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GREEN TEA EXTRACT INHIBITS EARLY ONCOGENIC RESPONSES IN MICE WITH NONALCOHOLIC STEATOHEPATITIS

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ABSTRACT

Nonalcoholic steatohepatitis (NASH) increases hepatocellular carcinoma (HCC) risk. We hypothesized that the hepatoprotective anti-inflammatory benefits of catechin-rich green tea extract (GTE) would protect against HCC progression by inhibiting NASH-associated liver injury and pro-oncogenic responses. We used an HCC model in high-fat (HF)-fed mice that mimics early oncogenic events during NASH without inducing tumorigenesis and premature mortality. Male C57BL/6J mice (4-wk old) were fed a HF diet containing GTE at 0% or 2%. Mice were administered saline or diethylnitrosamine (DEN; 60 mg/kg, i.p.) at 5-wk and 7-wk of age. NASH, inflammation, fibrosis, and oncogenic responses were assessed at 25-wk of age. Saline-treated mice showed prominent histopathological signs of steatosis and hepatocellular ballooning. Although DEN did not impact adiposity, steatosis, ballooning and hepatic lipid accumulation, these parameters were attenuated by GTE regardless of DEN. Hepatic lipid peroxidation and fibrosis that were increased by DEN were attenuated by GTE. Hepatic TLR4, MCP1 and TNFα mRNA levels were unaffected by DEN, whereas iNOS was increased by DEN. These transcripts were lowered by GTE. GTE attenuated the frequency of PCNA+ hepatocytes and mRNA expression of cyclin D1, MIB1 and Ki-67 that were otherwise increased by DEN. GTE increase APAF1 mRNA that was otherwise lowered by DEN. Relative to saline-treated mice, DEN increased mRNA levels of oncostatin M, gp130, c-Fos, c-Myc and survivin; each was lowered by GTE in DEN-treated mice. These findings indicate that GTE may protect against hepatic oncogenesis by limiting early steps in the carcinogenic cascade related to NASH-associated HCC.

KEYWORDS:

Cell proliferation; Diethylnitrosamine; Hepatocellular carcinoma; Green tea extract; Nonalcoholic steatohepatitis

INTRODUCTION

Nonalcoholic steatohepatitis (NASH), which is mediated by inflammation and oxidative stress, provokes the progression towards hepatocellular carcinoma (HCC).[1] The burden of HCC has been historically related to viral infection. However, evidence supports that NASH potentiates HCC risk and that its contribution towards HCC is outpacing that from hepatitis B and C, and alcoholic fatty liver disease.[2] Treatment options for advanced liver disease, including HCC, are limited due to the poor responses to a host of pharmacologic therapies and the risks associated with liver transplantation.[3, 4] Given the limited treatment options, preventive strategies focusing on dietary approaches have been emphasized to not only reduce the risk of NASH, but also its pathogenic progression towards HCC.

Experimental models of HCC include administration of carcinogens, intra-hepatic or -portal injections of tumor cells, genetic models or xenograft approaches.[5] These models are effective for studying overt HCC characterized by advanced tumorigenesis. However, the aggressive etiology of these models occurs in association with severe morbidity or premature mortality that limits an understanding of the early events driving the NASH-to-HCC development. Indeed, the progression from NASH to HCC is an early, but critical transition of liver injury characterized by altered molecular, genomic, and pathological signatures not shared with more advanced stages of liver disease.[6] Advanced fibrosis and cirrhosis are prerequisite for HCC development and both share signal transducer and activator of transcription (STAT)-mediated inflammatory and redox signaling pathways that trigger HCC development.[7] Thus, a need exists to study the pathologic progression of NASH to HCC in the absence of aggressive tumorigenesis to establish dietary approaches that prevent early initiating events leading to HCC.

The hepatocellular carcinogen diethylnitrosamine (DEN) induces irreversible HCC[8, 9] through a STAT3-dependent 'inflammation-fibrosis-cancer axis'.[10] Although NASH can be induced by a high-fat (HF) diet alone, it occurs with minimal fibrosis even after extended exposure (36-50 wk).[11] In contrast, fibrosis and hepatocellular proliferation are effectively induced by DEN in a

STAT3-dependent manner.[10] The administration of DEN in mice fed a HF diet has therefore been established as a model system to study the early, pre-oncogenic events implicated in the NASH-to-HCC transition.[12]

Findings from a population-based, case-control study suggest that chronic green tea consumption is associated with a lower risk of developing HCC.[13] A meta-analysis of prospective cohort studies also indicated that high green tea intakes are associated with a lower risk of liver cancer.[14] These benefits may be attributed to epigallocatechin gallate (EGCG), the major catechin in green tea, which was shown to selectively induce apoptosis and cell cycle arrest in a HCC cell line but not in non-cancerous hepatocytes.[15] Further, our report indicates that catechin-rich green tea extract (GTE) protects against NASH in obese mice by inhibiting hepatic inflammation consistent with a mechanism involving the gut-liver axis that limits metabolic endotoxemia and hepatic TLR4/NFκB activation.[16] Despite strong evidence that GTE alleviates NASH, it remains unclear whether its reported anti-inflammatory activities also protect against HCC progression otherwise induced by NASH. Thus, we hypothesized that GTE would prevent the progression towards HCC by limiting NASH-associated liver injury and pro-oncogenic responses. To test this, we adapted our well-established model of HF-induced NASH to incorporate DEN exposure. This permitted the present study examining GTE during NASH in association with DEN-induced HCC progression on hepatic injury, inflammation, and cell proliferation.

MATERIALS AND METHODS

Study Design

This study was approved by the Institutional Animal Care and Use Committee at The Ohio State University (Protocol #2012A00000156-R2) in accordance with the Animal Welfare Act and the guidelines of the Public Health Service as issued in the Guide for the Care and Use of Laboratory Animals. Forty, 3-wk old, male C57BL/6J mice (Jackson Laboratory) were acclimated for 1-wk. At 4-wk of age, equal numbers were randomized to an HF diet containing powdered GTE at 0% or 2% (w/w) until study termination at 25-wk of age. At 5-wk and 7-wk of age, equal numbers of mice within each dietary treatment were administered DEN (60 mg/kg body mass; i.p.) or saline vehicle once per week. At 25-wk of age, fasted mice (8-h) were sacrificed while under 2% isoflurane anesthesia. Liver and adipose tissues were excised, washed in phosphate buffered saline, snap-frozen in liquid nitrogen, and stored at -80°C. A portion of liver was also collected into 10% buffered formalin or RNAlater® (Sigma-Aldrich).

