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RESEARCH ARTICLE

Quercetin prevents liver carcinogenesis by inducing cell cycle arrest, decreasing cell proliferation and enhancing apoptosis

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Scope: Quercetin is the most abundant flavonoid in human diet. It has special interest as it holds anticancerous properties. This study aims to clarify the mechanisms involved in quercetin effects during the occurrence of preneoplastic lesions in rat liver.

Methods and results: Adult male Wistar rats were subjected to a two-phase model of hepatocarcinogenesis (initiated-promoted group). Initiated-promoted animals also received quercetin 10 and 20 mg/kg body weight (IPQ10 and IPQ20 groups, respectively). Antioxidant defenses were modified by quercetin administration at both doses. However, only IPQ20 group showed a reduction in number and volume of preneoplastic lesions. This group showed increased apoptosis and a reduction in the proliferative index. In addition, IPQ20 group displayed a reduction of cell percentages in G_1 and S phases, accumulation in G_2, and decrease in M phase, with reduced expression of cyclin D1, cyclin A, cyclin B, and cyclin-dependent kinase 1. Interestingly, peroxisome proliferator activated receptor-α levels were reduced in IPQ20 group.

Conclusion: The outcomes of this study represent a significant contribution to the current understanding on the preventive mechanisms of quercetin during the early stages of liver cancer development, demonstrating that in addition to its known proapoptotic characteristics, the flavonoid modulates the expression of critical cell cycle regulators and peroxisome proliferator activated receptor-α activity.

Keywords:
Apoptosis / Preneoplastic liver / Proliferation / Quercetin

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1 Introduction

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm and the third most frequent cause of cancer death, after lung and colon cancer [1]. In humans, liver preneoplastic lesions emerge weeks or months before the diagnosis of hepatocellular adenomas and HCCs [2]. A similar progression is reproduced by chemicals in laboratory rats [3], leading to the development of a number of systems to study liver neoplasia in vivo [4]. The initiation-promotion or two-stage model of cancer development mimics the events of the latent

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Abbreviations: 2-AAF, 2-acetylaminofluorene; **CAT**, catalase; **cdk1**, cyclin-dependent kinase 1; **DEN**, diethylnitrosamine; **GSH**, reduced glutathione; **GSSG**, oxidized glutathione; **HCC**, hepatocellular carcinoma; **IP**, initiated-promoted; **PCNA**, proliferating cell nuclear antigen; **PPAR-α**, peroxisome proliferator activated receptor-α; **rGST P**, pi class of rat glutathione S-transferase; **SOD**, superoxide dismutase; **TBARS**, thiobarbituric acid reactive substances

period of human hepatocarcinogenesis and represents a very useful tool to study molecular mechanisms occurring during the early stages of liver cancer. Diethylnitrosamine (DEN) is a genotoxic carcinogen widely used as chemical initiator in rodent models of experimental hepatocarcinogenesis [5]. DEN is a well-known contaminant present in tobacco smoke, water, preserved meats, fried meals, agricultural chemicals, cosmetics, and pharmaceutical products [6]. Following the initiation stage, the administration of promoting agents, such as 2-acetylaminofluorene (2-AAF), causes selective enhancement of the proliferation of initiated hepatocytes over noninitiated cells [7]. Although the coordination of cell replication and apoptosis is maintained in normal liver tissue, the disruption in this balance that takes place in precancerous and cancerous tissues is considered one of the hallmarks of cancer [8, 9].

Quercetin (3,3′,4′,5,7-pentahydroxylflavone) is the most abundant flavonoid present in the plant kingdom as a secondary metabolite. It is found in a variety of human foods such as grapes, apples, red onions, cherries, broccoli, berries, tea, citrus fruits, and capers. Average daily uptake varies between 10 and 100 mg depending on eating habits. Most quercetin is present in plants as hydrophilic glycosides that are not easily absorbed. Because quercetin is also available in highly purified extracts, daily uptakes of 500–1000 mg/day can be easily achieved using selected dietary supplements. In these purified forms, quercetin aglycone bioavailability is quite high [10]. Quercetin and related flavonoids have attracted much attention as potential anticarcinogens. The dose levels of quercetin required for anticancer activity are much higher than those achieved with daily intakes from the diet. These doses of quercetin would be attainable with the consumption of supplements containing the pure aglycone at high concentrations [10, 11].

It has been reported that quercetin has chemopreventive effects in a hepatocarcinogenic model, in part by reducing DEN-associated oxidative stress generation [12] and in part by inducing apoptosis of initiated cells at 24 h after DEN treatment [13]. Besides, in a medium-term bioassay of DEN-induced hepatic preneoplastic lesions, quercetin reduced the incidence of liver foci by reducing p53 expression and decreasing apoptosis [14]. Evidently, the molecular mechanisms involved in the preventive effects of quercetin in liver cancer appear to be complex. In the present study, we attempt to clarify additional mechanisms involved in quercetin anticarcinogenic effects during the early stage of neoplastic occurrence in rat liver, and we centered the focus on the balance between cell proliferation and apoptosis.

2 Materials and methods

2.1 Chemicals

DEN, 2-AAF, and quercetin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-pi class of rat glutathione S-transferase (rGST P) antibody was purchased from Stressgen Bioreagents (Ann Arbor, MI, USA). Cy3 fluorescent secondary antibody was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Antibodies against procaspase-3, Bax, Bcl-2, cytochrome c, proliferating cell nuclear antigen (PCNA), cyclin D1, cyclin E, cyclin A, cyclin B1, cyclin-dependent kinase 1 (cdk1), cdk2, and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-peroxisome proliferator activated receptor-α (PPAR-α) antibody was from Abcam Inc. (Cambridge, MA, USA). Pierce-enhanced chemiluminescence (ECL) Western Blotting Substrate was from Thermo Fisher Scientific (Rockford, IL, USA). All other chemicals were of the highest grade commercially available.

2.2 Animals and treatments

Adult male Wistar rats weighing 330–380 g were maintained in a room at constant temperature with a 12 h light–dark cycle, with food and water supplied ad libitum. Experimental protocols were performed according to the NIH "Guide for the Care and Use of Laboratory Animals" (Publication no. 25–28, revised 1996) and approved by the local animal care and use committee. Animals were divided in groups of —six to seven rats each. A scheme of the experimental protocol is shown in Fig. 1. Rats from the initiated-promoted (IP) group were subjected to a two-phase model of rat hepatocarcinogenesis, as described previously [15]. Briefly, animals received two intraperitoneal necrogenic doses of DEN (150 mg/kg body weight) 2 wk apart. One week after the last injection, the rats received 20 mg/kg body weight of 2-AAF by gavage for 4 consecutive days per week during 3 wk. Animals from IP group also received quercetin 10 and 20 mg/kg body weight (IPQ10 and IPQ20 groups, respectively), administered by gavage 2 h before each injection of DEN and 2 h before each week of promotion treatment. Quercetin was dissolved in an aqueous solution containing 0.5% carboxymethylcellulose. Additionally, control rats (C group) received only the vehicles of the drugs or quercetin at 10 and 20 mg/kg body weight (CQ10 and CQ20 groups, respectively). All animals were sacrificed at the end of the 6th wk and livers were removed and processed.

