pretreatment (Fig. 7).

Indirect antioxidant mechanisms of daidzein action also include up-regulation of antioxidant defense system. Daidzein dosage has been shown to up-regulate GSH concentrations [46,47] and Cu/Zn SOD activity [22]. The mechanism is not clear, but daidzein itself can exert a mild oxidative stress in vitro and it may in turn provoke the antioxidant defense systems to act against the GalN-induced oxidative injury [21]. However, we failed to demonstrate the enhancement of the antioxidant enzymes activities in the liver of daidzein-treated animals.

Alternatively, the weak estrogenic actions of daidzein may confer indirect protective effects. For example, daidzein has been shown to bind and interact with the estrogen receptor in vitro [48]. The importance of this pertains to studies where lipid peroxidation, induced by carbon tetrachloride, has been ameliorated by estrogen [49].

Daidzein may also act directly as a free radical scavenger to terminate lipid peroxidation. For example, the chemical structure of daidzein favors the donation of its hydrogen atoms to the superoxide anions in vitro, thus inhibiting damaging processes such as lipid peroxidation [50]. However, by using electron spin resonance spectroscopy, several studies have failed to demonstrate that daidzein is able to scavenge different types of free radical (such as hydroxyl and superoxide radicals) in vitro [18,51]. This suggests that the antioxidant property of daidzein is complicated and acts via multiple pathways.

Nevertheless, regardless of the etiologic mechanism, it is clear that both ATC and daidzein are protective in their own way.

5. Conclusion

In conclusion, this is the first study to demonstrate that daidzein can offer some protection against the formation of GalN-induced MDA-protein adducts in liver. α-Tocopherol also showed a potential protective effect against GalN-induced necrosis, and portal and lobular inflammations. However, the protective mechanism of both daidzein and ATC remains to be established but may be of potential clinical value.

Acknowledgment

We thank Rajkumar Rajendram and Ross Hunter for the technical support.

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