Mice and Diets

Only male mice were used in these studies because of their greater susceptibility to HF-induced obesity[17] and DEN-induced carcinogenesis.[18] The HF diet contained 60% of energy from fat (Research Diets) and was formulated with GTE (Unilever BestFoods). Complete dietary compositional details have been reported.[19] The catechin content of GTE was confirmed by HPLC-UV.[20] It contained 30% of total catechins (w/w) with the following distribution of specific catechins: 48% EGCG, 31% epigallocatechin, 13% epicatechin gallate, 8% epicatechin. GTE at 2% was used based on our reports that it alleviates NFκB activation and NASH without inducing hepatotoxicity.[16, 21] This level is also extrapolated from epidemiological observations suggesting that green tea (≥10 servings/d) lowers the risk for liver injury and metabolic abnormalities.[22]

DEN Administration

DEN is well-established to induce HCC, but its hepatoxicity is dependent on the interaction between dose (5-90 mg/d), frequency of administration, and basal diet composition.[9, 20] Younger

rodents (15-d old) are also more susceptible to HCC than older rodents (>6-wk old).[23] Based on our hypothesis that GTE would protect against NASH-associated HCC risk, we aimed to establish a model system focusing on early responses in the NASH-to-HCC transition without inducing premature mortality or overt tumorigenesis. For dose selection, a pilot study (n = 20) was conducted based on others[9, 24] in which 3-wk old mice were administered DEN one-time at 75 mg/kg (i.p.) in 100 µL saline. By 48-h, 40% mortality occurred, and all remaining mice experienced severe morbidity that required immediate euthanasia. The dose of DEN was therefore reduced to 60 mg/kg and administered once-weekly at 5- and 7-wk of age. Under these conditions, severe morbidity was averted, and 100% of mice survived until study termination at 25-wk of age. Further, gross pathological assessment indicated no evidence of HF- or DEN-induced hepatic tumors.

Metabolic Chemistries and Lipid Peroxidation

Serum alanine aminotransferase (ALT) and glucose were determined using clinical assays (Pointe Scientific). Serum insulin was measured by ELISA (Crystal Chem), and then insulin resistance (homeostatic model assessment of insulin resistance; HOMA-IR) was calculated.[25] Hepatic lipid was extracted as we described,[26] and then resolubilized in 1% (v/v) Triton X-100 to measure triglyceride by clinical assay (Pointe Scientific). Hepatic malondialdehyde (MDA), a lipid peroxidation biomarker, was measured by HPLC-FL and normalized to hepatic protein as we described.[27]

Histological Analysis

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin to assess NASH, as we described.[21] In brief, ten randomly selected fields (200x) from each section were scored in a blinded manner for liver steatosis, hepatocellular ballooning, and inflammatory infiltrates. A composite NASH activity score was calculated by summing steatosis, hepatocellular ballooning, and inflammatory infiltrate scores as described.[28] Hepatic fibrosis was

assessed following Masson's trichrome staining at 80x magnification.[28] Fibrotic lesions were quantified from 10 randomly selected fields using NIH ImageJ.

Immunohistochemical Analysis

Hepatocellular proliferation was assessed by quantifying proliferating cell nuclear antigen-positive foci (PCNA+). In brief, paraffin-embedded sections were deparaffinized in xylene, rehydrated, treated for antigen retrieval, and washed for 10-min with 3% (v/v) hydrogen peroxide prepared in methanol. Following serum-free protein blocking (10-min), sections were incubated for 30-min with PCNA primary antibody (1:200; Dako #M0879). After washing with wash buffer, and incubating (30-min) with biotinylated horse anti-mouse secondary antibody (1:200; Dako #BA-2000), sections were rinsed and incubated (30-min) with ABC Elite complex (Vector Labs). Sections were then treated with 3,3'-diaminobenzidine (Dako #SK-4100) for 5-min, counterstained with hematoxylin, treated with 1% ammonium hydroxide, and dehydrated in ethanol. PCNA+ hepatocytes were quantified in a blinded manner at 200x from 10 randomly selected fields and normalized per 100 hepatocytes.

Real Time q-PCR

Hepatic total RNA was extracted using Trizol (Thermo) and cDNA was synthesized using iScript reverse transcription kit (BioRad). RT-PCR analysis was performed using a SYBR Green PCR kit on a CFX384 instrument (Bio-Rad) using primers from Sigma-Aldrich (**Table 1**).[29] Target gene expression was quantified relative to 18S and HPRT using the $2^{-\Delta\Delta CT}$ method.[30]

Statistical Analysis

Data (means ± SE) were analyzed using GraphPad Prism (version 8). Two-way ANOVA was used to evaluate main effects due to DEN and GTE and their interaction. When a statistically significant DEN x GTE interaction was observed, Newman-Keuls post-hoc test was used to evaluate all potential group-wise differences. Mean differences were annotated such that those not sharing a common superscript (a>b>c) were significantly different from each other. Data not meeting assumptions for equal variance were log-transformed prior to analysis.

Pearson correlations were calculated by linear regression to define pair-wise relationships between variables. All analyses were statistically significant at $P \leq 0.05$.

RESULTS

GTE protects against HF-induced obesity

Neither DEN nor GTE affected energy intake throughout the 21-wk intervention (**Table 2**). However, body mass was attenuated by DEN and GTE, but without any DEN x GTE interactive effects. Adiposity was attenuated by GTE, but was unaffected by DEN. In agreement, only GTE attenuated subcutaneous and retroperitoneal adipose mass whereas both DEN and GTE attenuated mesenteric adipose mass. However, a DEN x GTE interaction indicated that GTE attenuated epididymal adipose mass only in DEN-treated mice. Liver mass was attenuated by DEN and was additionally lowered by GTE. Body mass was correlated with adiposity ($P<0.0001$; r = 0.93), suggesting that limiting adiposity was responsible for limiting body mass gain.

GTE attenuates histopathological changes associated with NASH

We reported that GTE supplementation for 8-wk protects against HF-induced NASH.[16, 21, 31, 32] In the present 21-wk intervention, GTE similarly protected against hepatic steatosis and hepatocyte ballooning (**Fig 1**). DEN did not affect these histopathologic NASH parameters and neither GTE nor DEN affected inflammatory infiltrates. Further, GTE regardless of DEN treatment attenuated the composite NASH score.

GTE lowers hepatic lipid accumulation, lipid peroxidation and hepatic injury

Histopathological observations were corroborated by direct assessment of hepatic lipid (**Fig 2A-B**). Although a DEN x GTE interaction was observed, post-hoc analysis indicated that hepatic total lipid was attenuated by GTE to a similar extent regardless of DEN treatment. However, hepatic triglyceride was attenuated by 12-20% in DEN-treated mice whereas its accumulation was 46-51% lower in GTE-supplemented mice. Serum ALT exhibited an interactive effect indicating that GTE attenuated the DEN-mediated increase in ALT to the

extent lowered among saline-treated controls that were provided GTE (**Fig 1C**). Adiposity correlated with hepatic total lipid (P<0.0001; r = 0.62) and triglyceride (P<0.0001; r = 0.82). Serum ALT also correlated with adiposity (P<0.0001; r = 0.77) and hepatic total lipid (P<0.0001; r = 0.79). These correlations suggest that limiting obesity helps to protect against NASH-associated liver injury.