2.3 Immunohistochemical observations

2.3.1 Confocal immunofluorescence analysis

Liver tissues were fixed in 10% v/v formalin solution and embedded in low-melting paraffin. Sections of 5 μm thickness were incubated with primary antibody (anti rGST P, 1:100) in a humidified chamber at 4°C overnight. Subsequently, fluorescent dye conjugated secondary antibody (dilution 1:100) was used. Nuclei were counterstained using 4′,6-diamidino-2-phenylindole (dilution 1:100). Detection of bound antibody was accomplished by immunofluorescence

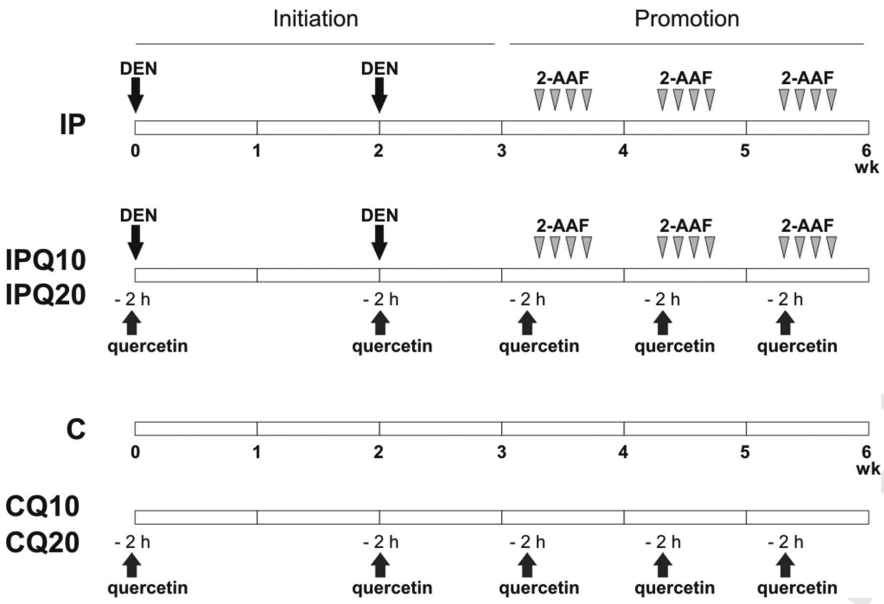

Figure 1. Scheme showing the experimental protocol used in this study. Male Wistar rats were subjected to a two-phase model of hepatocarcinogenesis. **IP** group received two intraperitoneal doses of DEN (150 mg/kg body weight) 2 wk apart. One week after the last injection, the animals received 20 mg/kg body weight of 2-AAF by gavage during four consecutive days per week during 3 wk. **IPQ10** and **IPQ20** groups were subjected to the same protocol and also received quercetin (10 and 20 mg/kg body weight, respectively) by gavage 2 h before each DEN injection and 2 h before each week of promotion treatment. Control (**C** group) received only the vehicles of the drugs or quercetin at 10 and 20 mg/kg body weight (**CQ10** and **CQ20** groups, respectively).

in a Nikon C1 Plus microscope (Nikon, Tokyo, Japan). Slides were also stained in the absence of primary antibodies to evaluate nonspecific secondary antibodies reactions.

2.3.2 Quantitation of preneoplastic foci

rGST P has been described as the most effective single marker of hepatic preneoplasia in the rat [16]. Consequently, immunohistochemical detection of this isozyme is the most widely used method for identification and quantitation of rat preneoplastic foci [17]. The stained liver images were quantified using ImageJ analysis software (U.S. National Institutes of Health, Bethesda, MD, USA). The number of preneoplastic foci per liver and the percentage of liver occupied by foci were calculated according to the modified Saltykov's method [18].

2.3.3 Determination of proliferative index

To investigate differences in proliferation activity among the experimental groups, liver slides were examined by immunohistochemical staining with anti-PCNA antibody, determined by the method of Greenwell et al. [19]. In order to differentiate proliferative cells inside the foci and in the surrounding tissue, consecutive section slides were stained with anti-rGST P [20]. The PCNA proliferative index was defined as the number of proliferative cells (in G_1, S, G_2, and M phases) per 100 hepatocytes counted in ten high-power fields.

The percentages of PCNA-positive preneoplastic hepatocytes in each phase of the cell cycle were also calculated [15].

2.4 Lipid peroxidation assay

Lipid peroxidation is considered as an indirect measure of oxidative stress [21]. The amount of aldehydic products generated by lipid peroxidation in liver homogenates was quantified by the thiobarbituric acid reaction according to the method of Ohkawa et al. [22] and measured by HPLC [23]. The amount of thiobarbituric acid reactive substances (TBARS) was expressed as percentage of C group.

2.5 Caspase-3 activity assay

Caspase-3 activity was determined using EnzChek1 Caspase-3 Assay Kit #1 (Molecular Probes Inc., Eugene, OR, USA), according to the manufacturer's suggestions. Briefly, cytosolic fractions from each sample were mixed with substrate solution. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm in a DTX 880 multimode detector (Beckman Coulter Inc., Fullerton, CA, USA).

2.6 Liver tissue antioxidant capacity

Reduced (GSH) and oxidized (GSSG) glutathione were determined in total liver homogenates according to the protocol described by Tietze [24], and GSH/GSSG ratio was calculated. Catalase (CAT) activity was determined by monitoring the rate of decomposition of H_2O_2 as a function of decrease in absorbance at 240 nm [20]. Superoxide dismutase (SOD) gel activity assay was based on the method of Donahue et al. [25]. Bands quantification was made by densitometry using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA) software.

2.7 Western blot analysis

Tissue samples were homogenized in 300 mM sucrose with protease inhibitors. Cytosolic, mitochondrial, and nuclear extracts were prepared as described previously [15, 26].

Protein concentration was determined by the Lowry method [27]. Equal amounts of protein were subjected to electrophoresis on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (PerkinElmer Life Sciences, Boston, MA, USA). Membranes were blocked with PBS-10% nonfat milk, washed and incubated overnight at 4°C with primary antibodies. Finally, membranes were incubated with peroxidase-conjugated secondary antibodies and bands were detected by the ECL detection system and quantified by densitometry using the Gel-Pro Analyzer software. Equal loading and protein transference were checked by Ponceau S staining of the membranes.

