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Research Paper

Chemopreventive and hepatoprotective effects of Epigallocatechin-gallate against hepatocellular carcinoma: role of heparan sulfate proteoglycans pathway

Mohamed M. Darweish[a], Ahmed Abbas[b], Mohamed A. Ebrahim[c] and Mohammed M.H. Al-Gayyar[a]

[a]Department of Clinical Biochemistry, [b]Deparment of Pharmacognosy, Faculty of Pharmacy and [c]Oncology Center, Mansoura University, Mansoura, Egypt

Keywords
caspase-3; epigallocatechin-gallate; fibroblast growth factor; heparan sulfate proteoglycans; syndecan-1

Correspondence
Mohammed M.H. Al-Gayyar, Clinical Biochemistry, Faculty of Pharmacy University of Mansoura, Mansoura, 35516 DK, Egypt.
E-mail: mhgayyar@yahoo.com

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Abstract

Objective Epigallocatechin-gallate (EGCG) claims a plethora of health benefits including protection against neoplastic diseases. Meanwhile, heparan-sulfate proteoglycans (HSPGs) have defensive role against tumour cell invasion. Therefore, the chemopreventive and hepatoprotective effects of EGCG were studied in hepatocellular carcinoma (HCC) *in vivo* and *in vitro* and compared with strong water soluble antioxidant, sodium ascorbate.

Methods HCC was induced in SD rats by thioacetamide (200 mg/Kg). Some rats were treated with EGCG (20 mg/Kg) or sodium ascorbate (100 mg/Kg). Liver impairment was assessed by measuring serum α-fetoprotein and investigating liver sections stained with H/E. Hepatic HSPGs, syndecan-1 and matrix metalloproteinase-9 (MMP-9) were measured by ELISA. Gene expression of fibroblast growth factor (FGF)-2 was measured. Cell death was assessed by caspase-3 activity. In addition, all markers were measured in human hepatocellular carcinoma cell line (HepG2).

Key findings EGCG increased the animal survival and decreased both α-fetoprotein and HepG2 viability. In addition, EGCG ameliorated fibrosis and massive hepatic tissue breakdown. EGCG restored HSPGs and reduced expression of MMP-9, syndecan-1 and FGF-2 *in-vivo* and *in-vitro*. Sodium ascorbate showed significantly lower results than EGCG.

Conclusions Besides antioxidant activity, other mechanisms are involved in the chemopreventive and hepatoprotective effects of EGCG including restoration of HSPGs receptors and inhibition of vascular invasion.

Introduction

The incidence of hepatocellular carcinoma (HCC) is becoming more and more significant both clinically and epidemiologically. HCC represents the fifth most common cancer in the world and the third most frequent cause of mortality among oncological patients (for review[1]). HCC is likely to rise from normal cells through the accumulation of multiple genetic changes, growth factors, nuclear factors and proinflammatory cytokines.[2]

Heparan sulfate proteoglycans (HSPGs) are present on the cell surface of most animal cells and are major elements of extracellular matrix (ECM).[3] HSPGs carry out a myriad of structural and signalling functions through their ability to bind to diverse protein ligands, including growth factors, growth factor receptors, cytokines, chemokines, proteases, matrix proteins and cell adhesion molecules.[4] HSPGs consist of a limited set of core proteins to which are covalently attached to one or more sugar chains called heparan sulfate chains. The chains are linear polysaccharides consisting of up to 200 repeating disaccharide units of uronic acid, glucuronic or iduronic acid, linked to glucosamine. Syndecan and glypican families are examples of cell surface HSPGs.[5] HSPGs function as anchor for lipoprotein lipase on the surface of capillary endothelial cells and participate in prevention of cell infiltration and intercellular adhesion.

HSPGs have very important defensive role against invasion of tumour cells. It has been reported that the activity of HSPGs degrading enzyme in high-invasive cancer cells was considerably higher than that in low-invasive cells.[6]

The role of chemotherapy in the treatment of patients with HCC remains controversial. There is no drug or protocol of treatment that can be recommended as standard therapy for HCC patients. Due to the lack of any effective systemic chemotherapy, there is an urgent need to investigate new drugs. Natural agents and herbal products are the main targets of this drug search, hoping that they would boost actions or reduce toxicity of conventional chemotherapeutic drugs.[7] One eligible such candidate is epigallocatechin-gallate (EGCG), a polyphenolic catechin that is abundant in green tea. EGCG claims a plethora of health benefits that include dietary protection against cardiovascular, neurological and neoplastic diseases.[8,9] The antineoplastic potential of EGCG has been evaluated in some animal models.[10–13] The multifaceted EGCG inhibition of the tumourigenesis is attributed to a unique combination of antioxidant, antiproliferative and proapoptotic effects.[14–16] In addition, EGCG showed antitumour activity against HCC through deactivation of growth factors like vascular endothelial growth factor and insulin growth factor.[17,18] However, no previous study investigated the antitumour activity of EGCG on HCC through HSPGs receptors pathway and its subsequent effect on fibroblast growth factor (FGF)-2. Therefore, we conducted the following study to evaluate the chemopreventive and hepatoprotective effects of EGCG *in vivo* and *in vitro* models of HCC. EGCG was compared with a strong water soluble antioxidant, sodium ascorbate, to elucidate if the antitumour activity of EGCG is attributed uniquely to its antioxidant activity or other mechanisms were involved. In addition, the effect of EGCG on HSPGs receptors and its subsequent effects on vascular invasion were studied.

Methods

Animals and their treatment outlines

The animal protocol was approved by ethical committee in Faculty of Pharmacy, University of Mansoura (protocol code no. 2012-32). Male Sprague Dawely rats weighing 180–200 g were used. All animals in the study were maintained under standard conditions of temperature 25°C, with regular 12 h light/12 h dark cycle and allowed free access to food and water. Rats were classified into the following groups with 10 rats in each group.

Control group

Rats received intraperitoneal (ip) injection of phosphate buffer saline (PBS, 10 mM, pH 7.4) and served as negative control throughout the study.

EGCG treated control group

Rats received ip injection of 20 mg/kg EGCG (Zhejiang Yixin Pharmaceutical Co., China) dissolved in PBS (10 mM, pH 7.4) twice per week for 16 weeks.

Sodium ascorbate treated control group

Rats were given ip injection of 100 mg/kg sodium ascorbate (Sigma Aldrich Chemicals Co., St. Louis, MO, USA) dissolved in PBS (10 mM, pH 7.4) twice per week for 16 weeks.

HCC group

Rats were injected with thioacetamide (Tocris, Bristol, UK) at a dose of 200 mg/kg, ip twice per week for 16 weeks.

HCC treated with EGCG

Rats received EGCG (20 mg/kg, ip) twice per week throughout the whole study and were injected with thioacetamide (200 mg/kg, ip) twice per week for 16 weeks starting from the second week after EGCG.