Based on our findings that GTE limits oxidative stress during NASH,[16] we hypothesized that GTE would limit DEN-mediated lipid peroxidation that is implicated in HCC progression.[33] Indeed, DEN increased hepatic MDA by 32-54% compared with saline-treated controls whereas GTE attenuated MDA by 25-36% regardless of DEN treatment (**Fig 2D**). Hepatic MDA also correlated with hepatic total lipid (P<0.01; r = 0.42) and serum ALT (P<0.0001; r = 0.61), suggesting that limiting liver steatosis protects against liver injury attributed to lipid peroxidation.

GTE limits DEN-induced hepatic fibrosis

Consistent with DEN potentiating liver fibrosis, which drives HCC development,[34] we hypothesized that GTE would protect against DEN-induced fibrogenesis. DEN-treated mice had marked evidence of portal and periportal fibrosis (**Fig 3A**). Although GTE had no effect on fibrosis in saline-treated mice (**Fig 3B**), GTE inhibited DEN-induced increases in fibrosis to the extent observed in saline-treated controls. Fibrosis severity also correlated with MDA (P<0.0001; r = 0.82) and ALT (P<0.001; r = 0.53), suggesting that oxidative stress contributes to fibrosis-associated liver injury.

GTE attenuates hepatic inflammation during NASH regardless of DEN treatment

We reported that GTE alleviates hepatic TLR4/NFκB-mediated inflammation that is otherwise increased by HF-feeding.[35] In agreement, GTE regardless of DEN administration attenuated TLR4, TNFα, and MCP1 mRNA expression by 34-79% (**Fig 4**); DEN had no effect on their expression. However, a DEN x GTE interaction indicated that DEN increased hepatic iNOS expression by 38% (**Fig 4**), whereas GTE in DEN-treated mice inhibited iNOS expression to that of the lowered levels observed in saline- and GTE-treated mice. Hepatic TLR4 mRNA

levels were correlated with serum ALT (P<0.01; r = 0.47) and fibrosis severity (P<0.01; r = 0.43), suggesting that pro-inflammatory signaling by TLR4 contributes to hepatic injury and fibrosis. Serum ALT also correlated with TNFα (P<0.001; r = 0.58), MCP1 (P<0.0001; r = 0.72), and iNOS (P<0.001; r = 0.59) mRNA levels in agreement with evidence that inflammation contributes to NASH-associated hepatic injury.[16, 31, 32]

GTE limits DEN-induced hepatocellular proliferation

HCC development is mediated by hepatocellular proliferation,[36] which is exacerbated by DEN in rodents fed a HF diet[12] Immunohistochemical studies indicated clear evidence that DEN treatment increased the number of PCNA+ hepatocytes (**Fig. 5A**). GTE regardless of DEN administration significantly attenuated PCNA+ hepatocytes by 48% in both saline and DEN treated mice. PCNA+ hepatocytes were correlated serum ALT (P<0.0001; r = 0.92) and MDA (P<0.0001, r = 0.61) as well as with mRNA levels of TLR4 (P<0.01; r = 0.46), TNFα (P<0.001, r = 0.59), MCP1 (P<0.0001; r = 0.69), and iNOS (P<0.01; r = 0.44). These findings support the concept that hepatocellular proliferation in association with liver injury is likely mediated by heightened inflammatory responses and oxidative damage.

GTE-mediated protection against DEN-induced hepatocellular proliferation was corroborated by assessing mRNA levels of cyclin D1, mindbomb E3 ubiquitin protein ligase-1 (MIB1), Ki-67 and the apoptotic protease activating factor 1 (APAF1) (**Fig 5B**). DEN increased cyclin D1, MIB1 and Ki-67 expression compared with saline-treated controls. GTE attenuated cyclin D1, MIB1 and Ki-67 expression by 33-46%, 40-43%, and 47-57%, respectively, compared with mice fed no GTE. Further corroborating these benefits, PCNA+ hepatocytes were correlated with cyclin D1 (P<0.001; r = 0.57), MIB1 (P<0.001; r = 0.58) and Ki-67 (P<0.0001; r = 0.72) mRNA levels. Hepatic fibrosis also correlated with mRNA levels of cyclin D1 (P<0.01; r = 0.42), MIB1 (P<0.001; r = 0.53) and Ki-67 (P<0.001; r = 0.52). Together, these correlative observations suggest that limiting hepatocellular proliferation helps to protect against fibrosis.

mRNA expression of APAF1, a mediator of apoptosis, that was otherwise lowered by DEN was also increased by GTE (**Fig 5B**).

GTE attenuates oncogenic signals otherwise increased by DEN

Because DEN-induces HCC in a STAT3-dependent manner,[10] we evaluated mRNA expression levels of pro-oncogenic signals upstream and downstream of STAT3 activation that mediate HCC. Oncostatin M and gp130 promote signal transduction to induce STAT3 activation.[37] In agreement, DEN increased mRNA expression levels of both gp130 and oncostatin M (**Fig 6A**). GTE regardless of DEN treatment limited their mRNA expression, and gp130 expression in DEN-treated mice was attenuated to extent observed among saline controls provided GTE. We also observed a significant correlation between PCNA+ hepatocytes and oncostatin M ($P<0.001$; r = 0.59) and gp130 ($P<0.01$; 0.56), which supports the concept that activating STAT3 increases hepatocellular proliferation.

The proto-oncogenes c-Fos, c-Met, and c-Myc and the anti-apoptotic gene survivin mediate hepatocellular proliferation downstream of STAT3 activation.[38-41] Based on our observation that GTE attenuated DEN-induced hepatocellular proliferation (**Fig 5**) and limited gene expression upstream of STAT3 activation (**Fig 6A**), we hypothesized that expression levels of c-Fos, c-Met, c-Myc and survivin also would be attenuated by GTE. Data in **Fig 6B** indicate that mRNA levels of c-Fos, c-Myc and survivin were increased by DEN; c-Met was increased by ~1.7-times but without any statistical significance. Consistent with our hypothesis, GTE attenuated c-Fos, c-Myc, and survivin mRNA levels, and both c-Fos and c-Myc were lowered to the extent observed in saline controls whereas survivin mRNA levels were lowered regardless of DEN treatment (**Fig 6B**). We also observed that survivin was inversely correlated with APAF1 ($P<0.01$; r = -0.45), supporting that improved apoptotic responses are associated with reduced oncogenesis.

DISCUSSION

The findings of this investigation in a well-established model of dietary HF-induced liver injury demonstrate that chronic GTE supplementation protects against DEN-mediated HCC risk by limiting NASH in association with reducing inflammation, oxidative damage, fibrosis and hepatocellular proliferation. Data show, in agreement with our reports,[16, 32] that GTE prevented NASH (i.e. steatosis, hepatocellular ballooning) consistent with a mechanism that limits TLR4/NFκB-mediated inflammation. GTE also protected against DEN-mediated increases in lipid peroxidation and fibrosis that occurred with increased hepatocellular proliferation markers. Further, GTE limited the expression of oncostatin M and gp130, which promote STAT3 activation, while also decreasing STAT3-dependent pro-oncogenic genes (c-Fos, c-Myc, survivin) that were otherwise increased in DEN-treated mice. Together, GTE likely protects against HCC development by limiting pathological responses leading to NASH, and their consequent pro-oncogenic signals that potentiate hepatocellular proliferation.