2.8 RNA isolation, cDNA synthesis, and real-time q-PCR

Total RNA was isolated from rat liver tissues by the TriZOL method (Life Technologies Inc., Gaithersburg, MD, USA) according to manufacturer's instructions. cDNA was made from 1 μg of the total RNA using an oligo-dT primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). The PCR reactions were performed using an Mx3000P Real-Time Thermocycler (Stratagene, La Jolla, CA, USA) with SYBR Green I (Invitrogen, Carlsbad, CA, USA) as a fluorescent dye. PCR reactions were initiated with incubation at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 40 s. For each sample, we analyzed glyceraldehyde-3P-dehydrogenase (*Gapdh*) expression to normalize target gene expression. Primer sequences, with their corresponding amplification efficiency, are available online as Supporting Information Table 1. Gene-specific amplification was confirmed by a single peak in the melting curve analysis. Relative changes in gene expression were determined using the 2-ΔΔCt method [28].

2.9 Statistical analysis

Results were expressed as mean ± SE. Significance in differences was tested by one-way ANOVA, followed by Tukey's test. Differences were considered significant when the p value was < 0.05.

3 Results

Since CQ10 and CQ20 groups did not show any differences between them throughout the study, from now on, we will refer to both quercetin control groups as a single group (CQ).

3.1 Effect of quercetin on number and volume of preneoplastic foci

We assessed the effect of quercetin treatments on number and volume of rGST P-positive foci. As expected, neither preneoplastic foci nor initiated hepatocytes were observed in control rats treated with quercetin or with the vehicles (data not shown). On the other hand, animals subjected to the initiation-promotion model displayed preneoplastic lesions that were quantified. Representative images from IP, IPQ10, and IPQ20 groups are shown in Fig. 2A. Quercetin at the dose of 10 mg/kg body weight administered to IP animals did not induce any changes in number or volume of liver foci. However, both parameters were significantly decreased in IPQ20 group as compared to IP animals. Quercetin treatment at 20 mg/kg body weight reduced the total number of initiated cells capable of developing into focal clones (–50%, Fig. 2B) and the growth rate and total cellular population of preneoplastic foci (–52.5%, Fig. 2C).

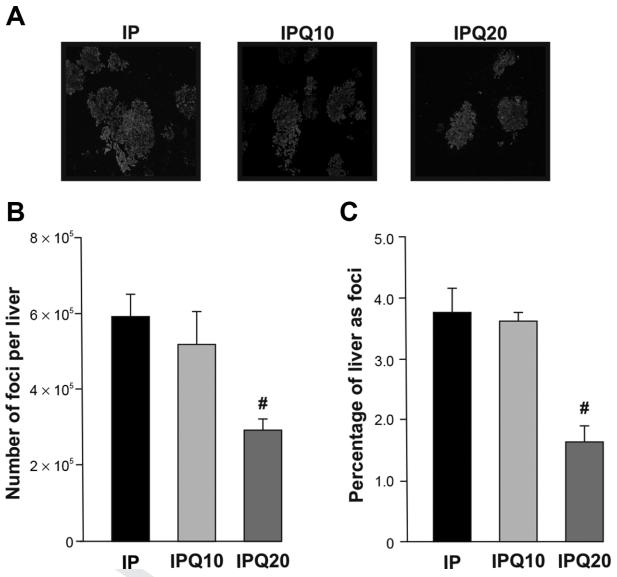

Figure 2. Effect of quercetin on number and volume of liver preneoplastic foci. (A) Representative images of rGST P-positive preneoplastic foci obtained by confocal microscopy (objective: 10×). C and CQ livers displayed no rGST P staining (data not shown). Changes in (B) number of liver lesions per liver and (C) volume percentage of the liver occupied by preneoplastic foci are represented for IP, IPQ10, and IPQ20 groups. IP: rats with liver preneoplasia; IPQ10: IP rats treated with quercetin 10 mg/kg body weight; IPQ20: IP rats treated with quercetin 20 mg/kg body weight. Data are expressed as mean ± SE; $n \geq 4$. $^{\#}p < 0.05$ versus IP.

3.2 Analysis of lipid peroxidation and antioxidant capacity

Preneoplastic treatment induced a 30% increase in the generation of TBARS as compared to control animals. In animals carrying hepatic lesions induced by DEN/2-AAF treatment, quercetin administration produced a decrease in TBARS levels (both IPQ10 and IPQ20 groups); reaching control values (Fig. 3A). Also, GSH/GSSG ratio, and CAT and Cu/Zn SOD activities were analyzed. No changes in GSH/GSSG ratio