HCC treated with sodium ascorbate

Rats received sodium ascorbate (100 mg/kg, ip) twice per week throughout the whole study. Rats were given thioacetamide (200 mg/kg, ip) twice per week for 16 weeks starting from the second week after EGCG.

The doses and time course of experiments used for EGCG and sodium ascorbate in this study were in the range of those used in other studies applied for the same animal species.[10,19] In addition, the dose was determined after appropriate preliminary experiments (Supporting Information Figures 1–3).

Animal sacrifice and collection of samples

The animals were sacrificed by decapitation. Rat trunk blood was collected and centrifuged at 3000 rpm for 5 min, and serum samples were separated and stored at −80°C. The whole liver was dissected, and the number of tumour nodules on the surface of the liver was counted. Rat livers were cleaned with ice-cold saline. A piece of the liver was fixed in 10% buffered formalin for subsequent morphologic analysis. Another part was homogenized in a 10-fold volume of ice-cold sodium potassium phosphate buffer (0.01 M, pH 7.4) containing 1.15% KCl. The homogenates were centrifuged at 600 g for 10 min at 4°C. The supernatant was stored at −80°C till used.

Morphologic analysis of hepatic tissue

The liver was cut, fixed in 10% buffered formalin and embedded in paraffin. Five micrometre thickness sections

were cut and stained with Mayer's H&E for examination of cell structure by light microscope. Hepatic specimens were anonymously coded and examined in a masked manner. The morphologic changes were photographed using digital camera-aided computer system (Nikon Digital Camera, Japan).

Immunohistochemistry

Immunohistochemical analyses were performed on 5-μm thick paraffin sections cut from a paraffin block of liver. Sections were incubated with monoclonal anti-HSPGs (Antibodies Online Inc, Atlanta, GA, USA) at 4°C. Slides were counterstained with haematoxylin and examined using Nikon Digital Camera.

Measuring liver function

Serum alanine aminotransferase (ALT) activity as well as albumin and bilirubin concentrations were measured by standard methodologies using commercially available kits provided by Biodiagnostic Company (Giza, Egypt).

Assessment of oxidative stress

It was estimated through the following parameters:

Hepatic malondialdhyde (MDA) concentration was measured by thiobarbituric acid as described previously by our group.[20,21] In brief, after precipitation of proteins by trichloroacetic acid, thiobarbituric acid reacts with MDA to form thiobarbituric acid-reactive substance that is measured at 532 nm.

Hepatic superoxide anion concentration was measured by nitroblue tetrazolium (NBT) method.[22] It depends on the ability of the superoxide anion to reduce NBT to an insoluble formazan that can be measured at 560 nm.

Hepatic myeloperoxidase (MPO) activity was determined using tetramethylbenzidine (TMB) method.[23] It depends on the measurements of the colour change of TMB over time at 405 nm.

ELISA

The levels of biochemical parameters were measured by ELISA assay using a commercially available matrix metalloproteinase-9 (MMP-9) (R&D Systems Inc., Minneapolis, MN, USA), HSPGs, α-fetoprotein (USCN Life Science Inc. Houston, TX, USA) and syndecan-1 ELISA kits (Cusabio, Wuhan, Hubei Province, China) in accordance with the manufacturer's instructions.

Estimation of caspase-3 activity

Caspase-3 enzyme activity assay was measured colourimetrically using commercially available kits (GenScript, Piscataway, NJ, USA) following the manufacture procedure.

Quantitative real-time PCR

Total RNA was isolated from euthanized rat liver using RNeasy Mini kit (Qiagen, USA). The amount of RNA was quantified by using a Maxima SYBR Green/Fluorescein qPCR Master Mix (Fermentas, USA). One microgram of total RNA was reverse transcribed into single-stranded complementary DNA (cDNA) by using QuantiTect Reverse Transcription Kit (Qiagen, USA). FGF-2 mRNA levels in different rat liver tissues and human hepatocellular carcinoma (HepG2) cells were determined using Maxima SYBR Green/Fluorescein qPCR Master Mix by Rotor-Gene Q (Qiagen, USA). In the meanwhile, rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and an internal reference control. Gene-specific PCR primers (Table 1) were designed using Primer Express 3.0 (Applied Biosystems, USA) according to the nucleotide sequence obtained from the Gene Bank.

Tissue culture

Cell lines

The HepG2 cell line was used. They were purchased from American Type Culture Collection (ATCC, Manassas, VA, US) and given to us as a kind gift from The Holding

Table 1 The primers set used for the detection of human and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and fibroblast growth factor (FGF)-2

Name	Sequence	Reference sequence	Amplicon size	Annealing temperature
Human GAPDH F369	5′-AGAAGGCTGGGGCTCATTTG-3′	BC004109.2	258 bp	58°C
Human GAPDH R626	5′-AGGGGCCATCCACAGTCTTC-3′			
Rat GAPDH 165F	5′-CCATCAACGACCCCTTCATT-3′	NM_017008.3	193 bp	58°C
Rat GAPDH 358R	5′-CACGACATACTCAGCACCAGC-3′			
Human FGF-2 F2896	5′-AAAGCAGGAGGATCGCTTGA-3′	NM_002006.4	94 bp	58°C
Human FGF-2 R2989	5′-CCACCATGCCTGGCTATTTT-3′			
Rat FGF-2 70F	5′-ATTTCCAAAACCTGACCCGAT–3′	NM_019305.2	98 bp	60°C
Rat FGF-2 168R	5′-AACTTTCTCCCTTCCTGCCTTT–3′			

Company for Biological Products & Vaccines (Vacsera, Cairo, Egypt), where the HepG2 cells were examined for identity and absence of bacteria, mycoplasma and viruses. HepG2 cells were grown in DMEM supplemented with 10% foetal bovine serum and 1% streptomycin and penicillin, and incubated for 24 h at 37°C in a 5% CO_2 incubator to allow the cells to grow.

MTT assay

1×10^4 cells were plated in each well of 96-well plates, and were placed in the humidified 5% CO_2 incubator at 37°C to allow them to grow for 24 h period. Cells were transferred to serum-free medium and exposed to different concentrations of EGCG and sodium ascorbate (10, 50, 100 and 200 μM) and placed in the humidified 5% CO_2 incubator for 48 h. Cells incubated in culture medium alone served as a control for cell viability (untreated wells). Cell viability was determined using the MTT assay as described previously by our group.[24] In brief, viability of HepG2 cells was determined by incubating cells for 4 h at 37°C with 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich Chemicals Co.) in PBS. MTT, a yellow dye, is reduced to purple formazan in living cells, which was dissolved in acid isopropanol (1 : 9 of 1 N HCl/isopropanol). Optical density was measured at 540 nm and 690 nm using microplate reader (Bio-Tek Instruments, VT, USA).