Persons with NASH compared to those with simple steatosis have increased susceptibility to HCC and greater risk of mortality.[42] Indeed, NASH provokes advanced fibrosis and cirrhosis in association with increased hepatocellular proliferation.[43] The development of NASH is mediated by TLR4/NFκB inflammatory responses along with increased oxidative distress.[44] Under these circumstances, STAT3 becomes activated, which promotes tumorigenesis through a reciprocal interaction between pro-inflammatory and oncogenic signaling in agreement with evidence that HCC patients have elevated STAT3 expression.[45] Thus, persistent oxidative stress and pro-inflammatory responses in NASH-afflicted livers together with increased hepatocellular proliferation creates a microenvironment that favors carcinogenesis. DEN-induced hepatocellular carcinogenesis closely recapitulates the ‘inflammation-fibrosis-carcinogenesis’ response that characterizes the pathogenesis of HCC.[10] Further, a HF diet exacerbates DEN-mediated HCC by inducing NASH and its milieu of inflammation and oxidative damage. Thus, our approach in HF mice exposed to low-dose DEN provided the most suitable model to test our

hypothesis focusing on GTE to prevent the NASH-to-HCC transition that is marked by fibrosis and increased hepatocellular proliferation. Indeed, the low-dose of DEN used did not cause any tumors, but this does not preclude the possibility of any preneoplastic lesions, which requires separate study.

NASH-associated HCC is driven by inflammation and oxidative stress that provoke dysregulated cellular proliferation.[36] Our study specifically focused on these early events that are implicated in the NASH-to-HCC progression. Indeed, the frequency of DEN administration and age of mice in combination with HF-feeding for 21-wk resulted in a model that lacked overt tumorigenesis or pre-mature mortality but had marked NASH with inflammation, oxidative stress, fibrosis, and hepatocellular proliferation. This contrast reports in which observable HCC is induced by DEN at a higher dose and/or greater frequency to younger rodents.[24] Nonetheless, in the present model system, data demonstrated that chronic GTE supplementation, similar to our earlier works in relatively acute models,[35] alleviates NASH by limiting TLR4/NFκB inflammation. Thus, the benefits of GTE along the gut-liver axis that limit endotoxin-mediated increases in TLR4/NFκB inflammation[35] likely help to alleviate DEN-induced HCC risk.

Oxidative stress is established to drive NASH development,[46] but DEN independent of HF-feeding also increases oxidative modification of DNA.[33] Although STAT1 signaling mediates NASH and fibrosis, whereas STAT3 mediates HCC, both pathways are commonly induced by oxidative distress.[47] We reported previously that GTE in obese mice with NASH attenuated hepatic lipid peroxidation and improved endogenous antioxidant defences.[48] In agreement, the present study shows that GTE attenuated hepatic MDA that was otherwise increased in DEN-treated mice compared with saline-treated controls. This supports that the GTE-mediated decrease in hepatic oxidative stress helps to lower the progression towards HCC consistent with oxidative stress being recognized to potentiate hepatocellular proliferation by inducing fibrosis.[10]

Our prior reports indicate that GTE attenuates HF-induced NASH by limiting hepatic NFκB activation and its downstream pro-inflammatory responses (e.g. TNFα, MCP-1) consistent with a mechanism of downregulating TLR4/MyD88 signalling.[16, 49, 35] In agreement, GTE in the present study attenuated TLR4 and NFκB-dependent genes (i.e. TNFα, MCP1, iNOS) regardless of DEN administration. Interestingly, DEN increased hepatic lipid peroxidation in association with increased iNOS, but not other NFκB-dependent genes. This is likely attributed to iNOS being regulated by redox-sensitive transcription factors other than NFκB.[50] Indeed, although STAT3 mediates oncogenesis, it also functions to transcriptionally regulate iNOS.[51] Further, that DEN did not increase hepatic TNFα or MCP1 in the present study is likely attributed to differences in model systems. Specifically, relatively mild inflammation was observed in the present study in which DEN was administered once-weekly on two occasions among 5-7 wk old mice whereas others show that a lower dose of DEN administered twice-weekly for 11-wk markedly increased hepatic inflammation in association with tumorigenesis.[10] Thus, in addition to differences in DEN dose and frequency that likely limited hepatic inflammation in the present study, younger rodents also show greater susceptibility to DEN-induced inflammation and hepatoxicity.[23]

Advanced fibrosis provokes the progression from NASH to HCC.[52] iNOS mediates fibrogenesis by increasing oxidative stress and inducing extracellular matrix production, which highlights therapies that target of iNOS to manage fibrosis.[53] DEN induces iNOS expression independent of HF-feeding[54] whereas a HF-diet alone does not induce marked fibrosis.[11] Our data show that DEN exacerbated fibrosis in HF mice, which was alleviated by GTE to levels of saline controls that had limited histological evidence of fibrosis. Although a comprehensive investigation of the anti-fibrotic mechanism of GTE was beyond the scope of this study, GTE has been reported to attenuate DEN-induced increases in hydroxyproline and collagen accumulation.[55] Thus, limiting DEN-induced fibrosis by GTE likely contributes to its

hepatoprotection against HCC risk consistent with fibrosis mediating the transition of NASH to HCC.[7]

We also evaluated hepatocellular proliferation as a critical driver of HCC. An increased hepatic PCNA+ phenotype predominates in patients with liver cancer.[56] We show that HF and DEN-treated mice had a greater frequency of PCNA+ hepatocytes that was attenuated by GTE to the extent reduced by GTE in saline controls. These effects are likely due to GTE limiting NFκB- and STAT3-dependent signaling that otherwise increases cell proliferation and susceptibility towards HCC.[57] In agreement with GTE limiting PCNA+ hepatocytes, it also attenuated cell proliferation markers (cyclin D1, MIB1, Ki-67) and restored an apoptotic marker (APAF1) that were increased and decreased, respectively, by DEN. These data are consistent with reports that cyclin D1 overexpression is associated with DEN-induced HCC,[58] whereas DEN causes tumorigenesis by inhibiting APAF1-mediated apoptosis.[59] Thus, these findings suggest that GTE protects against the progression of NASH to HCC by favorably altering cellular proliferation and apoptosis.

