

Inhibition of diethylnitrosamine-initiated alcohol-promoted hepatic inflammation and precancerous lesions by flavonoid luteolin is associated with increased sirtuin 1 activity in mice

Bruna Paola Murino Rafacho^{1,2}, Camilla Peach Stice¹, Chun Liu¹, Andrew S. Greenberg³, Lynne M. Ausman^{1,4}, Xiang-Dong Wang^{1,4}

¹Nutrition and Cancer Biology Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging (HNRCA) at Tufts University, Boston, MA, USA; ²Department of Internal Medicine, Botucatu School of Medicine, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil; ³Obesity and Metabolism Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging (HNRCA) at Tufts University, Boston, MA, USA; ⁴Friedman School of Nutrition Science and Policy, Tufts University, Boston, MA 02111, USA

Correspondence to: Xiang-Dong Wang, MD, PhD. Nutrition and Cancer Biology Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Room 514, Boston, MA 02111, USA. Email: xiang-dong.wang@tufts.edu.

Background: Chronic and excessive alcohol consumption is an established risk for hepatic inflammation and carcinogenesis. Luteolin is one of the most common flavonoids present in plants and has potential beneficial effects against cancer. In this study, we examined the effect and potential mechanisms of luteolin supplementation in a carcinogen initiated alcohol-promoted pre-neoplastic liver lesion mouse model.

Methods: C57BL/6 mice were injected with diethylnitrosamine (DEN) [i.p. 25 mg/kg of body weight (BW)] at 14 days of age. At 8 weeks of age mice were group pair-fed with Lieber-DeCarli liquid control diet or alcoholic diet [ethanol (EtOH) diet, 27% total energy from ethanol] and supplemented with a dose of 30 mg luteolin/kg BW per day for 21 days.

Results: DEN-injected mice fed EtOH diet displayed a significant induction of pre-neoplastic lesions, a marker associated with presence of steatosis and inflammation. Dietary luteolin significantly reduced the severity and incidence of hepatic inflammatory foci and steatosis in DEN-injected mice fed EtOH diet, as well the presence of preneoplastic lesions. There was no difference on hepatic protein levels of sirtuin 1 (SIRT1) among all groups; however, luteolin supplementation significantly reversed alcohol-reduced SIRT1 activity assessed by the ratio of acetylated and total forkhead box protein O1 (FoxO1) and SIRT1 target proliferator-activated receptor gamma, coactivator 1 alpha (PGC1 α).

Conclusions: Dietary intake of luteolin prevents alcohol promoted pre-neoplastic lesions, potentially mediated by SIRT1 signaling pathway.

Keywords: Luteolin; diethylnitrosamine (DEN); liver cancer; inflammation; sirtuin 1 (SIRT1)

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Introduction

Liver cancer is the third leading fatal cancer in the world (1). Chronic and excessive alcohol consumption is a major risk factor for liver cancer in the United States and it has been estimated that 32-45% of all cases are due to alcohol abuse (2). Alcohol readily diffuses across membranes and distributes through all cells and interacts with proteins and

cell membranes (3), disrupts fatty acid oxidation, thereby contributing to steatosis onset and liver injury (4). We and others have shown that alcohol has a promoter action on the development of chemically induced hepatic cancer (2,5,6). Although the molecular mechanisms of alcohol induced carcinogenesis are not fully understood, increasing evidence suggests a link between alcohol metabolism and cancer

(7-10). Alcohol metabolism involves the enzymes aldehyde dehydrogenase (ALDH), alcohol dehydrogenase (ADH), cytochrome P4502E1 (CYP2E1), and catalase, generating acetaldehyde and reactive oxygen species (ROS) (3). Acetaldehyde adducts and ROS can activate the adaptive immune response and the production of proinflammatory cytokines (4,11,12), leading to inflammation of the liver (7,13). Indeed, cellular and molecular abnormalities due to inflammatory condition might represent an early step of hepatocarcinogenesis and is considered a key factor in the development of cancer (11,14).