Cytotoxicity with LDH

Cell cytotoxicity was performed following the protocol discussed previously.[25] Supernatant was collected from each well and centrifuged for 5 min at 700*g* to remove cell debris. Supernatant was plated in a second 96-well plate. Lactate dehydrogenase (LDH) reaction mixture (Sigma Aldrich Chemicals Co.) was added to each well including controls and cell-free wells. The plate was allowed to develop for 20 min in the dark at room temperature. Cytotoxicity with LDH was determined by subtracting the normalized absorbance at 680 nm of the cell-free wells from the normalized absorbance of wells with cells using Bio-Tek Instruments, VT, USA. Relative cytotoxicity was determined by normalizing against the positive cytotoxicity control, 1% Triton-X 100.

Statistical analysis

For descriptive statistics of quantitative variables, the mean ± standard error was used. Normality of the sample distribution of each continuous variable was tested with the Kolmogorov–Smirnov (K–S) test. One-way analysis of variance was used to compare means between groups. Once the differences exist among the means, post-hoc Bonferroni correction test was calculated. Rats' survival was estimated using the Kaplan–Meier method. Statistical computations were done on a personal computer using the computer software SPSS version 13 (Chicago, IL, USA). Statistical significance was predefined as $P \leq 0.05$.

Results

Chemopreventive effect of EGCG against HCC *in vivo* and *in vitro*

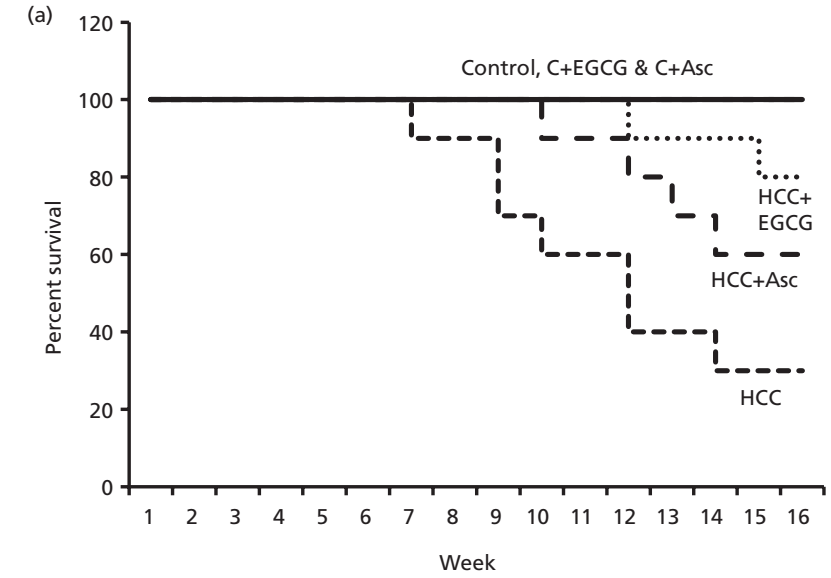
Animal survival was calculated after 16 weeks following different drug treatments at the end of the experiment. The survival of rats was 30% in HCC group (Figure 1a). In addition, HCC resulted in elevation of the average number of nodules per nodule bearing liver (Figure 1b). In parallel, we found 2.8-fold increase serum α-fetoprotein in HCC group as compared with control group (Figure 1c). Treatment with EGCG (20 mg/kg, ip, twice/week) for 16 weeks increased the animal survival to 80%, resulted in 70% reduction in the average number of nodules per nodule bearing liver and blocked the increase in serum α-fetoprotein level in HCC group and did not affect the control group. Regarding in-vitro studies, treatment with EGCG showed dose-dependent reduction in the viability (Figure 1d) and elevation of cytotoxicity of HepG2 (Figure 1e). Treatment with sodium ascorbate *in vivo* and *in vitro* showed little chemopreventive effect as compared with EGCG.

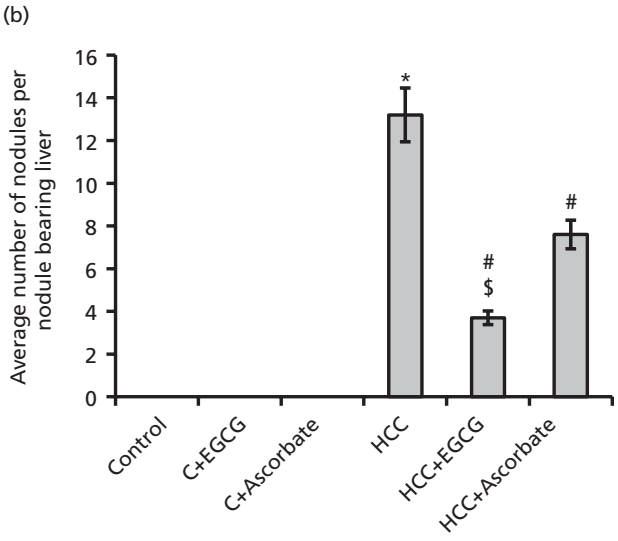
Hepatoprotective effect of EGCG against HCC *in vivo*

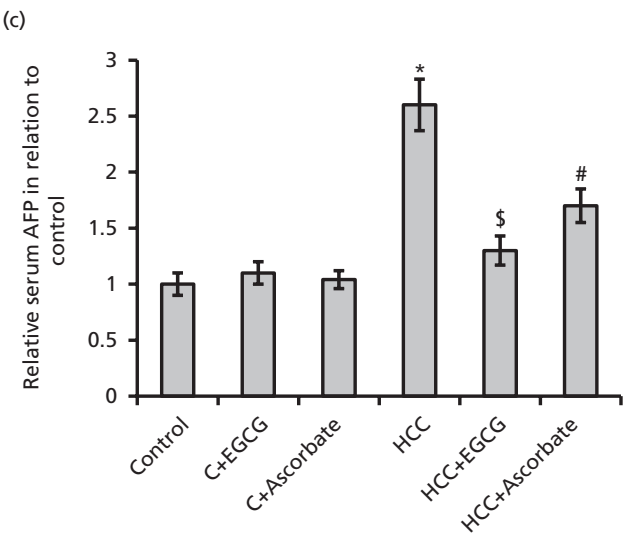
Next, we examined the hepatoprotective effect of EGCG against HCC *in vivo*. HCC rats showed 2.9- and 2.1-fold increase in serum ALT and bilirubin, respectively as well as 47% reduction in serum albumin level in HCC group as compared with the control group (Figure 2a). In addition, liver sections stained with H/E showed marked cellular infiltrate in the portal tract, fibrosis and massive breakdown of hepatic tissues. However, sections from HCC rats treated with EGCG showed nearly normal appearance of hepatic lobule (Figure 2b). Treatment with sodium ascorbate showed little hepatoprotective effect as compared with EGCG.