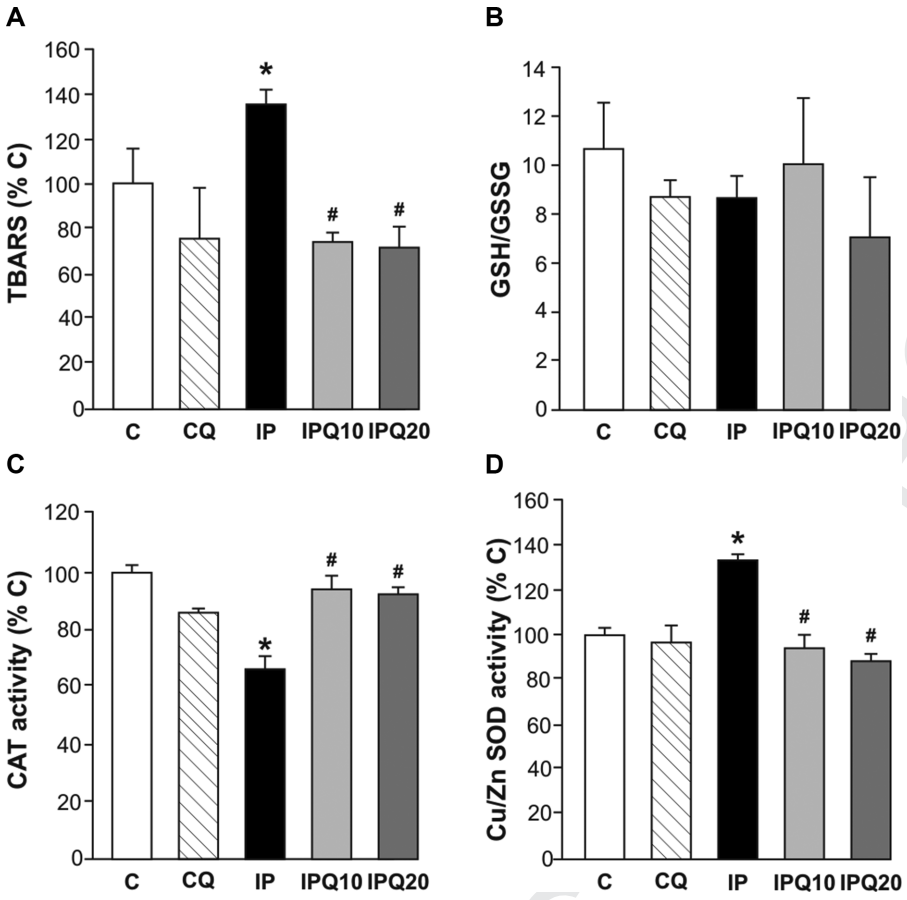

Figure 3. Analysis of lipid peroxidation and liver antioxidant capacity. (A) Lipid peroxidation was determined by quantification of the amount of thiobarbituric acid reactive substances (TBARS). (B) Determination of GSH/GSSG ratio in liver homogenates from the experimental groups. Analysis of (C) catalase (CAT) and (D) Cu/Zn superoxide dismutase (SOD) activities in total liver homogenates. C: control rats; CQ: C rats treated with quercetin; IP: rats with liver preneoplasia; IPQ10: IP rats treated with quercetin 10 mg/kg body weight; IPQ20: IP rats treated with quercetin 20 mg/kg body weight. Data are expressed as percentage of C group and are mean ± SE; $n \geq 4$. $^{*}p < 0.05$ versus C; $^{\#}p < 0.05$ versus IP.

were observed between treatments (Fig. 3B). On the other hand, CAT activity was significantly decreased (Fig. 3C) and Cu/Zn SOD activity significantly increased (Fig. 3D) in IP animals whereas quercetin treatment abolished these changes.

3.3 Effect of quercetin on programmed cell death

Apoptosis is orchestrated by activation of the caspase cascade and effector caspase-3 is the ultimate responsible for the majority of the effects [29]. Figure 4A shows that caspase-3 activity was significantly increased in IP group with respect to C animals. This result was expected, since rates of apoptosis are higher in the preneoplastic liver than in the normal tissue [8]. Moreover, when quercetin was administered at 20 mg/kg body weight, apoptosis was enhanced as caspase-3 activity was significantly increased in IPQ20 group relative to IP animals. In association with these results, levels of procaspase-3 (inactive precursor of caspase-3) were measured by Western blot and showed a decrease in IPQ20 rats (Fig. 4A, right panel).

Since the fate of the cells (survival or death) is largely dependent on the mitochondrial Bax/Bcl-2 ratio [31], we measured mitochondrial Bax and Bcl-2 levels and calculated the aforementioned ratio. Figure 4B shows that Bax/Bcl-2 ratio was significantly increased in IP animals as compared to C group. Treatment with quercetin at 20 mg/kg body weight significantly augmented Bax/Bcl-2 ratio in IPQ20 relative to IP group. Furthermore, levels of cytosolic cytochrome c were increased in IPQ20 group compared to IP animals (Fig. 4C). Together, these results support the fact that apoptosis is enhanced in rats treated with quercetin at 20 mg/kg body weight and the mitochondrial pathway is involved in this process.

3.4 Effect of quercetin on the proliferative status of liver foci

Figure 5A shows representative images for immunohistochemical detection of PCNA-positive cells. As shown in Fig. 5B, control livers (both C and CQ groups) showed a very low incidence of cell replication, in line with the quiescent status of normal adult liver [33]. In animals carrying hepatic lesions, proliferative indexes were determined both inside and surrounding the foci. As expected, proliferative status inside the foci was notably increased with respect to the surrounding tissue. In addition, whereas the IPQ10 group did not show differences in the proliferative index with respect to the IP group, treatment with quercetin at 20 mg/kg body weight induced a significant diminution of proliferative

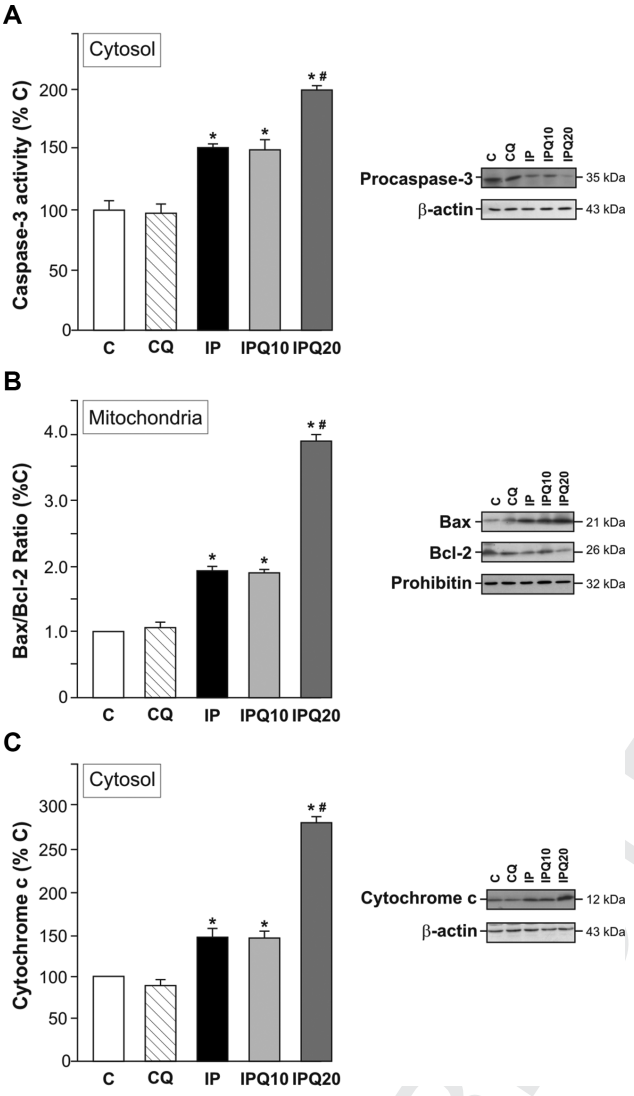

Figure 4. Effect of quercetin on apoptotic cell death. (A) Caspase-3 activity was determined in cytosolic fractions and expressed as percentages, being C group considered 100%. Additionally, procaspase-3 levels were evaluated by Western blot (right panel). (B) Mitochondrial levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins were analyzed by Western blot. After densitometric quantitation, Bax/Bcl-2 ratio was calculated, and results were expressed as percentage of C group. (C) Release of cytochrome c was determined by Western blot in cytosolic extracts from each experimental group. C: control rats; CQ: C rats treated with quercetin; IP: rats with liver preneoplasia; IPQ10: IP rats treated with quercetin 10 mg/kg body weight; IPQ20: IP rats treated with quercetin 20 mg/kg body weight. Results are expressed as percentage, considering control as 100%. β-Actin and prohibitin were probed as loading control in cytosolic and mitochondrial extracts, respectively. Data are mean ± SE; $n \geq 4$. $^{*}p < 0.05$ versus C; $^{\#}p < 0.05$ versus IP.