Sirtuin 1 (SIRT1), the mammalian ortholog of yeast sirtuin silent information regulator 2, is a conserved NAD⁺-dependent protein deacetylase expressed in various tissues including the liver (15). It has been implicated in various biological processes, and its expression and activity in the liver can be altered by alcohol intake (16,17). SIRT1 activation has been described as a protective factor in many diseases (18-20), including high fat diet promoted hepatocarcinogenesis (15,21). Despite this evidence, the role of SIRT1 and liver cancer is controversial, reported both as a tumor promoter and as a tumor suppressor protein (22-24). SIRT1 has also been a target for dietary intervention in many models, including liver injury (23), addressing questions to whether dietary compounds can modulate SIRT1 expression and/or activity and exhibit anti-carcinogenic effects.

Epidemiological studies have shown that a diet rich in plant-derived foods is consistently associated with reduced risk of developing chronic diseases, supporting recommendations to increase the consumption of plant-derived foods (25,26). Although it is not clear which compounds in plant foods are responsible for this preventive effect, evidence suggests that flavonoids may participate in this activity (26,27). Luteolin is one of the most common flavonoids present in edible plants, with celery, parsley, broccoli, peppers, cabbages, and herbal spices as the main food sources of luteolin (28). It has been shown that luteolin exhibits anti-inflammatory and anticancer properties by inhibiting pro inflammatory cytokines, inducing apoptosis and preventing DNA damage and carcinogen metabolic activation (25,29-31), but most of the studies were conducted *in vitro*. One previous study described anti-carcinogenic potential of luteolin against hepatocellular carcinoma in rats (32). So far to our knowledge, there is no *in vivo* study addressing whether luteolin protection against alcohol induced hepatic carcinogenesis.

The present study investigated a dietary intervention with luteolin at 30 mg/kg of body weight for 21 days in DEN-initiated ethanol-promoted hepatic carcinogenesis in mice. The presence of steatosis, inflammation, and preneoplastic lesions and the involvement of SIRT1 pathway and CYP2E1 in this model were examined.

Methods

Study design

The experimental protocol was adapted from a well-established animal model to study liver carcinogenesis (6,33-35). All animal protocols were approved by the Institutional Animal Care and Use Committee at the Jean Mayer-USDA Human Nutrition Research Center on Aging at Tufts University. Male C57BL6 mice were distributed into four groups by weight-matching, according to diet and treatment. Diethylnitrosamine (DEN, Sigma Aldrich, St. Louis, MO; i.p. 25 mg/kg of BW) was injected into two DEN treated groups at 2 weeks of age. After an adaptation period, all mice were group pair-fed with Lieber-DeCarli (Dyets Inc., Bethlehem, PA, USA) liquid control diet or alcoholic diet (EtOH diet, 27% total energy from ethanol) and supplemented with a dose of 30 mg luteolin/kg BW per day for a treatment period of 21 days. In the control diet, ethanol was replaced by an isocaloric amount of maltodextrin (Purina Test diets, Richmond, IN, USA). Therefore, the animal groups were: (I) control group (no DEN injection or ethanol feeding); (II) control + DEN (DEN injected, no ethanol feeding); (III) DEN + EtOH (DEN injected and ethanol feeding); (IV) DEN + EtOH + L (DEN injected and ethanol feeding + luteolin treatment). After the treatment period, all mice were terminally exsanguinated under anesthesia and liver tissue was collected and frozen for analysis. The study design is presented in *Figure 1*.

Luteolin supplementation

Luteolin commercial compound was purchased from Sigma Aldrich (St. Louis, MO, USA), and was incorporated directly into the diet to achieve a homogenous diet mixture. The rationale for luteolin dose of 30 mg/kg of body weight was based on previous studies reporting an anti-carcinogenic action of luteolin (36,37), daily human intake of luteolin, and data about luteolin absorption in rodents and humans

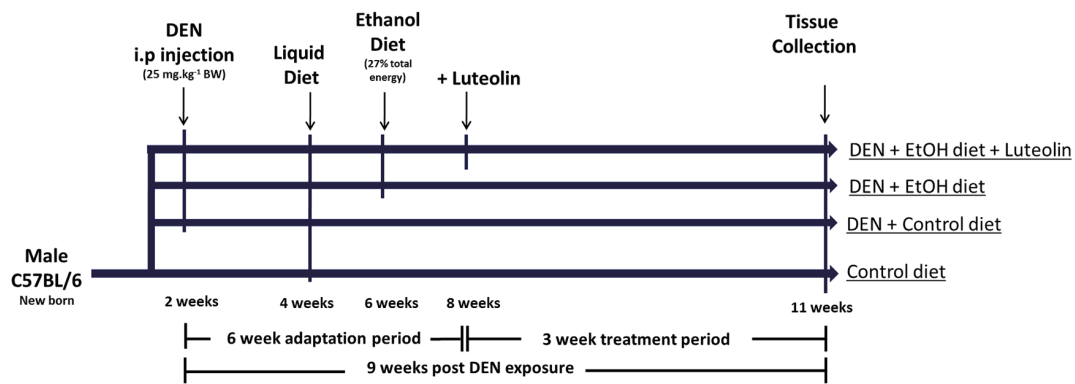


Figure 1 Study design. DEN, diethylnitrosamine; BW, body weight; EtOH, ethanol.