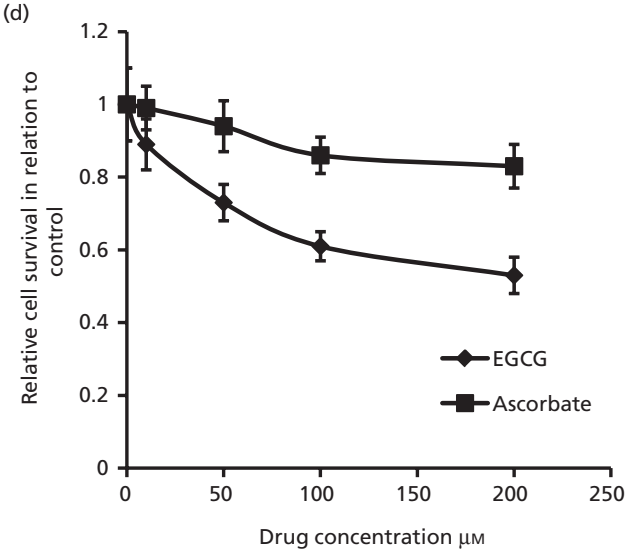
EGCG blocked HCC-induced elevation of oxidative stress markers

It is known that the oxidative stress status has a key role in HCC development and progression.[26] However, we found 2.5-, 2.2- and 4.6-fold increase in hepatic superoxide anion, MPO and MDA levels, respectively in HCC group as compared with the control group (Figure 3a, 3c and 3e). Treatment with EGCG significantly reduced the elevated oxidative stress markers in HCC group and did not affect the control group. In parallel, treatment of HepG2 cell with









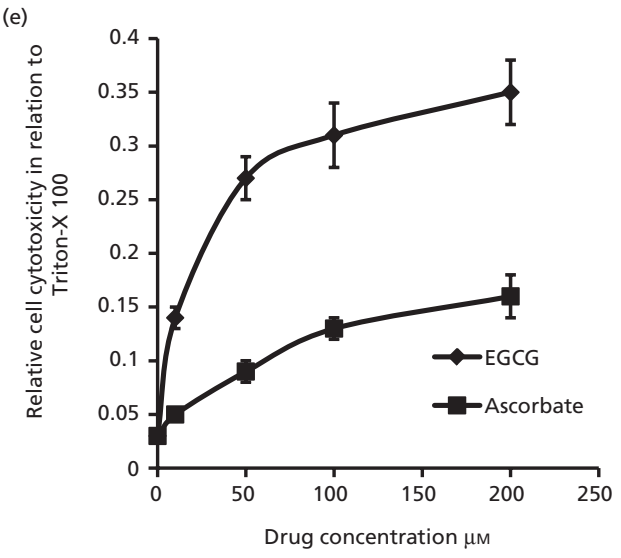


Figure 1 Chemopreventive effect of epigallocatechin-gallate against hepatocellular carcinoma *in vivo* and *in vitro*. (a) Kaplan–Meier survival curves of rats. Statistical analysis of average number of nodules per nodule bearing liver (b) and serum α-fetoprotein (c) in hepatocellular carcinoma rats treated with epigallocatechin-gallate and sodium ascorbate. Statistical analysis of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (d) and lactate dehydrogenase assay (e) of human hepatocellular carcinoma cells treated with different doses of epigallocatechin-gallate and sodium ascorbate. *: significant difference as compared with the rest of the groups at $P < 0.05$. #: significant difference as compared with the control groups at $P < 0.05$.$: significant difference as compared with the hepatocellular carcinoma+ascorbate groups at $P < 0.05$. C, control; AFP, α-fetoprotein; EGCG, epigallocatechin-gallate; HCC, hepatocellular carcinoma.

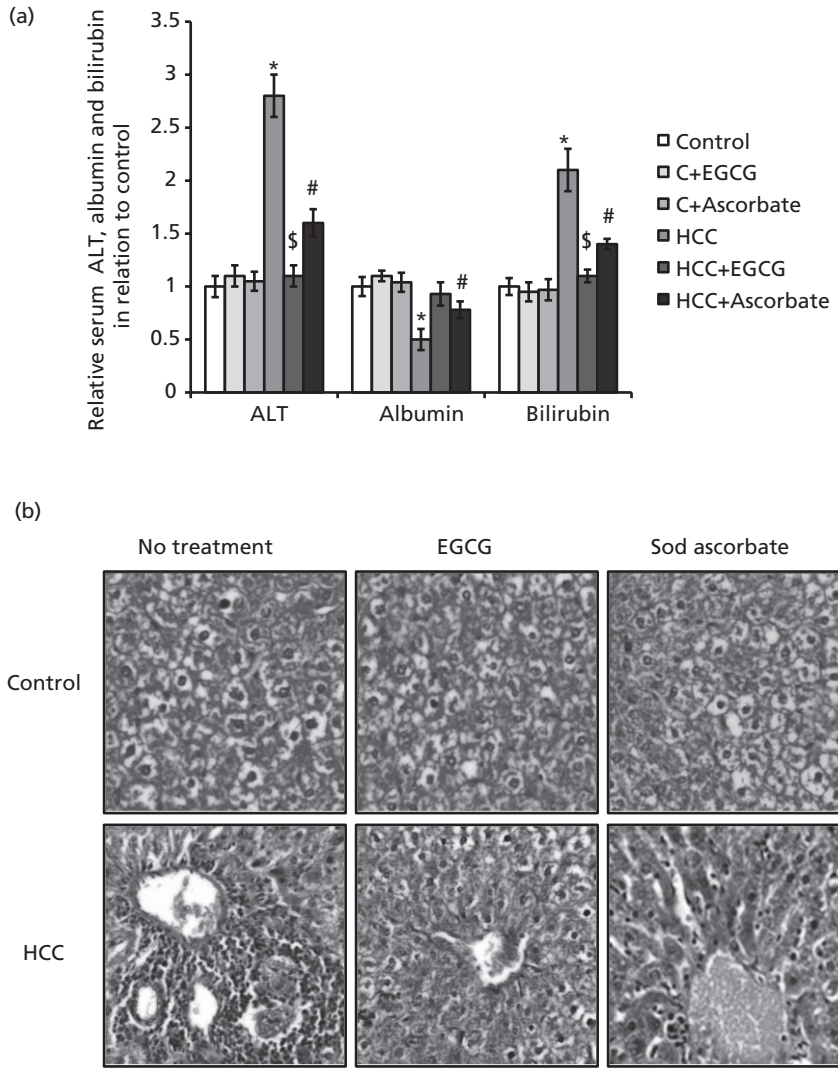


Figure 2 Hepatoprotective effect of epigallocatechin-gallate against hepatocellular carcinoma *in vivo*. (a) Statistical analysis of liver function markers in hepatocellular carcinoma rats treated with epigallocatechin-gallate and sodium ascorbate. (b) Representative images of liver sections stained with haematoxylin/eosin. *: significant difference as compared with the rest of the groups at $P < 0.05$. #: significant difference as compared with the control groups at $P < 0.05$. $: significant difference as compared with the hepatocellular carcinoma +ascorbate groups at $P < 0.05$.