Not only does STAT3 inhibition protect against HCC, it inhibition also alleviates NASH to likely mediate its benefits on HCC development.[60] In support that GTE protects against NASH-associated HCC in a STAT3-dependent manner, our findings show that GTE inhibited signal transducers (gp130, oncostatin M)[37] that promote STAT3 activation while also downregulating STAT3-dependent genes (c-Fos, c-Myc, survivin) that promote tumorigenesis. Thus, our findings support a complementary mechanism by which STAT3 activation could be inhibited in addition to prior evidence *in vitro* indicating that EGCG directly binds STAT3 to prevent its phosphorylation.[61]

CONCLUSION

The global prevalence of NASH-associated HCC is expected to increase.[1] Due to the poor prognosis of and lack of treatment for HCC, prophylactic dietary strategies that manage NASH

risk are needed to prevent the progression towards HCC. Our findings support that catechin-rich GTE protects against DEN-induced oncogenesis in a NASH model that is highly susceptible to HCC. The protective benefits of GTE on NASH-associated HCC risk are likely attributed to lowering NFκB- and STAT3-dependent signaling that induce inflammation, cell survival, hepatocellular proliferation, and oncogenesis that collectively potentiate HCC.[62, 63] Thus, these data can help to establish a timely dietary strategy against HCC and are expected to support future trials in persons at risk for developing HCC. This would ultimately lead to evidence-based dietary recommendations that promote optimal health by limiting NASH-associated oncogenesis.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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AUTHOR CONTRIBUTIONS

PD, JBK, CC, JL, GYS, BDO, KLS and JMT performed the experiments and analyzed the data; CC, JMT, SKC and RSB conceived the study design; PD and RSB wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Table 1. Primers used for mRNA expression studies using RT-qPCR.[1]

Gene	Forward (5' to 3')	Reverse (5' to 3')	Accession #
18S	CGCTTCCTTACCTGGTTGAT	GAGCGACCAAAGGAACCATA	NM_001013237
APAF1	CACACATCCTGTTGCTCGTC	GAGTGGCCTGACTTTTGACCT	NM_001042558
c-Fos	CGAAGGGAACGGAATAAGATG	GCTGCCAAAATAAACTCCAG	NM_010234
c-Met	GACTTCAGCCATCCCAATGT	GGTGAACTTCTGCGTTTGC	XM_006505002
c-Myc	TGAAGTTCACGTTGAGGGG	AGAGCTCCTCGAGCTGTTTG	NM_001177353
Cyclin D1	AGGCAGCGCGCGTCAGCAGCC	TCCATGGCGCGGCCGTCTGGG	XM_006507413
gp130	GAGCTTCGAGCCATCCGGGC	AAGTTCGAGCCGCGCTGGAC	NM_010560
HPRT	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC	NM 013556
iNOS	TTCTGTGCTGTCCCAGTGAG	TGAAGAAAACCCCTTGTGCT	NM_010927
Ki-67	CATCAGCCCATGATTTTGCAAC	CTGCGAAGAGAGCATCCATC	XM_006507413
MCP1	TGATCCCAATGAGTAGGCTGGAG	ATGTCTGGACCCATTCCTTCTTG	NM_011333
MIB1	GTCATCCCAGTCTCCAGGATTCTGAA	GGACCAAAAGCCTAACAATCTGGGT	NM_001364997
Oncostatin M	GCAGCTGTGGCTTTCTCTGG	TCGTCCCATTCCCTGAAGAC	XM_030245653
TNFα	CTCCAGGCGGTGCCTATG	GGGCCATAGAACTGATGAGAGG	M11731
Survivin	ATCCACTGCCCTACCGAGAA	CTTGGCTCTCTGTCTGTCCAGTT	NM009689

[1]Abbreviations: APAF1, apoptotic protease activating factor 1; HPRT, hypoxanthine phosphoribosyltransferase; c-Myc, avian myelocytomatosis virus oncogene cellular homolog; c-Met, mesenchymal to epithelial transition; gp130, glycoprotein 130; iNOS, inducible nitric oxide synthase; MCP1, monocyte chemoattractant protein-1; MIB1, mindbomb E3 ubiquitin protein ligase-1; TNFα, tumor necrosis factor-α

Table 2. Phenotypic characteristics of mice fed a HF diet containing GTE at 0% or 2% for 21 wk and treated with DEN (60 mg/kg) or saline vehicle at 5-wk and 7-wk of age.[1,2]

	Saline	Saline + GTE	DEN	DEN + GTE	DEN	GTE	DEN × GTE
Initial body mass (g)	19.5 ± 0.3	19.8 ± 0.3	19.8 ± 0.3	19.3 ± 0.4	*P*>0.05	*P*>0.05	*P*>0.05
Final body mass (g)	47.9 ± 0.7	35.6 ± 1.0	44.6 ± 1.6	32.8 ± 0.9	*P*<0.05	*P*<0.0001	*P*>0.05
Energy intake (kcal/d)	12.4 ± 0.2	11.9 ± 0.2	12.4 ± 0.2	12.0 ± 0.2	*P*>0.05	*P*>0.05	*P*>0.05
Subcutaneous adipose (g)	3.7 ± 0.2	1.6 ± 0.2	3.7 ± 0.4	1.2 ± 0.1	*P*>0.05	*P*<0.0001	*P*>0.05
Epididymal adipose (g)	2.2 ± 0.2^{b}	2.2 ± 0.2^{b}	2.8 ± 0.1^{a}	1.7 ± 0.2^{b}	*P*>0.05	*P*<0.01	*P*<0.01
Retroperitoneal adipose (g)	1.6 ± 0.1	0.8 ± 0.0	1.2 ± 0.1	0.7 ±0.1	*P*>0.05	*P*<0.0001	*P*>0.05
Mesenteric fat (g)	1.3 ± 0.1	0.4 ±0.0	0.9 ± 0.1	0.3 ± 0.1	*P*<0.01	*P*<0.0001	*P*>0.05
Adiposity (%)[3]	19.1 ± 0.9	14.5 ± 0.9	19.9 ± 1.0	12.5 ± 0.9	*P*>0.05	*P*<0.0001	*P*>0.05
Liver mass (g)	2.1 ± 0.1^{a}	1.1 ± 0.0^{c}	1.6 ± 0.1^{b}	1.0 ± 0.0^{c}	*P*<0.01	*P*<0.0001	*P*<0.05

[1]Abbreviations: DEN, diethylnitrosamine; GTE, green tea extract; HF, high-fat.

[2]Data (means ± SEM, n = 10 mice/group) were analyzed by 2-way ANOVA. *P*-values for main effects due to DEN and GTE, and their interaction are reported in the table. Newman-Keuls post-test was used to assess any significant interactions. Groups not sharing a superscript are different (*P*≤0.05).

[3] Adiposity was calculated by dividing total adipose mass (sum of epidydimal, retroperitoneal, subcutaneous, and mesenteric fat depots) by terminal body mass.