that demonstrated that luteolin has similar pharmacokinetics in the two species (38,39). Luteolin intake in the American population is relatively low, varying from 0.01 to 8.88 mg/d, with an average of 0.1 mg/d (27,40,41). Using an established equation to calculate the dosage equivalence for human consumption (42,43), the dose of 30 mg/kg of body weight in mice is equivalent to 0.081 mg/kg of body weight for humans, meaning a dose within the physiologic range.

Histopathological evaluation

5 μ m sections of formalin-fixed, paraffin-embedded liver tissue were stained with H&E. Liver histopathology was graded according to steatosis magnitude (both macro- and micro-vesicular fat accumulation), and the degree of liver inflammation severity as described previously (15,44). Briefly, the degree of steatosis was graded 0-4 (grading 0 \leq 5%, 1=5-25%, 2=26-50%, 3=51-75%, 4>75%), based on the average percent of fat-accumulated hepatocytes per field at 100 \times magnification under H&E staining in 20 random fields. Inflammatory foci which mainly constitute mononuclear inflammatory cells were evaluated by the number of inflammatory-cell clusters in 20 random fields at 100 \times magnification, as reported as number of inflammatory foci per cm². Preneoplastic lesions were evaluated and presented by the incidence and number of basophilic and eosinophilic foci per cm² in 20 random fields at 100 \times magnification (45).

Protein isolation and western blotting

Liver sections were lysed in cold whole cell lysis buffer (25 mM Hepes at Ph =7.5, 300 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1% Triton X-100)

containing protease inhibitors. The lysates were centrifuged at 15,000 \times g for 10 min at 4 $^{\circ}$ C, and the supernatant used. The protein concentration of the supernatant was measured by the Coomassie Plus protein quantification method (Thermo Fisher Scientific, USA). For western blotting analysis, equal amounts of protein (50 μ g) of each sample were boiled in reducing Laemmli sample buffer and resolved by 8-10% SDS-PAGE. Proteins were then transferred onto Immobilon-P membranes (Millipore Corporation, MA, USA) blocked with 5% non-fat milk in TBST buffer, and incubated with selected primary antibody: acetylated-FoxO1, forkhead box protein O1 (FoxO1), SIRT1, proliferator-activated receptor gamma, coactivator 1 alpha (PGC1 α) (Santa Cruz, TX, USA), NF- κ B, phosphorylated NF- κ B and CYP2E1 (Cell signaling, MA, USA), and respective second antibodies (Santa Cruz, TX, USA). Proteins were detected by a horseradish peroxidase-conjugated secondary antibody (Bio-Rad, CA, USA). The specific bands were visualized by a Super Signal West Pico Chemiluminescent Substrate Kit (Pierce, IL, USA) according to the manufacturer's instructions. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used as internal control. Intensities of protein bands were quantified using GS-710 Calibrated Imaging Densitometer (Bio-Rad, CA, USA).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from frozen liver sections with Trizol reagent (Invitrogen, NY, USA), as previously described (15). cDNA was prepared from the RNA samples using M-MLV reverse transcriptase (Invitrogen, NY, USA) and an automated thermal cycler PTC-200

Table 1 Body weight, liver weight, and liver weight and final body weight ratio¹

Groups	Control	DEN + Control	DEN + EtOH	DEN + EtOH + L
Number of animals	11	14	13	10
Initial body weight (g)	21.5±0.5 ^a	20.6±0.6 ^a	20.3±0.3 ^a	20.7±0.3 ^a
Final body weight (g)	28.6±0.8 ^a	27.4±0.9 ^a	23.8±0.2 ^b	23.7±0.4 ^b
Liver weight (g)	1.19±0.06 ^a	1.36±0.06 ^{a,b}	1.42±0.02 ^b	1.47±0.03 ^b
LW/FBW ²	4.1±1.1 ^a	4.9±0.4 ^b	5.9±0.4 ^c	6.2±0.5 ^c

¹, all data are expressed as mean ± SD; ², ratio between liver weight (LW) and final body weight (FBW). ^{a,b,c}, different letters represent significant difference (P<0.05) between groups. DEN, diethylnitrosamine; EtOH, ethanol; L, luteolin.