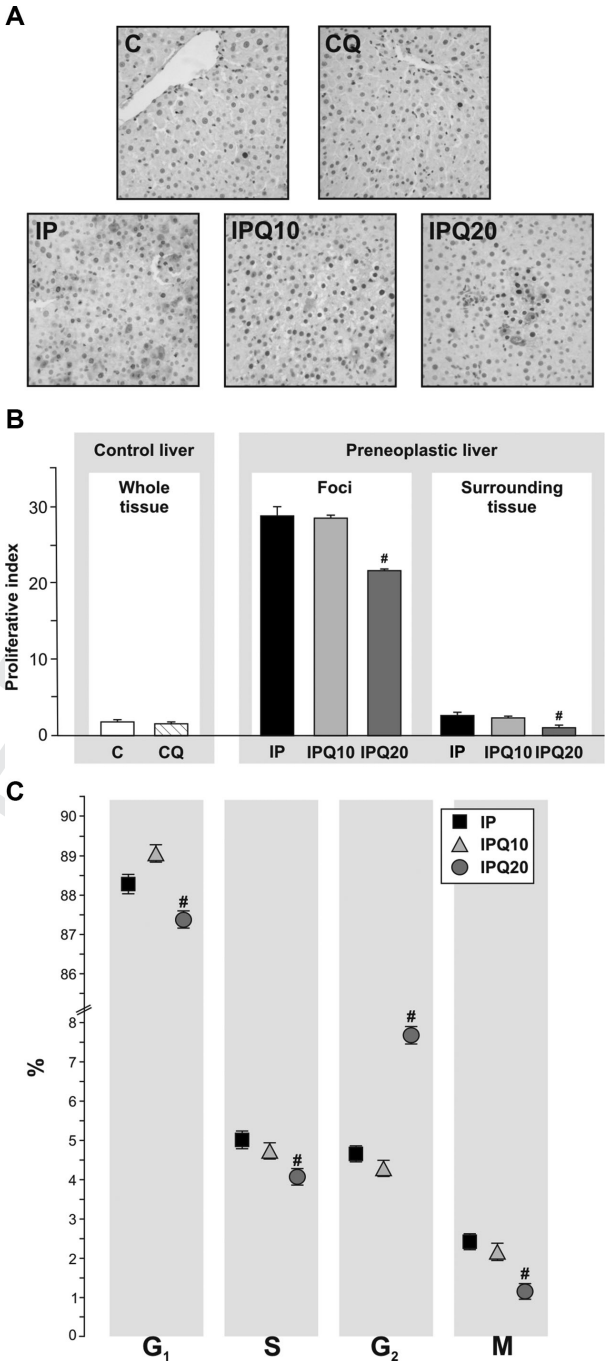

Figure 5. Effect of quercetin on proliferative status of liver foci. (A) Representative images of proliferating cell nuclear antigen (PCNA)-positive cells obtained by optical microscopy (objective: 20×). (B) Changes in the proliferative index in the whole liver (C and CQ groups), differentiated between the foci and the surrounding tissue (IP, IPQ10, and IPQ20 groups). (C) Determination of the percentage of preneoplastic hepatocytes in each phase of the cell cycle. C: control rats; CQ: C rats treated with quercetin; IP: rats with liver preneoplasia; IPQ10: IP rats treated with quercetin 10 mg/kg body weight; IPQ20: IP rats treated with quercetin 20 mg/kg body weight. Data are expressed as mean ± SE; $n \geq 4$. $^{\#}p < 0.05$ versus IP.

index both inside the foci (–25%) and in the surrounding tissue (–50%). Changes in nontumor host tissue support the fact that this tissue is far from being "normal," and it might even play an important role in the pathogenesis of the disease or it may contribute to the tumor-suppressive effects of some anticancer agents [34].

Additionally, we determined the percentages of preneoplastic hepatocytes in each phase of the cell cycle (Fig. 5C). We observed a significant diminution of the percentages of cells in G_1 and S phases in the IPQ20 group, which could be explained by a lower entrance of preneoplastic cells into the cell cycle. In addition, a significant accumulation of cells in G_2 phase was also observed in the IPQ20 group. Accordingly, this group showed a diminution in the percentage of cells in M phase.

3.5 Effect of quercetin on the expression of cell cycle related genes

Real-Time q-PCR studies revealed several fold increments of cell cycle related genes in rats carrying liver preneoplasia compared to control animals, in accordance with the proliferative status of preneoplastic liver and the quiescent status of normal liver (Fig. 6A). In animals with hepatic lesions, treatment with quercetin at 20 mg/kg body weight significantly prevented the increased expression of genes encoding for cyclin D1 (*Ccnd1*), cyclin A2 (*Ccna2*), cyclin B1 (*Ccnb1*), and *cdk1*. In addition, treatment with quercetin at 20 mg/kg body weight induced a significant increment in mRNA levels of the cell cycle regulator p53 (*Tp53*) relative to IP group. IPQ10 group did not show any significant differences in gene expression with respect to IP group (Fig. 6A).

In accordance with these results, Western blot studies revealed reduction of cyclin D1, cyclin A, cyclin B1, and cdk1 proteins levels and a significant increase in p53 protein level in IPQ20 group relative to IP group (Fig. 6B).

3.6 Effect of quercetin on peroxisome proliferator activated receptor-α (PPAR-α)

In the past few years, it has been demonstrated that quercetin is a selective inhibitor of PPAR-α that binds directly to the receptor and competes with its ligands for binding [35]. Thus, we hypothesized that quercetin treatment may affect PPAR-α expression as another mechanism that contributes to the preventive effects of the flavonoid.

Results showed that hepatic PPAR-α protein expression was increased in animals with liver preneoplasia, compared to control rats. In addition, when quercetin was administered at a dose of 10 mg/kg body weight (IPQ10), no effect in PPAR-α protein levels was observed; however, treatment with quercetin at 20 mg/kg body weight produced a significant attenuation of PPAR-α protein levels (Fig. 7A). We also studied hepatic PPAR-α mRNA (*Ppara*) expression and observed a similar profile as for protein expression, with a significant increment of *Ppara* in IP group with respect to control animals and a diminution in IPQ20 rats (Fig. 7B).