FIGURE LEGENDS

Fig 1. GTE limits NASH in HF-fed mice regardless of DEN treatment. Male C57BL/6J mice (4-wk old) were fed a HF diet containing GTE at 0% or 2% for 21-wk. At 5-wk and 7-wk of age, equal numbers of mice within each dietary treatment were administered saline or DEN (i.p.; 60 mg/kg). Liver sections were stained with hematoxylin and eosin, and images (100x) were evaluated for NASH. Hepatic steatosis, hepatocyte ballooning and inflammatory infiltrates as well as their sum (i.e. NASH activity score) were determined. Data (means ± SE, n = 10/group) were analyzed by two-way ANOVA with Newman-Keuls post-test as appropriate to evaluate effects due to DEN, GTE or their interaction. Groups not sharing common superscript are different ($P \leq 0.05$). Abbreviations: DEN, diethylnitrosamine; GTE, green tea extract; HF, high-fat; NASH, nonalcoholic steatohepatitis.

Fig 2. GTE prevents hepatic lipid accumulation, lipid peroxidation and liver injury in mice fed a HF diet regardless of DEN treatment. Male C57BL/6J mice (4-wk old) were fed a HF diet containing GTE at 0% or 2% for 21-wk. At 5-wk and 7-wk of age, equal numbers of mice within each dietary treatment were administered saline or DEN (i.p.; 60 mg/kg). **A)** GTE in HF-fed mice lowered hepatic total lipid regardless of DEN administration. **B)** GTE-supplemented mice had lower hepatic triglyceride relative to mice fed no GTE regardless of DEN administration. **C)** DEN administration increased serum ALT in HF mice, whereas GTE attenuated serum ALT to a similar extent regardless DEN administration. **D)** Hepatic MDA was increased by DEN in HF mice. GTE attenuated hepatic MDA in HF mice regardless of DEN administration. Data (means ± SE, n = 10/group) were analyzed by two-way ANOVA with Newman-Keuls post-test as appropriate to evaluate effects due to DEN, GTE or their interaction. Groups not sharing common superscript are different ($P \leq 0.05$). Abbreviations: ALT, alanine aminotransferase; DEN, diethylnitrosamine; GTE, green tea extract; HF, high-fat; MDA, malondialdehyde.

Fig 3. GTE attenuates DEN-induced hepatic fibrosis in HF-fed mice. Male C57BL/6J mice (4-wk old) were fed a HF diet containing GTE at 0% or 2% for 21-wk. At 5-wk and 7-wk of age, equal numbers of mice within each dietary treatment were administered saline or DEN (i.p.; 60 mg/kg). **A)** Masson trichrome stained liver sections were assessed for fibrosis (80x magnification). Representative images at 80x and 400x (inset) magnification are shown. **B)** DEN administration increased fibrosis in HF mice compared with saline controls. GTE protected against fibrosis in DEN-treated mice. Data (means ± SE, n = 10/group) were analyzed by two-way ANOVA with Newman-Keuls post-test as appropriate to evaluate effects due to DEN, GTE or their interaction. Groups not sharing common superscript are different ($P \leq 0.05$). Abbreviations: DEN, diethylnitrosamine; GTE, green tea extract; HF, high-fat.

Fig 4. GTE attenuates hepatic inflammation. Male C57BL/6J mice (4-wk old) were fed a HF diet containing GTE at 0% or 2% for 21-wk. At 5-wk and 7-wk of age, equal numbers of mice within each dietary treatment were administered saline or DEN (i.p.; 60 mg/kg). Unlike iNOS, DEN did not increase hepatic mRNA levels of TLR4, TNFα, or MCP1. GTE regardless of DEN lowered TLR4, TNFα, MCP1 and iNOS expression. Data (means ± SE, n = 10/group) were analyzed by two-way ANOVA with Newman-Keuls post-test as appropriate to evaluate effects due to DEN, GTE or their interaction. Groups not sharing common superscript are different ($P \leq 0.05$). Abbreviations: DEN, diethylnitrosamine; GTE, green tea extract; HF, high-fat; iNOS, inducible nitric oxide synthase; MCP1, monocyte chemoattractant protein 1; TLR4, toll-like receptor-4; TNFα, tumor necrosis factor-α.

Fig 5. GTE limits hepatocellular proliferation in mice fed HF-diet regardless of DEN treatment. Male C57BL/6J mice (4-wk old) were fed a HF diet containing GTE at 0% or 2% for 21-wk. At 5-wk and 7-wk of age, equal numbers of mice within each dietary treatment were administered saline or DEN (i.p.; 60 mg/kg). **A)** PCNA+ cells were quantified in a blinded-manner from 10 randomly selected fields (200x) and normalized per 100 hepatocytes. DEN

increased the number of hepatic PCNA+ cells in HF mice, whereas GTE lowered PCNA+ cells in both saline and DEN treated mice. **B)** mRNA expression of hepatic cell-proliferation markers cyclin D1, MIB1 and Ki-67 were increased whereas, APAF1, inducer of apoptosis was lowered by DEN compared with saline controls. Cyclin D1, MIB1 and Ki-67 expression were lowered by GTE regardless of DEN treatment. GTE prevented the DEN-induced loss of APAF1 expression. Data (means ± SE, n = 10/group) were analyzed by two-way ANOVA with Newman-Keuls post-test as appropriate to evaluate effects due to DEN, GTE or their interaction. Groups not sharing common superscript are different ($P \leq 0.05$). Abbreviations: APAF1, apoptotic peptidase activating factor-1; DEN, diethylnitrosamine; GTE, green tea extract; HF, high-fat; MIB1, mindbomb E3 ubiquitin protein ligase-1; PCNA, proliferating cell nuclear antigen.

Fig 6. GTE attenuates DEN-induced increases in proto-oncogenic signals. Male C57BL/6J mice (4-wk old) were fed a HF diet containing GTE at 0% or 2% for 21-wk. At 5-wk and 7-wk of age, equal numbers of mice within each dietary treatment were administered saline or DEN (i.p.; 60 mg/kg). **A)** mRNA expression of oncostatin M and gp130 were increased by DEN and attenuated by GTE regardless of DEN treatment. **B)** mRNA expression of c-Fos, c-Myc and survivin were increased by DEN. GTE attenuated c-Fos and c-Myc only in DEN-treated mice whereas GTE lowered survivin regardless of DEN treatment. Data (means ± SE, n = 10/group) were analyzed by two-way ANOVA with Newman-Keuls post-test as appropriate to evaluate effects due to DEN, GTE or their interaction. Groups not sharing common superscript are different ($P \leq 0.05$). Abbreviations: c-Myc, avian myelocytomatosis virus oncogene cellular homolog; c-Met, mesenchymal to epithelial transition; DEN, diethylnitrosamine; GTE, green tea extract; HF, high-fat; gp130, glycoprotein 130.