(MJ Research, USA). qRT-PCR was performed using FastStart Universal SYBR Green Master (ROX) (Roche, USA). Relative gene expression was determined using the 2- $\Delta\Delta$ CT method. The sequences for TNF α , IL-6 and IL-1 β were: tumor necrosis factor alpha (Tnf- α) forward-CAAACCACCAAGTGGAGGAG, reverse-CGGACTCCGCAAAGTCTAAG; IL-6 forward-GGATACCACTCCCAACAGACCT, reverse-GCCATTGCACAACCTCTTTTCTC; IL-1 β forward-AGCCAAGCTTCCCTTGTGCAAGTGT, reverse-GCAGCCCTTCATCTTTTGGGGTCC. GAPDH was used as internal control, and the sequences were: forward 5'-ATCACCATCTTCCAGGAGCGA-3' and reverse 5'-AGCCTTCTCCATGGTGGTGAA-3'.

Hepatic NAD/NADH ratio evaluation

Quantification of oxidized nicotinamide adenine dinucleotide (NAD) and reduced nicotinamide adenine dinucleotide (NADH) in the liver was carried out using NAD/NADH assay kit from BioVision (Milpitas, CA, USA), according to the manufacturer's instructions. Briefly, livers were snap frozen in liquid nitrogen and extracted at 4 °C with extraction buffer provided with the NAD/NADH assay kit. NAD and NADH values were normalized by protein concentration.

Statistical analysis

Graph Pad Prism (Graph Pad Software, CA, USA) was used to perform the statistical analysis, and resulting values were expressed as mean ± SEM or mean ± SD, as indicated in the footnotes. Group means were compared using one-way ANOVA analysis with adjustments for multiple comparisons. P value was set at 0.05 for the comparisons to reach statistical significance. Different letters indicate significant differences between groups.

Results

Luteolin inhibited steatosis and DEN-initiated ethanol promoted hepatic carcinogenesis

Ethanol fed groups had significantly lower final body weight and significantly higher liver weight in comparison to Control and Control + DEN groups (Table 1). Luteolin had no significant effect on final body weight or liver weight when comparing DEN + EtOH and DEN + EtOH + L groups (Table 1).

Regarding pathology data, all mice in DEN + EtOH group developed steatosis (62% in grade 1 and 39% in grade 2, Figure 2). Luteolin inhibited steatosis in DEN + EtOH + L group, in which 20% of mice presented steatosis in grade 2, 70% with grade 1 and 10% had no steatosis (Figure 2). In DEN injection group without ethanol feeding, one animal presented steatosis in grade 1 (DEN + Control, Figure 2) and steatosis was not detected in the Control group. Similarly, preneoplastic foci, markers of precancerous lesions, were not observed in the absence of ethanol in DEN-injection group (DEN + Control, Figure 3A). DEN + EtOH group showed incidence of 54% of preneoplastic foci (Figure 3A). Preneoplastic foci were not detected after luteolin supplementation (DEN + EtOH + L group and in Control and DEN + Control groups (Figure 3A).

Luteolin reduces hepatic inflammation in DEN-initiated EtOH-promoted hepatic carcinogenesis

DEN + EtOH group had a significantly greater number of inflammatory foci as compared to Control and DEN + Control groups (by approximately 5 fold, Figure 3B). Luteolin supplementation significantly reduced by 50% the number of hepatic inflammatory foci in comparison to DEN + EtOH group (Figure 3B). Regarding inflammatory