Additionally, we measured the transcription levels of PPAR-α-target genes: *Acox1*, *Acsl1*, and *Cpt1a* [36]. Results showed that the hepatic expression of these genes was induced in IP animals. This induction was reverted in IPQ20 group, reaching control levels (Fig. 7C).

4 Discussion

Quercetin, the most abundant flavonoid in the diet, is considered one of the most promising compounds for disease prevention and therapy; however, many of its effects still need to be studied [10, 11]. In addition, both cytoprotective and cytotoxic effects of quercetin were described in diverse cell culture models [37, 38]. Regarding cell proliferation, a dual effect of quercetin has been reported: the flavonoid stimulates or inhibits cell proliferation in vitro, at physiologically relevant concentrations [39]. These differential effects may depend on the presence of estrogen receptor α and its ratio to the estrogen receptor β, a parameter that varies between different tissues [40]. So, given both beneficial and adverse effects of quercetin, it is assumed that its prevalent action depends on the cell type or tissue, the presence of a stressor, and the dose of the flavonoid used.

Particularly in cancer, considerable in vitro and in vivo data indicate that quercetin acts as an anticarcinogen agent [11,41]. Numerous in vitro investigations have reported the effects of quercetin on signal transduction pathways associated with the process of carcinogenesis. They include effects on cell cycle regulation, apoptosis, proinflamatory protein induction, and angiogenesis [11]. Thus, the molecular mechanisms involved in the preventive effects of quercetin in cancer appear to be complex [12–14]. For this reason, we attempt to provide new data on these mechanisms in early liver carcinogenesis, focusing on the balance between cell proliferation and apoptosis.

The present study was conducted using quercetin aglycone. This is not the form in which the flavonoid is available in the regular diet (usually as glycosides), but it is the main form present in purified dietary supplements. It has been reported that the effects of quercetin as an anticarcinogen are generally observed for the aglycone, but not for the glycoside form [11]. We tested two doses of quercetin (10 and 20 mg/kg body weight) and we observed that in the IPQ10 group there was no reduction of preneoplastic foci. However, the IPQ20 group showed a significant reduction in both number and volume of hepatic lesions. Other studies have reported quercetin effectiveness in reducing hepatic lesions with doses of the aglycone form ranging from 5 to 25 mg/kg body weight [12, 14]. It has been postulated that prevention of DEN-induced liver foci development by quercetin is because the flavonoid reduces lipid peroxidation levels and strengthens the endogenous antioxidant defense system during the

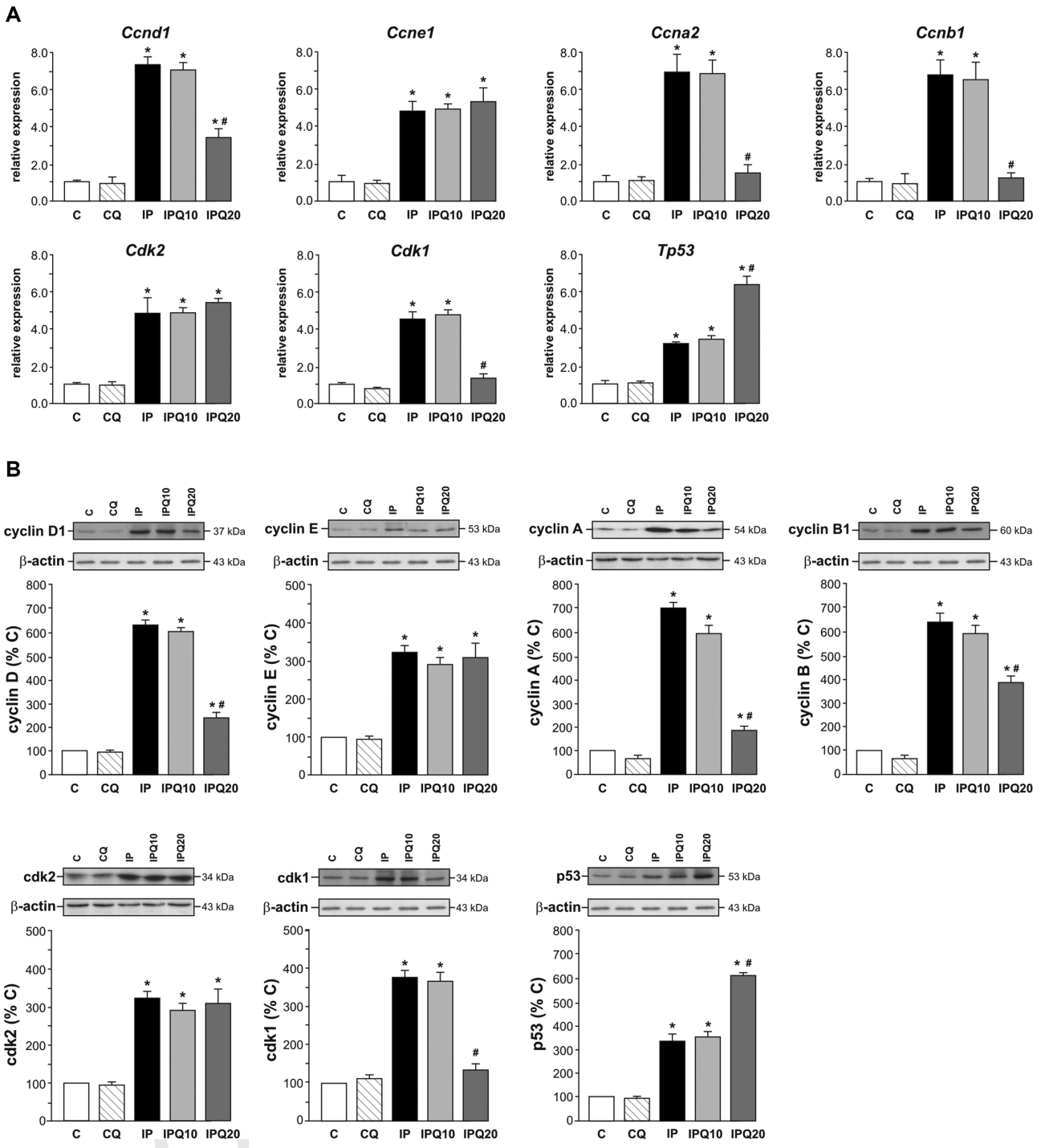

Figure 6. Effect of quercetin on the expression of some cell cycle related genes. (A) Real-time q-PCR analysis of genes encoding for: cyclin D1 (*Ccnd1*), cyclin E1 (*Ccne1*), cyclin A2 (*Ccna2*), cyclin B1 (*Ccnb1*), cyclin-dependent kinase 2 (*Cdk2*), cyclin-dependent kinase 1 (*Cdk1*), and tumor protein p53 (*Tp53*). (B) Western blot analysis of cell cycle related proteins: cyclin D1, cyclin E, cyclin A, cyclin B1, cdk2, cdk1, and p53. C: control rats; CQ: C rats treated with quercetin; IP: rats with liver preneoplasia; IPQ10: IP rats treated with quercetin 10 mg/kg body weight; IPQ20: IP rats treated with quercetin 20 mg/kg body weight. Data are expressed as ratio/percentage of C group and are mean ± SE; $n \geq 4$. $^{*}p < 0.05$ versus C; $^{\#}p < 0.05$ versus IP.