EGCG showed dose-dependent reduction in superoxide anion, MPO and MDA (Figure 3b, 3d and 3f). In addition, treatment with sodium ascorbate exerts more significant decrease in the oxidative stress markers as compared with EGCG *in vivo* or *in vitro*.

EGCG blocked HCC-induced reduction in HSPGs

As shown in Figure 4, HCC in rats caused 37% reduction in hepatic HSPGs as compared with the control group.

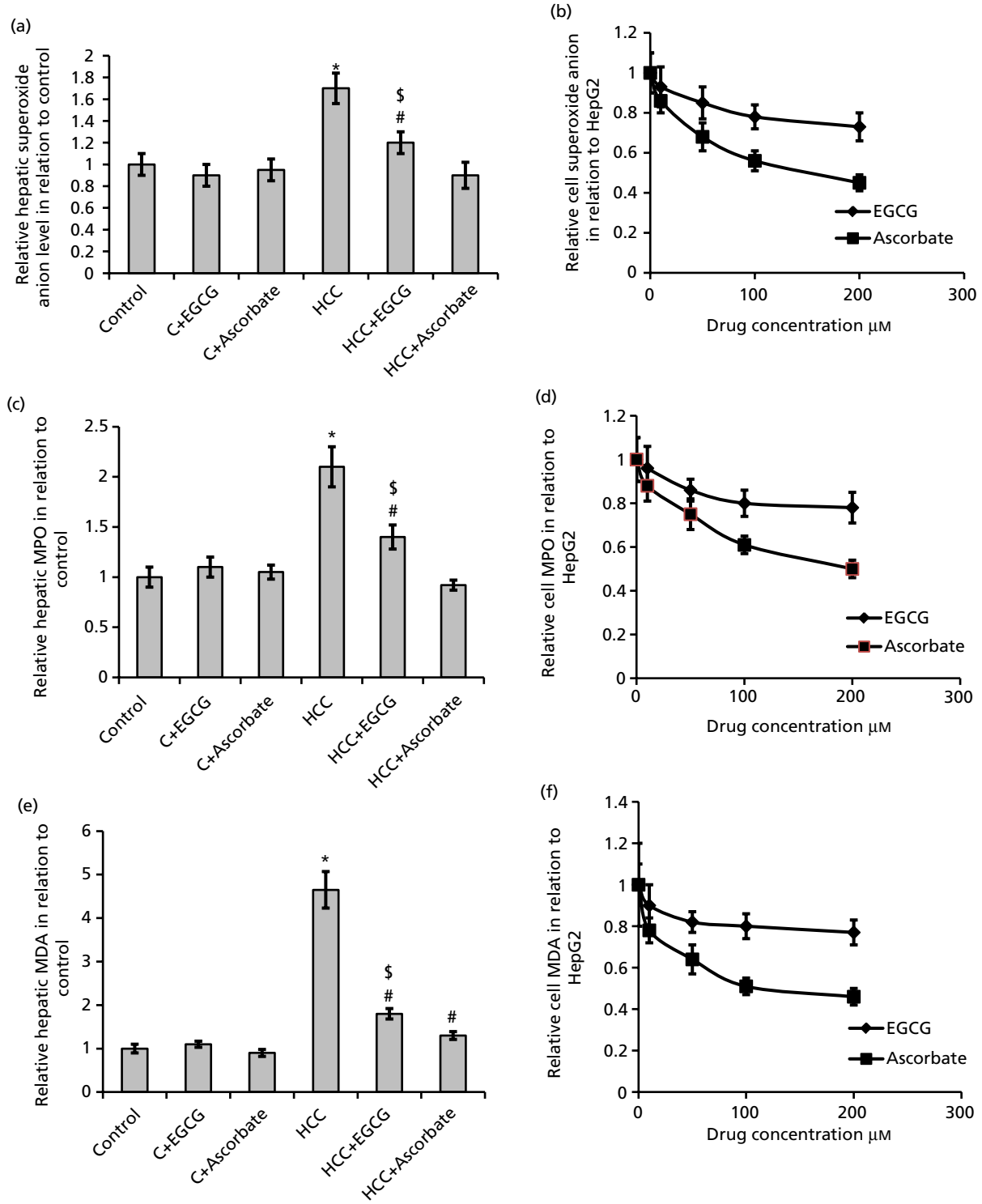


Figure 3 Epigallocatechin-gallate blocked hepatocellular carcinoma-induced elevation of oxidative stress markers. Statistical analysis of rat liver homogenate for detection of superoxide anion (a), myeloperoxidase (c) and malondialdhyde (e) levels in hepatocellular carcinoma rats treated with epigallocatechin-gallate and sodium ascorbate. In parallel, human hepatocellular carcinoma cell lysate were analysed for determination of superoxide anion (b), myeloperoxidase (d) and malondialdhyde (f). *: significant difference as compared with the rest of the groups at $P < 0.05$. #: significant difference as compared with the control groups at $P < 0.05$. $: significant difference as compared with the hepatocellular carcinoma+ascorbate groups at $P < 0.05$.