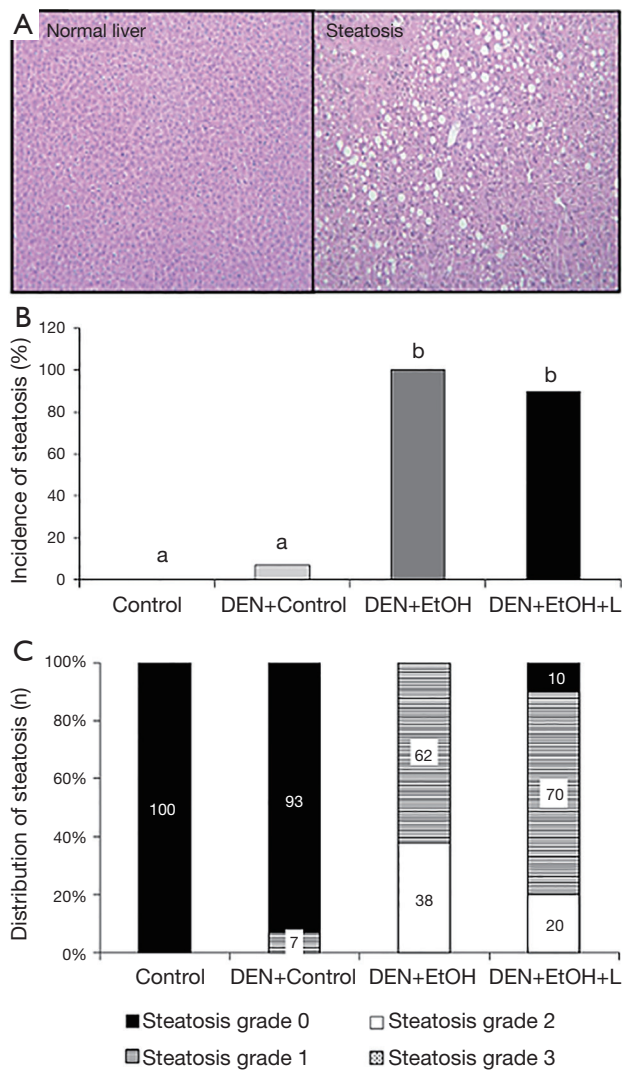


Figure 2 (A) Representative histopathology figure of steatosis observed in DEN + EtOH group; (B) incidence of steatosis between groups. Data represented in percentage; (C) steatosis grade distribution between groups determined by histopathology of liver. Data are expressed in percentage. DEN, diethylnitrosamine; EtOH, ethanol; L, luteolin. ^{a,b}, different letters represent significant difference ($P < 0.05$).

cytokines, no changes in $Tnf-\alpha$ and $Il-6$ mRNA expression were detected between all groups. mRNA expression of $Il-1\beta$ was significantly higher in DEN + Control group in comparison to Control group. DEN + EtOH + L group presented significantly lower $Il-1\beta$ mRNA expression as compared to DEN + Control group, as shown in *Figure 4*. Western blot analysis was conducted to evaluate total $NF-\kappa B$ protein expression, but no differences were observed among the groups (data not shown).

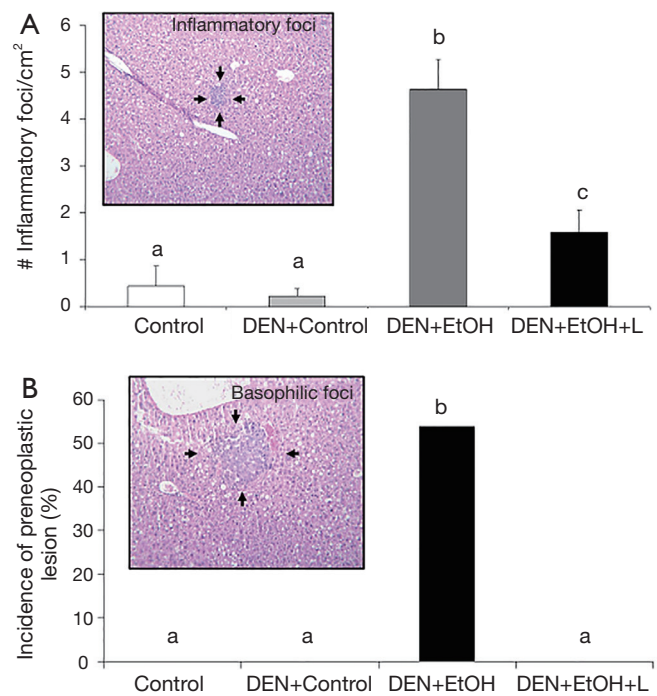


Figure 3 (A) Number of inflammatory foci in the liver with representative histopathology figure of inflammatory foci; (B) incidence of preneoplastic lesions in the liver with a representative histopathology figure of basophilic foci, marker of preneoplastic lesion. Data are expressed as mean \pm SEM. DEN, diethylnitrosamine; EtOH, ethanol; L, luteolin. ^{a,b,c}, different letters represent significant difference ($P < 0.05$).