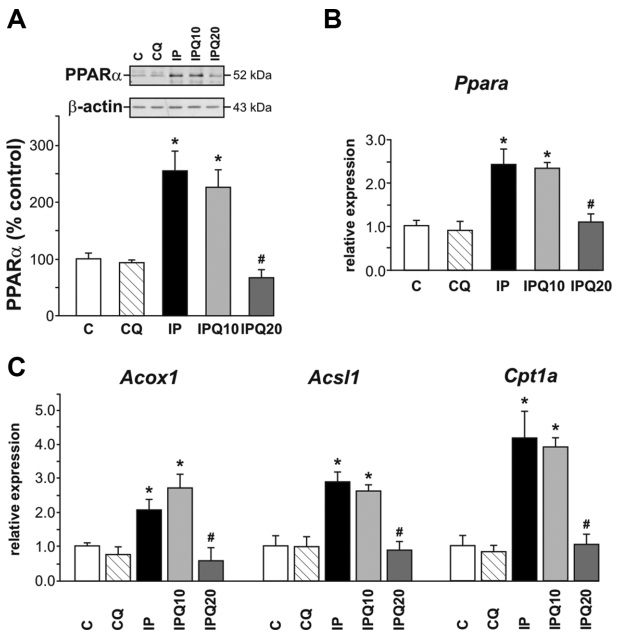

Figure 7. Effect of quercetin on hepatic PPAR-α expression. (A) Expression levels of hepatic PPAR-α protein by Western blot analysis. (B) mRNA levels of PPAR-α (*Ppara*) by real-time q-PCR. (C) Transcription levels of PPAR-α-target genes: acyl-coenzyme A oxidase 1, palmitoyl (*Acox1*), acyl-CoA synthetase long-chain family member 1 (*Acsl1*), and carnitine palmitoyltransferase 1a, liver (*Cpt1a*). C: control rats; CQ: C rats treated with quercetin; IP: rats with liver preneoplasia; IPQ10: IP rats treated with quercetin 10 mg/kg body weight; IPQ20: IP rats treated with quercetin 20 mg/kg body weight. Data are expressed as ratio/percentage of C group and are mean ± SE; $n \geq 4$. $^{*}p < 0.05$ versus C; $^{\#}p < 0.05$ versus IP.

initial hours after DEN injection [12, 42]. In our work, we studied lipid peroxidation levels and antioxidant enzymes activities at the end of the experimental protocol. Given the prooxidant nature of the genotoxic treatment, it is reasonable that IP animals showed alterations in TBARS levels and in the antioxidant capacities, with respect to control rats. Administration of quercetin (both IPQ10 and IPQ20) maintained these parameters similar to those in control rats. Similar results have been previously published [12]. Notably, quercetin at 10 mg/kg body weight maintained its antioxidant properties but lacked its antiproliferative and proapoptotic activities; these properties are key in decreasing preneoplastic foci growth.

Apoptosis is another mechanism that could be modulated by quercetin. Numerous in vitro studies in HCC cell lines have reported the modulatory action of quercetin on several key components of signaling pathways linked to apoptotic cell death [32, 43, 44]. In vivo studies in liver preneoplasia models have shown that quercetin induced apoptosis by increasing caspases-9 and -3 at 24 h post DEN initiation [13], whereas in a medium-term assay the flavonoid inhibited apoptotic cell death [14]. These discrepancies about the effect of quercetin on apoptosis could depend on the experimental models. In the present work, quercetin at 20 mg/kg body weight increased caspase-3 activity, indicating that the programmed cell death is activated with the treatment. Furthermore, quercetin induced an increase in mitochondrial Bax/Bcl-2 ratio. It is known that this ratio determines cell survival or cell death after apoptotic stimuli [31]. Due to the translocation of proapoptotic Bax to the mitochondrial membrane in the IPQ20 group, cytochrome c is released from mitochondria to cytosol, leading to executioner caspases activation. In the present work, we describe the ability of quercetin to modulate proteins of the Bcl-2 family in vivo, and it seems that this mechanism is operating as a preventive action of the flavonoid during the development of liver lesions.

Premalignant lesions and even some neoplastic tumors are highly dependent of chemical promotion and responsive to treatments that decrease proliferation and increase apoptosis [45]. Therefore, targeting one or both of these events may result in the reversion of cell growth. In this context, we analyzed whether quercetin was able to affect cell proliferation by targeting cyclins and cdks that command each phase of the cell cycle, as another possible feature of the preventive action of the flavonoid. IPQ20 group showed a significant reduction in the proliferative status both inside the foci and in the surrounding tissue. Cell cycle distribution of preneoplastic hepatocytes displayed a diminution of cells in G_1 and S phases of the cell cycle, which may be attributed in part to a minor entrance of preneoplastic cells into the cell cycle, and in part to the increase of apoptosis. Induction of cyclin D1 regulates the progression through G_1 phase and also G_1/S transition; therefore, this protein is considered the "rate-limiting" step in hepatocyte proliferation [46]. In line with previous studies in different cell types [47–49], the increased cyclin D1 expression observed in animals with proliferative foci was prevented by quercetin administration, resulting in diminution of preneoplastic cells in G_1 and S phases. We have also observed that quercetin induced a significant accumulation of preneoplastic cells in G_2 phase, indicating a major effect in G_2/M transition. This action of quercetin has only been described in cultured cells [50–52]. The initiation of mitosis is controlled by the activation of the cyclin B/cdk1 complex, so reduced cyclin B1 protein levels can induce G_2 arrest [53], and inhibition of cyclin B1 transcription causes cell cycle arrest. Treatment with quercetin during the development of liver preneoplastic lesions prevented the increases of both cyclin B1 and cdk1. Although only mRNA and protein levels of cyclin B1 and cdk1 were measured, it is clear that the lack of increment in IPQ20 group with respect to IP animals does not favor cyclin B/cdk1 complex formation. In addition, prevention of the increase of cyclin A mRNA and protein levels was also observed. Cyclin A is particularly interesting among the cyclin family because it functions in both S phase and mitosis, depending on its cdk partner [54, 55]. So, the prevention of cyclin A increase by quercetin may have a double impact on preneoplastic cell proliferation: there could be a reduction in the passage into the S phase and also a decrease into M phase entry.