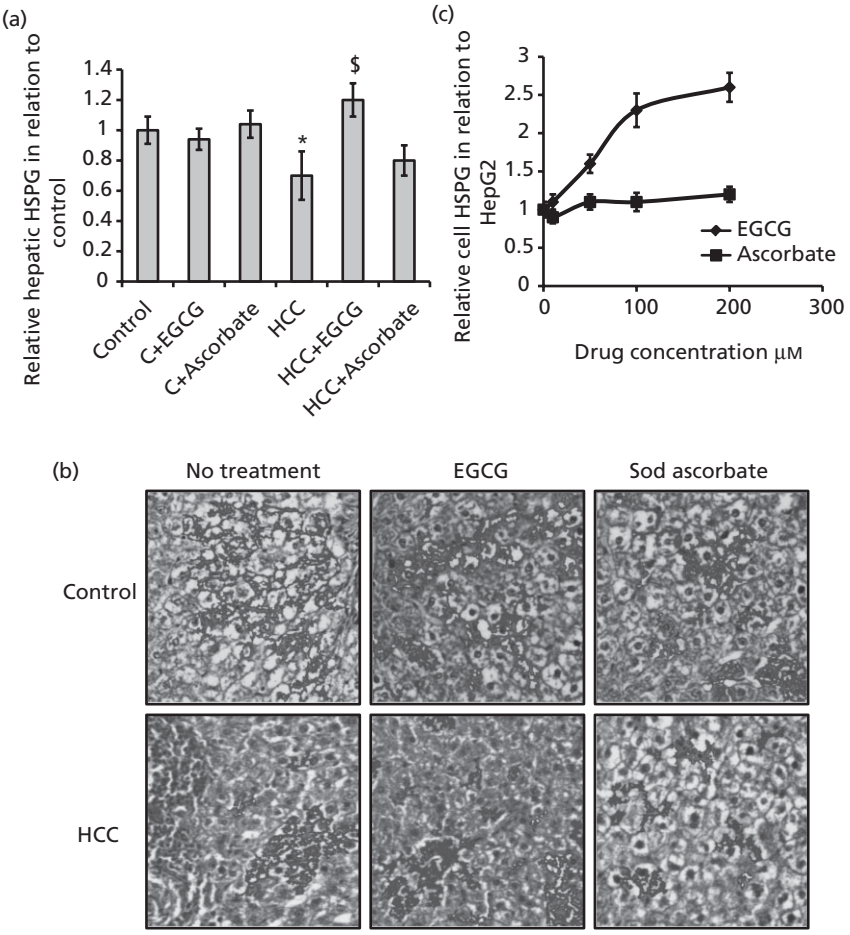


Figure 4 Epigallocatechin-gallate blocked hepatocellular carcinoma-induced reduction in heparan-sulfate proteoglycans. Statistical analysis (a) and representative images of heparan-sulfate proteoglycans (b) in hepatocellular carcinoma rat livers**.** Statistical analysis of heparan-sulfate proteoglycans in human hepatocellular carcinoma cell lysate (c) treated with epigallocatechin-gallate and sodium ascorbate. *: significant difference as compared with the rest of the groups at $P < 0.05$. #: significant difference as compared with the control groups at $P < 0.05$. $: significant difference as compared with the hepatocellular carcinoma+ascorbate groups at $P < 0.05$.

Treatment with EGCG attenuated the reduced HSPGs levels in HCC rats and did not affect the control rats. In addition, treatment of HepG2 cells with EGCG caused dose-dependent increase in HSPGs. However, treatment with sodium ascorbate did not affect the HSPGs levels *in vivo* or *in vitro*.

EGCG blocked HCC-induced expression of tumour invasion markers

Highly invasive tumour cells are expected to secrete large amounts of proteolytic enzyme such as MMP-9. Moreover, we have previously demonstrated increased serum level of syndecan-1 in patients with HCC as compared with the control.[27] Analysis of hepatic homogenate showed 2- and 2.4-fold increase in MMP-9 and syndecan-1 levels, respectively in HCC rats as compared with control rats (Figure 5a and 5c). In addition, we found 3.5-fold increase in the gene expression of hepatic FGF-2 in HCC rats as compared with control rats (Figure 5e). However, treatment with EGCG blocked the increase in the expression of MMP-9, syndecan-1 and FGF-2 in HCC group and did not affect the control group. Regarding HepG2 cell-line, EGCG caused dose-dependent reduction in lysate level of MMP-9 and syndecan-1 (Figure 5b and 5d) and the gene expression of FGF-2 (Figure 5e). Treatment with sodium ascorbate reduced expression of invasion markers, but its effect is significantly lower than that of EGCG *in vivo* and *in vitro*.

Effect of EGCG on HCC cell death

Finally, we examined the effect of EGCG on HCC induced cell death. We found 2.3-fold increase in caspsase-3 enzyme activity in HCC rats as compared with control rats (Figure 6a). Treatment with 20 mg/kg EGCG did not

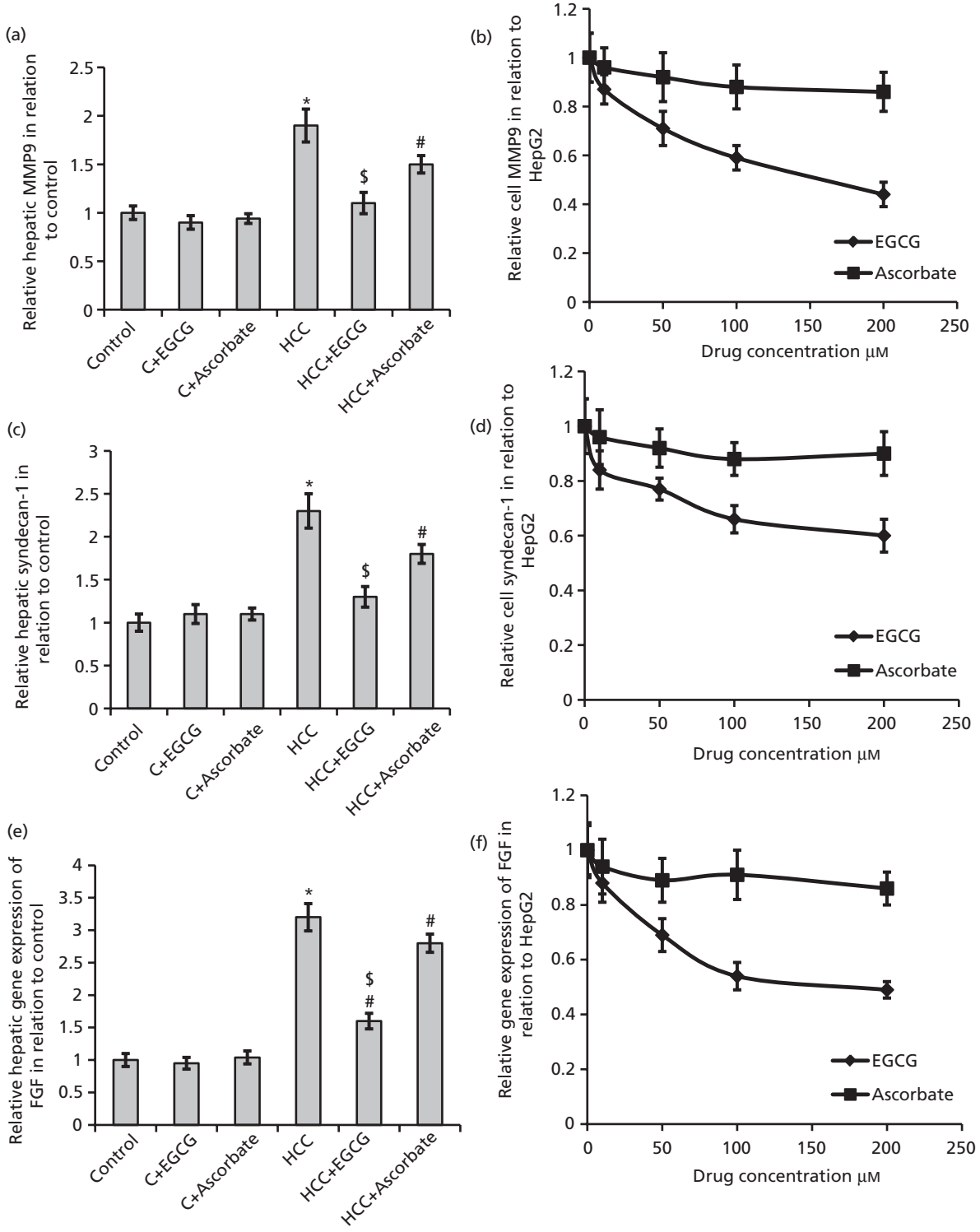


Figure 5 Epigallocatechin-gallate blocked hepatocellular carcinoma-induced expression of tumour invasion markers. Statistical analysis of hepatic homogenate for protein expression of matrix metalloproteinase-9 (a) and syndecan-1 (c) and gene expression of fibroblast growth factor-2 (e) in hepatocellular carcinoma rats**.** human hepatocellular carcinoma cell lysate was examined for protein expression of matrix metalloproteinase-9 (b) and syndecan-1 (d) and gene expression of fibroblast growth factor-2 (f). *: significant difference as compared with the rest of the groups at $P < 0.05$. #: significant difference as compared with the control groups at $P < 0.05$. $: significant difference as compared with the hepatocellular carcinoma+ascorbate groups at $P < 0.05$. MMP-9, matrix metalloproteinase-9; FGF, fibroblast growth factor.