Reduction in DEN-initiated EtOH-promoted hepatic carcinogenesis by luteolin was associated with restored SIRT1 activity and increased PGC1 α protein expression

Western blot analyses were conducted to evaluate SIRT1 protein expression and activity in the liver, as well as its downstream target PGC1 α . To evaluate SIRT1 activity, the acetylation of FoxO1 was determined. No difference in SIRT1 protein expression was observed among the groups (*Figure 5A*). DEN-EtOH group showed decreased SIRT1 deacetylase activity (*Figure 5B*) in comparison to Control group, as represented by the increased acetylated FoxO1 ratio, and diminished PGC1 α protein expression (*Figure 5C*). Luteolin supplementation significantly increased SIRT1 deacetylase activity and PGC1 α protein expression in DEN + EtOH + L group as compared to DEN + EtOH group (*Figure 5B,C*). No changes in $NAD^+/NADH$ ratio in the liver were observed among all groups (data not shown).

CYP2E1 protein expression was significantly increased in the liver of both ethanol fed groups (DEN + EtOH and

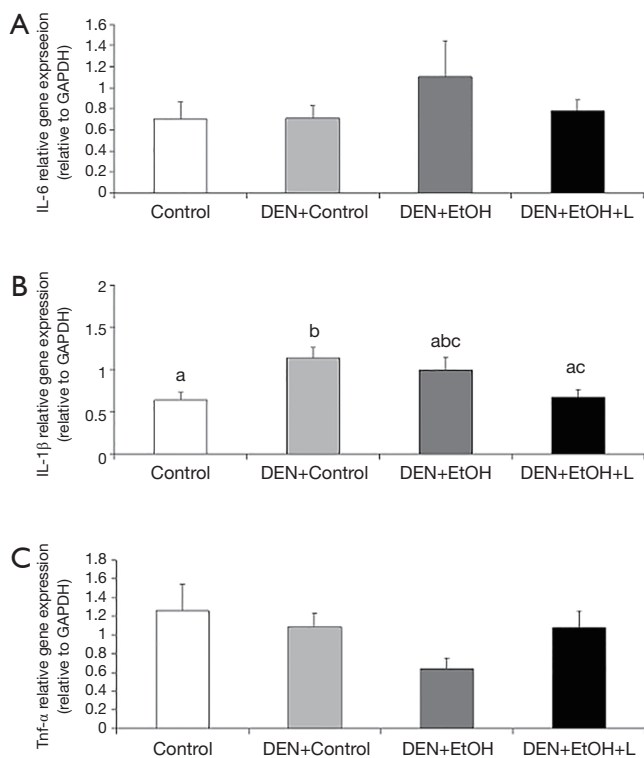


Figure 4 Gene expression of inflammatory cytokines relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the liver. mRNA from liver tissue (n=10-12 per group) were analyzed by real-time PCR and GAPDH was used as loading control. (A) Relative gene expression of IL-6; (B) relative gene expression of IL-1β; (C) relative gene expression of TNF-α. Data shown are mean ± SEM. DEN, diethylnitrosamine; EtOH, ethanol; L, luteolin. ^{a,b,c}, different letters represent significant difference (P<0.05).

DEN + EtOH + L) in comparison with Control and DEN + Control groups, and luteolin did not affect its expression (data not shown).

Discussion

In the present study, ethanol treatment caused a significant increase in preneoplastic lesions associated with steatosis and inflammatory foci after 9 weeks of exposure in DEN-initiated mice. The absence of preneoplastic lesions in the Control mice given the same dose of the DEN carcinogen alone suggests the promoting effect of alcohol intake on hepatic carcinogenesis in this model. This notion was supported by our previous study in a rat model (35). Furthermore, the significantly decreased final body weight