Fig. 1

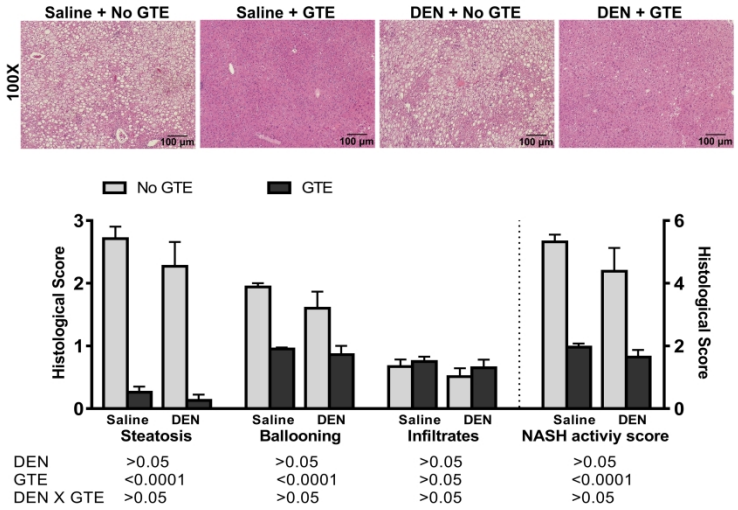

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Fig. 2

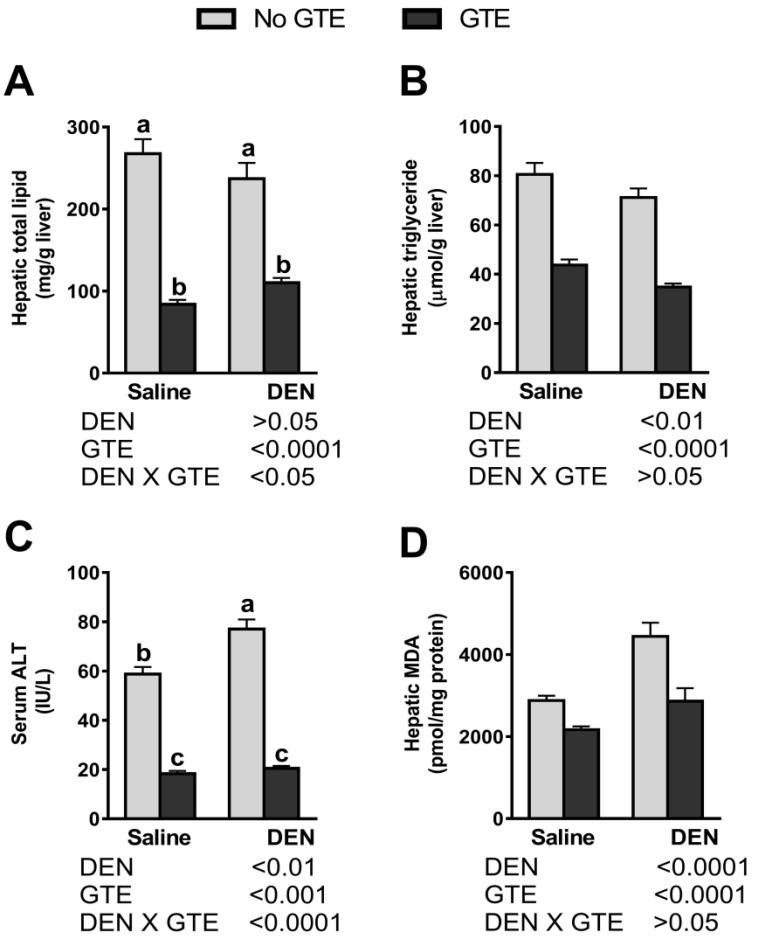

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Fig. 3

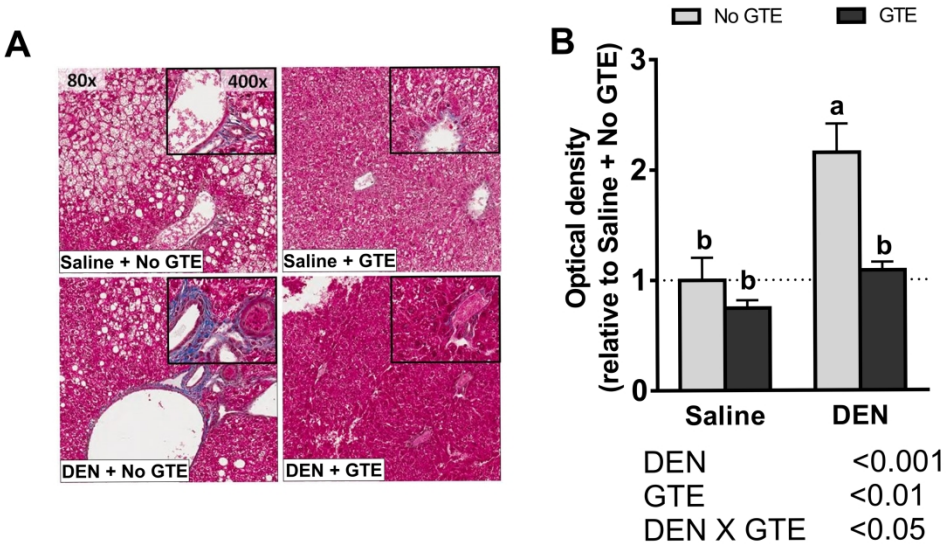

Fig 3. GTE attenuates DEN-induced hepatic fibrosis in HF-fed mice. Male C57BL/6J mice (4-wk old) were fed a HF diet containing GTE at 0% or 2% for 21-wk. At 5-wk and 7-wk of age, equal numbers of mice within each dietary treatment were administered saline or DEN (i.p.; 60 mg/kg). A) Masson trichrome stained liver sections were assessed for fibrosis (80x magnification). Representative images at 80x and 400x (inset) magnification are shown. B) DEN administration increased fibrosis in HF mice compared with saline controls. GTE protected against fibrosis in DEN-treated mice. Data (means ± SE, n = 10/group) were analyzed by two-way ANOVA with Newman-Keuls post-test as appropriate to evaluate effects due to DEN, GTE or their interaction. Groups not sharing common superscript are different (P≤0.05). Abbreviations: DEN, diethylnitrosamine; GTE, green tea extract; HF, high-fat.

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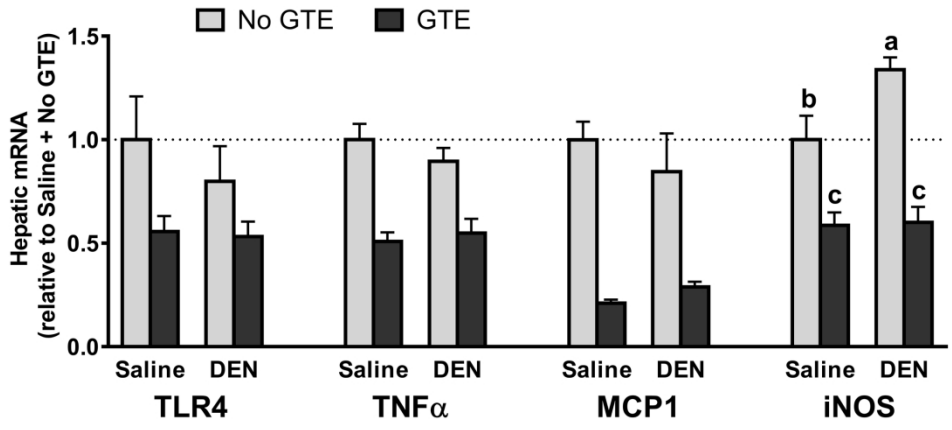
Fig. 4