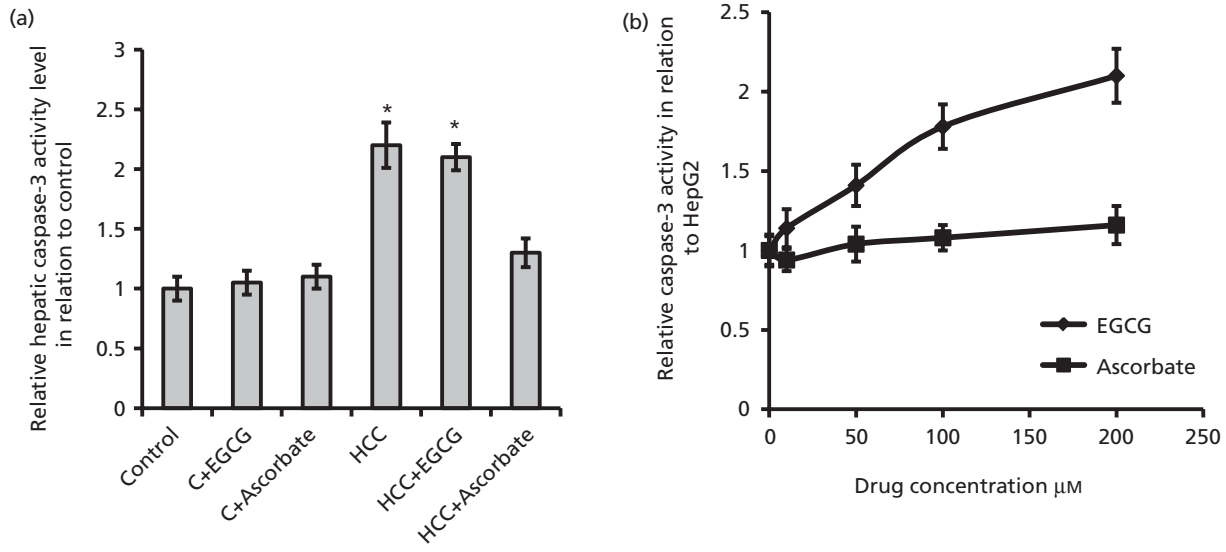


Figure 6 Effect of epigallocatechin-gallate on hepatocellular carcinoma cell death. Statistical analysis of caspase-3 activity *in vivo* (a) and *in vitro* (b). *: significant difference as compared with the rest of the groups at $P < 0.05$.

significantly affect the activity of caspase-3 in HCC. On the other hand, treatment of HepG2 with EGCG produced dose-dependent increase in the activity of caspase-3 (Figure 6b). Moreover, treatment with sodium ascorbate did not affect caspase-3 levels *in vivo* and *in vitro*.

Discussion

Primary liver cancer represents a major health burden and the main cause of cancer-related mortality in the world. HCC is a very aggressive cancer with a dismal outcome; patients usually survive less than 1 year after diagnosis.[28] Although a wide range of therapeutic options is available, the efficacy of these methods and the survival of patients with HCC remain poor. Surgical resection is currently the most effective treatment for early-stage HCC patients with preserved liver function and without distant metastasis.

Epidemiological and preclinical studies have demonstrated that catechins derived from green tea have profound chemopreventative and antitumour effects.[29–31] EGCG is the most abundant and active polyphenolic compounds in green tea.[32,33] However, we found that EGCG elevated the survival of rats from 30% in HCC group to 80% and reduced the average number of nodules per nodule bearing liver. In addition, treatment with EGCG ameliorates the altered hepatocyte structure as well as it reduced the elevated liver enzymes and α-fetoprotein in HCC without affecting the control group. Meanwhile, EGCG showed dose-dependent reduction in the viability of HepG2, human HCC cell-line. Zhang and colleagues illustrated that HepG2 cell cultured in serum-free medium showed 45% reduction in cell viability with 100 μM EGCG and 90% reduction with 240 μM after 36 h,[34] while we showed about 40% reduction in cell viability with 100 μM EGCG and 55% reduction with 200 μM after 48 h. It has been reported previously that EGCG showed antitumour activity against HCC through suppression of prostanoid EP(1),[13] deactivation of vascular endothelial growth factor,[17] deactivation of insulin growth factor,[18] inhibition of cell cycle progression through activation of AMP-activated protein kinase[35] and inhibition of Bcl-2 family proteins.[36]

It is known that the oxidative stress status has a key role in HCC development and progression as it can affect cell proliferation, apoptosis, cell cycle arrest and cell senescence.[26] We found significant increase in hepatic level of superoxide anion, MPO and MDA in HCC rats. However, an increased level of ROS causes oxidative stress and creates a potentially toxic environment to the cells. Studies of mechanisms of oxidative stress have shown that it can affect mitogen-activated protein kinases with subsequent serious influence on regulation of cell growth and transformation processes.[37] Oxidative stress also activates hepatic stellate cells that represent the main connective tissue cells in the liver, involved in formation of extracellular matrix and required for normal growth and differentiation of cells during liver damage.[38] However, we found that treatment with EGCG attenuated the elevated levels of oxidative stress markers in the HCC group and did not affect the control group. It has been reported previously that EGCG inhibited hepatic stellate cells activation by inhibiting cell proliferation through attenuation of oxidative stress.[39,40]

HSPGs are important extracellular matrix components that can influence the cell behaviour. HSPGs have been well described to be involved in a vast number of cell–cell and

cell–matrix interactions.[41] We found a significant decrease in hepatic HSPGs in HCC group as compared with the control group. HSPGs are intricately involved in a variety of physiological processes such as morphogenesis, wound healing and regulation of cell differentiation and growth.[42] In particular, its complex formation with growth factors and their receptors is indispensable for proper receptor activation and signalling (for examples, review[43]). However, treatment of HCC rats with EGCG restores HSPGs levels.