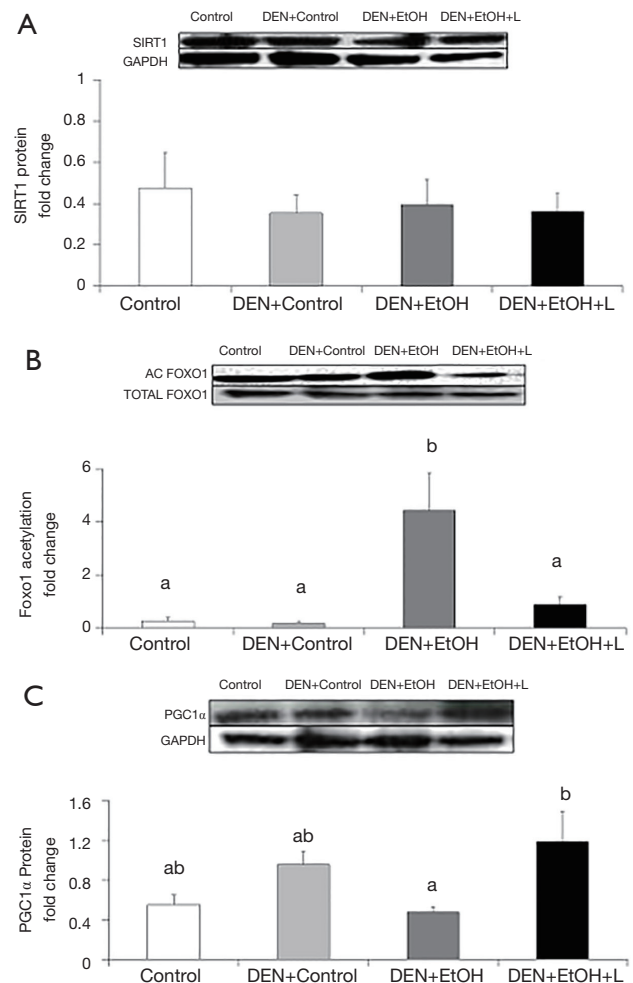


Figure 5 Protein expression in liver lysates analyzed by western blotting (n=10-12 per group) with representative western blots with 1 sample per group in order: Control, DEN + Control, DEN + EtOH and DEN + EtOH + L in: (A) graphical representation of fold changes in sirtuin 1 (SIRT1); (B) graphical representation of fold changes in FoxO1 acetylation (total FoxO1 as loading control), representing SIRT1 activity; (C) graphical representation of fold changes in proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α). GAPDH was used as loading control unless specified otherwise. Data shown are mean ± SEM. DEN, diethylnitrosamine; EtOH, ethanol; L, luteolin. ^{a,b}, different letters represent significant difference (P<0.05).

and significantly increased liver weight found in DEN initiated ethanol fed groups provided evidence of the deleterious effect of alcohol intake in growth and liver function in this model.

To our knowledge this is the first report showing that treatment with luteolin significantly inhibited DEN-

initiated alcohol-promoted liver pre-cancerous lesions associated with inhibition of steatosis and inflammatory foci in mice. Besides the promotion of carcinogenesis, the effects of long-term alcohol intake include a number of biochemical and molecular alterations and pathological lesions such as steatosis (4,35). The anti-steatosis effect of luteolin reported in the present study is supported by previous reports showing that luteolin and herb extracts containing luteolin can inhibit lipid accumulation in the liver (46,47). Our findings also complement previous studies with cell lines and different murine models showing that luteolin has anti-cancer (29,32,48-50) and anti-inflammatory properties (29,51,52). It is also important to note that the protective effect of luteolin was not associated with changes in the body weight of mice, suggesting minimal toxicity of luteolin intake.

It is well known that carcinogenesis is a process that involves the transition of a normal cell into a preneoplastic lesion that might develop into a malignant tumor, but the precise mechanism of alcohol-associated carcinogenesis is not fully understood. In our findings, the presence of preneoplastic lesions in DEN + EtOH group was associated with a significant increase in the number of inflammatory foci and IL-1 β , suggesting the role of inflammation in the progression of cancer in this model (11). To support this concept, hepatic inflammatory foci have been shown to have a good correlation with carcinogenicity (45,53). Luteolin treatment inhibited inflammatory foci and reduced gene expression of IL-1 β and IL-6 in comparison to DEN + EtOH group. TNF- α expression was not increased in DEN + EtOH group, but it is important to note that luteolin restored its gene expression to levels similar to the control group. Therefore, the anti-inflammatory properties of luteolin could be related to the anti-carcinogenic effect observed in this work. This notion is supported by Lin *et al.* (28) that postulated that the anti-carcinogenic potential of luteolin is related to reduced TNF α and IL-1 by inhibition of NF- κ B signaling. In this study, we did not detect changes in NF- κ B protein expression and the effect found in inflammatory cytokine expression, such as TNF α was marginal. This could be due to the use of homogenates of hepatic tissue with varying sites of lesions that might make the differences difficult to detect.