In addition, p53 (both transcript and protein) was augmented in quercetin-treated animals carrying preneoplastic foci, a result that was predictable since p53 has a pivotal role in cell cycle regulation and apoptosis. Additional studies are required to define if changes in critical cell cycle regulators induced by quercetin are a consequence of a direct effect of the flavonoid or an indirect action via p53 induction.

Finally, quercetin can act as an inhibitor of PPAR-α by binding directly to the receptor and competing with its ligands [35]. We analyzed the effects of quercetin on PPARα expression and activation and observed a significant inhibition of PPAR-α activation in the IPQ20 group. Interestingly, the 10 mg/kg body weight dose did not reduce preneoplastic foci incidence and neither did inhibit PPAR-α activation. We made some additional experiments using MK886, a noncompetitive inhibitor of PPAR-α [55] and found that MK886 effectively blocked PPAR-α activation, reduced number and volume of preneoplastic foci, and decreased cell proliferation by inducing cell cycle arrest in G_2/M (data not shown). These results led us to assume that inhibition of PPAR-α activation is a key event in the preventive action of quercetin on genotoxic neoplastic occurrence in rat liver. Additional studies using PPAR-α-knock-down animals are necessary to confirm this hypothesis.

In conclusion, the present work provides new data concerning the mechanisms involved in the preventive actions of quercetin during the development of liver cancer. In addition to its proapoptotic properties, quercetin is able to modulate the expression of critical cell cycle regulators and PPAR-α activation. These actions affect the proliferation of preneoplastic hepatocytes that finally conduct to a reduction of liver lesions.

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The authors have declared no conflict of interest.

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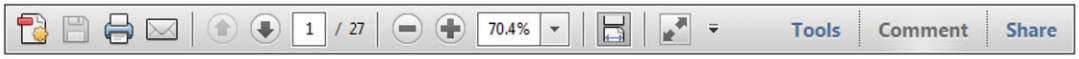

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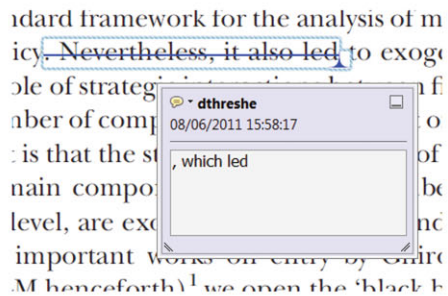

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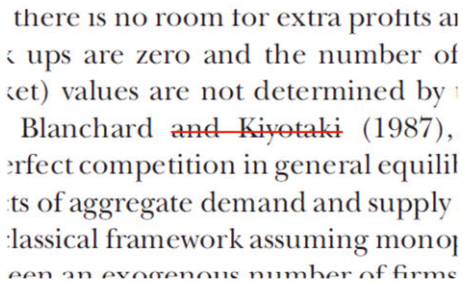

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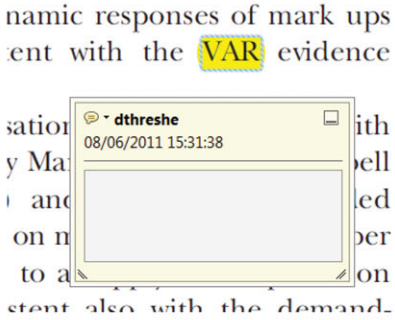

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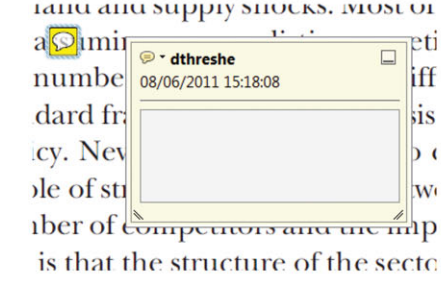

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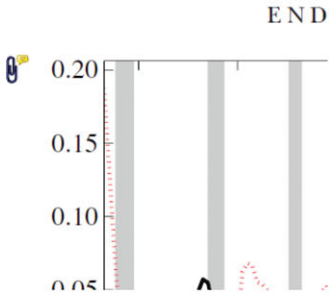

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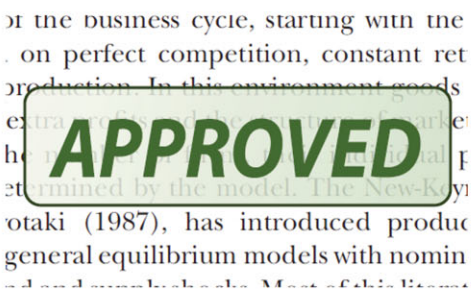

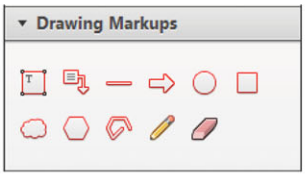

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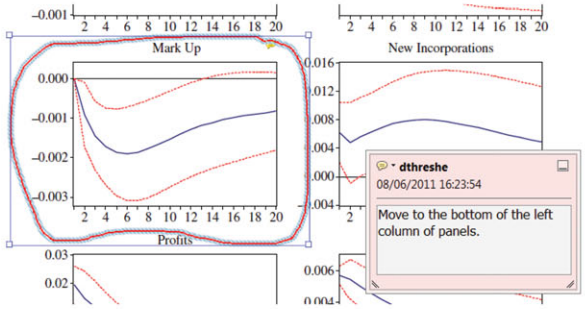

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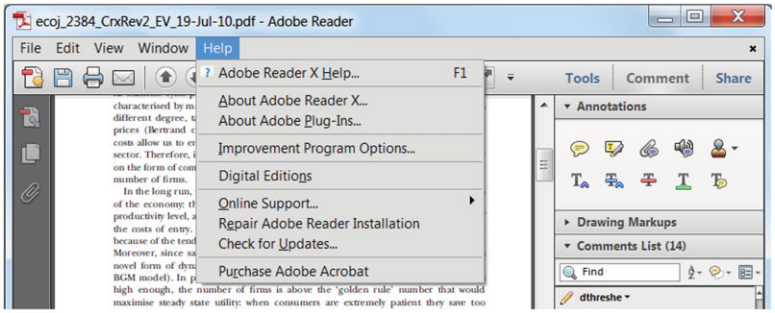

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