Recent discoveries indicated that HSPGs localized within the tumour microenvironment can be attacked by enzymes that alter proteoglycan structure resulting in dramatic effects on tumour growth and metastasis.[44] MMPs are a family of secreted or transmembrane proteins that are capable of digesting extracellular matrix and basement membrane. MMP-9 is believed to be capable of degrading type IV collagen, which is a major constituent of basement membrane.[45,46] Meanwhile, syndecan-1, the main heparan sulfate present on the cell surface, is a modulator of proteolytic activity and chemokine functions *in vivo*, which regulates leukocyte recruitment and tissue remodelling during inflammation and wound repair.[47] Proteolytic conversion of syndecan-1 from a membrane-bound into a soluble molecule marks a switch from a proliferative to an invasive tumour.[48] Syndecan-1 binds to extracellular matrix proteins and to growth factors such as FGF family, the most potent angiogenic stimulators.[49] The results presented here indicated that MMP-9/syndecan-1/ FGF axis expression was elevated more frequently in HCC tissues than in corresponding non-tumourous ones. Many studies illustrated the increase in FGF levels in HCC.[50–52] Moreover, we have previously demonstrated elevated serum level of syndecan-1 in patients with HCC as compared with the control.[27] Many members of the FGF family, such as FGF-1 and FGF-2, are immobilized in the ECM bound to HSPGs and released from this storage site by proteases such as MMP-9.[53] The tumours with high level of MMP-9 expression were more susceptible to relapse or metastasis than those with low level of MMP-9 expression. These results revealed that MMP-9 is an important molecule which participates in the invasion of HCC. It has been reported previously that, biological consequences of increased FGF signalling include increased motility and invasiveness, increased angiogenesis and enhanced metastasis.[2,54] Off note, we found that treatment with EGCG significantly reduce the gene expression of FGF-2 in HCC rats which is accompanied with significant elevation in the hepatic level of HSPG.

Increased oxidative stress and MMP-9/syndecan-1/FGF-2 axis activates apoptosis pathway in different models.[55–57] Therefore, we next evaluated the effects of EGCG on apoptosis in HCC rat livers. Our results showed no significant difference in caspase-3 activity in HCC rats treated with EGCG as well as increasing caspase-3 activity in HepG2. However, the inability of EGCG to elevate caspase-3 in the in-vivo model can be attributed to the presence of normal hepatic tissues besides the timorous one as well as the presence of many factors that affect EGCG bioavailability like the rate of EGCG absorption, metabolism and excretion, which are not present in the in-vitro model. Kuo and Lin have shown that EGCG inhibits proliferation in HepG2 by inducing apoptosis and blocking cell cycle progression in the G1 phase.[58]

The current study investigated the chemopreventive and hepatoprotective effects of two different compounds in HCC rats and HepG2 cell lines: (1) the strong water soluble antioxidant, sodium ascorbate and (2) the green tea polyphenol, EGCG. Although the results of the two drugs were in parallel, the hepatoprotective effects of EGCG were superior to those of sodium ascorbate, a fact possibly attributable to the pleiotropic effects of EGCG, including antioxidant effects. However, the antioxidant activity of sodium ascorbate against HCC-induced oxidative stress is superior to those of EGCG indicating that EGCG works via other mechanisms besides antioxidant activity. One of these mechanisms includes EGCG effects on HSPGs, MMP-9, sydecan-1 and FGF-2. While previous studies examined the mechanisms of hepatoprotective effects of EGCG *in vivo* and *in vitro* through prostanoid EP1,[13] vascular endothelial growth factor[59] and insulin growth factor,[18] our study

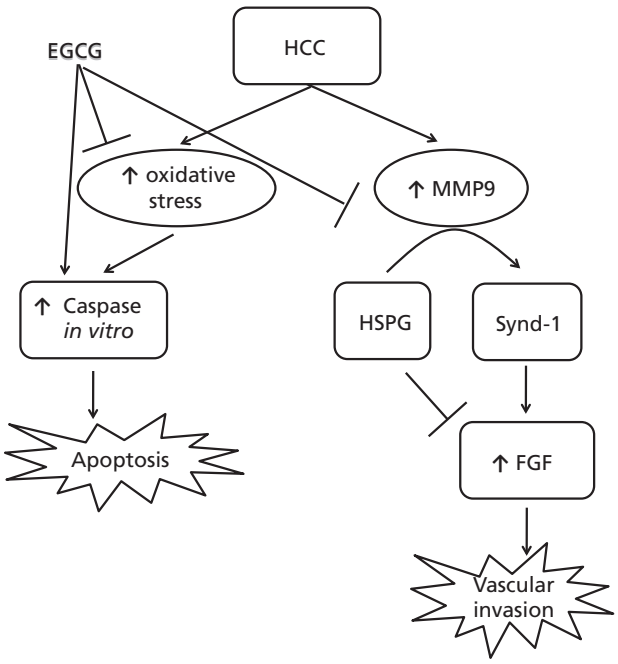


Figure 7 Schematic representation of chemopreventive and hepatoprotective mechanism of epigallocatechin-gallate against hepatocellular carcinoma.

is the first to demonstrate the chemopreventive and hepatoprotective effects of EGCG in HCC through HSPGs receptors pathway.

Conclusions

The main findings of the current study are that EGCG possesses chemopreventive and hepatoprotective effects, which can be partially explained by the antioxidant activity of EGCG as indicated by reducing the levels of MDA, superoxide anion and myeloperoxidase. However, other mechanisms are involved including: (1) restoration of HSPGs; (2) inhibition of vascular invasion markers, as demonstrated by reduced expression of MMP-9, syndecan-1 and FGF-2 *in vivo* and *in vitro*; and (3) activation of caspase-3 apoptotic pathway. The mechanism of action was summarized in Figure 7. To the best of our knowledge, our study demonstrates for the first-time chemopreventive and hepatic-protection role of EGCG in the in-vivo and in-vitro models of HCC.

Declarations

Conflict of interest

All authors disclose that they do not have any commercial association that might pose a conflict of interest in connection with the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Kaplan–Meier survival curves of hepatocellular carcinoma (HCC) rats treated with different doses of epigallocatechin-gallate (EGCG, A) or sodium ascorbate (B).

Figure S2 Relative serum α-fetoprotein (AFP) in hepatocellular carcinoma (HCC) rats treated with different doses of epigallocatechin-gallate (EGCG, A) or sodium ascorbate (Asc, B). *: significant difference as compared with the rest of groups at $P < 0.05$. #: significant difference as compared with the control groups at $P < 0.05$.

Figure S3 Relative serum heparan sulfate proteoglycans (HSPGs) in hepatocellular carcinoma (HCC) rats treated with different doses of epigallocatechin-gallate (EGCG, A) or sodium ascorbate (Asc, B). *: significant difference as compared with the rest of the groups at $P < 0.05$. #: significant difference as compared with the control groups at $P < 0.05$.