In order to explore the mechanisms by which luteolin prevents alcohol induced liver injury, we evaluated the SIRT1 protein expression and deacetylase activity. In the present study, DEN-EtOH group presented an increase in

FoxO1 acetylation and a decrease in SIRT 1 downstream target PGC1 α protein expression, suggesting decreased SIRT1 deacetylase activity. This finding is supported by a previous work of our laboratory showing that alcohol intake impairs the function of SIRT1 (16). Interestingly, the protective effect of luteolin was accompanied by induction of SIRT1 activity, demonstrated by decreased acetylation of FoxO1 and increased PGC1 α protein expression.

Decreased SIRT1 activity has been linked to chronic inflammation, and it was previously reported in liver cancer (54). SIRT1 regulates many cellular processes through deacetylating FoxO family transcription factors, such as FoxO1 (55). Decreased SIRT1 deacetylation leads to increased FoxO1 expression, enhancing inflammation by affecting the transcription of pro-inflammatory genes like TNF α and IL-6 (54). Our results are in accordance with these observations, since DEN + EtOH group showed increased acetylated FoxO1 in association with inflammatory response. Luteolin seems to inhibit inflammation through SIRT1 activation, since the FoxO1 acetylation and inflammatory foci were decreased in luteolin treated mice. No previous studies have been found about luteolin and sirtuins, but similar results are described for resveratrol, a flavonoid considered a SIRT1 activator, with anti-inflammatory and anti-carcinogenic action (56-59).

PGC-1 α is the master regulator of mitochondrial biogenesis and function (60,61). SIRT1 deacetylates PGC-1 α , enhancing PGC1 α activity (60,62). In the present study, decreased SIRT1 activity observed in the DEN + EtOH group was accompanied by decreased PGC1 α protein expression. This observation is supported by previous reports showing that overexpression of SIRT1 in livers of mice significantly increases the expression of genes that are under the control of PGC-1 α , whereas SIRT1 knockdown attenuated this effect (63). Deletion of SIRT1 can alter fatty acid metabolism in the liver and PGC1 α can activate the oxidative metabolism by increasing mitochondrial biogenesis and increasing fatty acid oxidation (61,63-65). In the present study, the diminished SIRT1-PGC1 α signaling could be associated to the increased steatosis found after ethanol feeding in DEN initiated mice. Decreased PGC1 α expression has also been reported in a variety of cancers, and PGC1 α seems to regulate a crosstalk between energy metabolism and inflammation (66). Some reports suggest that overexpression of PGC1 α can suppress the secretion of pro inflammatory cytokines (65,67). The increased SIRT1 activity induced by luteolin could activate PGC1 α , reduce steatosis and inflammation caused by DEN and ethanol, thus contributing

to the anti-tumorigenic action found in this study. Similarly, Wang and colleagues described that activation of SIRT1 by resveratrol treatment reduced tumorigenesis in mice, associated with increased levels of PGC1 α (68).

In order to investigate how luteolin can affect SIRT1 activity, we evaluated NAD⁺/NADH ratio in the liver. SIRT1 function is tightly regulated by NAD⁺ availability (54), and this availability can be reduced by alcohol oxidation (3,69). However, the decreased SIRT1 activity found in our study seems to be mediated by a different mechanism, since no differences on NAD⁺/NADH ratio was observed between all groups.

Since CYP2E1 is an important pathway of ethanol metabolism, and its induction by chronic alcohol intake results in an enhanced activation of procarcinogens to carcinogens and release of inflammatory mediators (70), we evaluated the role of CYP2E1 expression in the present study. DEN initiated ethanol fed mice showed significantly increased protein expression of the enzyme as compared to control diet groups, but luteolin did not reverse CYP2E1 expression. These data support our notion that the potential protective effect of luteolin is related to SIRT1 signaling.

Conclusions

This study demonstrated that luteolin prevents alcohol promoted hepatic carcinogenesis potentially mediated by a SIRT1 signaling pathway. Our findings support the potential use of dietary means for preventing alcohol induced liver lesions and open a new avenue for research on interaction between luteolin and SIRT1.

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