	TLR4	TNFα	MCP1	iNOS
DEN	>0.05	>0.05	>0.05	<0.05
GTE	<0.001	<0.0001	<0.0001	<0.0001
DEN X GTE	>0.05	>0.05	>0.05	<0.05

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Fig. 5

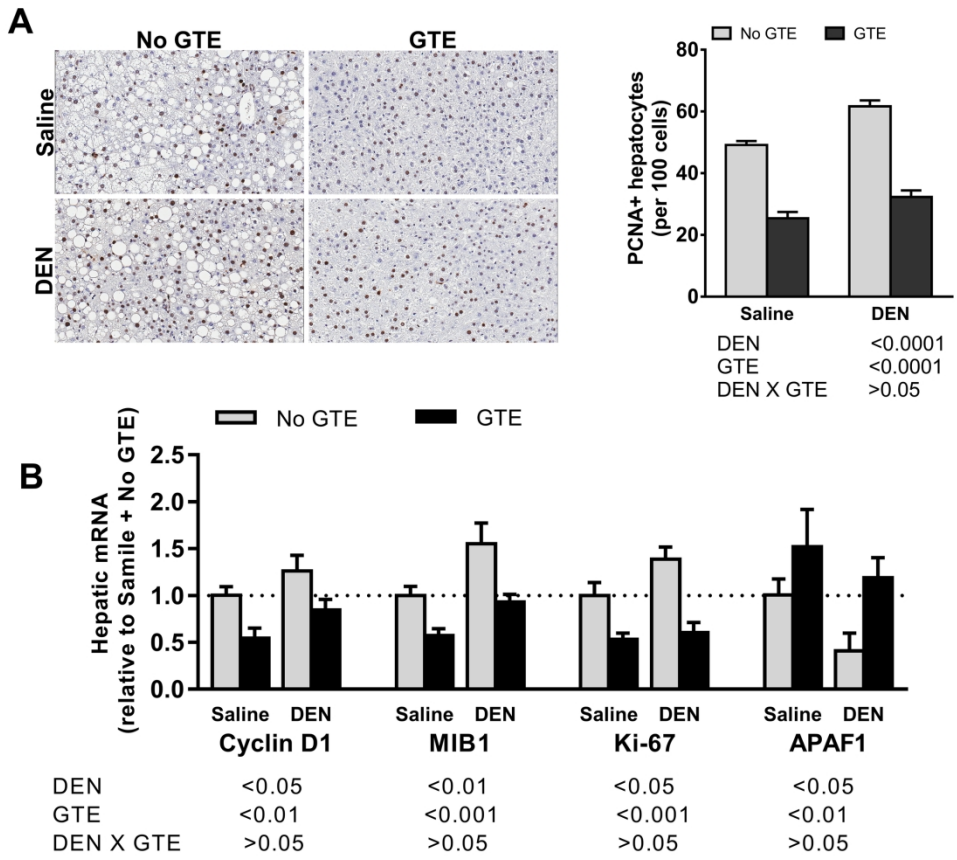

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Fig. 6

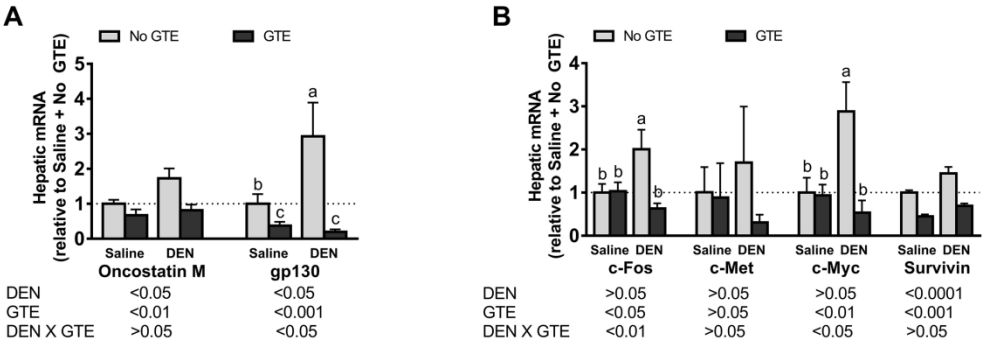

Fig 6. GTE attenuates DEN-induced increases in proto-oncogenic signals. Male C57BL/6J mice (4-wk old) were fed a HF diet containing GTE at 0% or 2% for 21-wk. At 5-wk and 7-wk of age, equal numbers of mice within each dietary treatment were administered saline or DEN (i.p.; 60 mg/kg). A) mRNA expression of oncostatin M and gp130 were increased by DEN and attenuated by GTE regardless of DEN treatment. B) mRNA expression of c-Fos, c-Myc and survivin were increased by DEN. GTE attenuated c-Fos and c-Myc only in DEN-treated mice whereas GTE lowered survivin regardless of DEN treatment. Data (means ± SE, n = 10/group) were analyzed by two-way ANOVA with Newman-Keuls post-test as appropriate to evaluate effects due to DEN, GTE or their interaction. Groups not sharing common superscript are different ($P \leq 0.05$). Abbreviations: c-Myc, avian myelocytomatosis virus oncogene cellular homolog; c-Met, mesenchymal to epithelial transition; DEN, diethylnitrosamine; GTE, green tea extract; HF, high-fat; gp130, glycoprotein 130.

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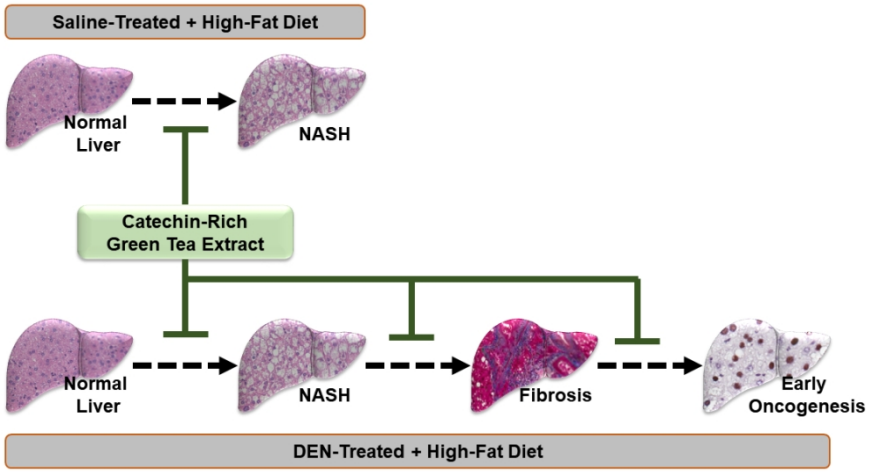

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