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Involvement of catalase in the apoptotic mechanism induced by apigenin in HepG2 human hepatoma cells

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ABSTRACT

Apigenin has been reported to inhibit proliferation of cancer cells; however, the mechanism underlying its action is not completely understood. Here, we evaluated the effects of apigenin on the levels of expression and activity of antioxidant enzymes, and the involvement of ROS in the mechanism of cell death induced by apigenin in HepG2 human hepatoma cells. Upon treatment with apigenin, HepG2 cells displayed a reduction in cell viability in a dose- and time-dependent manner, and some morphological changes. In addition, apigenin treatment induced ROS generation and significantly decreased the mRNA levels and activity of catalase and levels of intracellular GSH. On the other hand, apigenin treatment did not alter the expression or activity levels of other antioxidant enzymes. Addition of exogenous catalase significantly reduced the effects of apigenin on HepG2 cell death. We also demonstrated that HepG2 cells are more sensitive to apigenin-mediated cell death than are primary cultures of mouse hepatocytes, suggesting a differential toxic effect of this agent in tumor cells. Our results suggest that apigenin-induced apoptosis in HepG2 cells may be mediated by a H_2O_2-dependent pathway via reduction of the antioxidant defenses.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer worldwide, especially in regions where hepatitis B is endemic, such as China, southeast Asia, and southern Africa. The incidence of HCC has tripled in the United States over the past two decades as a result of an increase in hepatitis C infections [1,2]. Current treatment options for HCC remain limited, therefore, development of more-effective therapeutic tools and strategies is greatly desirable [3]. In order to develop an effective drug against several types of cancer, considerable attention has been devoted to identifying plant-derived compounds displaying anti-tumor activity, such as the flavonoids [4].

Apigenin (5,7,4′-trihydroxyflavone), a naturally occurring plant flavonoid, is abundant in vegetables and fruits [5]. Apigenin is a bioactive flavonoid with anti-inflammatory, antioxidant, and anticancer properties against several human-cancer cell lines [6], including prostate carcinoma [7,8], colon carcinoma [9], breast cancer [10], leukemia cells [11], cervical carcinoma [12], lung cancer [13], and hepatoma [14,15].

The cellular effects of apigenin are associated with cell-cycle arrest and apoptosis in a wide range of malignant cells [11,16,17]. Exposure of a wide array of malignant cells to apigenin induces a reversible G2/M and G0/G1 arrest by inhibiting $p34^{cdc2}$ kinase activity [9], and increasing p53 protein stability [18,19]. Apigenin is able to increase p21 levels, resulting in arrest of the cell cycle and apoptosis in several cancer cell lines, regardless of the Rb status and p53 involvement [20,21,12,14]. Apigenin also induces apoptosis by alteration of the Bax/Bcl-2 ratio, release of cytochrome c, and induction of Apaf-1, leading to caspase activation and PARP-cleavage [21,13,11,12,22].

Accumulated evidence indicates that apigenin can suppress the growth of hepatocarcinoma cells by arresting the cell cycle, which is associated with an accumulation of p53 tumor-suppressor protein and induction of p21 expression [14] and caspase-mediated apoptosis [23], also causing a dose-dependent elevation of intracellular ROS[1] levels.

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[1] The abbreviations used are: ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; NADPH, nicotinamide adenine dinucleotide phosphate; BHA, butylated hydroxy anisole; BHT, butylated hydroxy toluene; EtOH, ethanol; Tris, tris(hydroxymethyl)aminomethane; NaCl, sodium chloride; PCR, polymerase chain reaction; DCFDA, 2′,7′-dichlorofluorescin diacetate; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; Gred, glutathione reductase; BSO, buthionine sulfoximine.

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Treatment of human prostate cancer R22v1 cells with apigenin resulted in mitochondrial membrane depolarization, intracellular ROS generation, activation of caspases (-3, -9 and -8) and induction of apoptosis [22]. Apigenin also induces apoptosis in prostate epithelial cells through ROS generation, which interferes with mitochondrial functions and leads to mitochondrial permeability transition [8]. In addition, treatment with various inhibitors of NADPH oxidase significantly abrogated both the generation of ROS and the induction of apoptosis by apigenin. These results suggest that ROS generated through the activation of NADPH oxidase may play an essential role in the apigenin-induced apoptosis in HepG2 cells [15].

Intracellular generation of H_2O_2, the most stable ROS, has been considered an important mediator of apoptosis. H_2O_2 can also alter protein activity through oxidative modification of regulatory protein complexes (e.g., the Nrf2/Keap1 system). The Nrf2 transcription factor regulates the expression of antioxidant enzymes and xenobiotic detoxification. Nrf2 promotes activation of genes containing antioxidant response elements (AREs), such as the phase-2 genes encoding heme oxygenase, quinine oxidoreductase, γ-glutamylcysteine synthase, glutathione *S*-transferase (GST), catalase, superoxide dismutase (MnSOD) and other antioxidant-function genes, their expression being induced upon cells' exposure to oxidative stress [24]. Flavonoids have recently been demonstrated to favor both Nrf2 nuclear translocation and eventually ARE activation in several cell lines.

GSH is involved in several important cellular functions, such as chemical detoxification and antioxidant defense. The GSH to GSSG cellular ratio, which under physiological conditions is around 100 to 1 in the liver, decreases during oxidative stress and apoptosis. Altered GSH redox state triggers the unfolding protein response and apoptosis [25].

Although prior studies have shown that apigenin-induced ROS-dependent apoptosis occurs in liver cancer and other malignancies, the pathways whereby apigenin leads to ROS generation and execution of the apoptosis program are still not well characterized.

The main purpose of the present study was to investigate whether the mRNA levels and activity of different antioxidant enzymes (such as SOD, catalase, GPx, GRed), GSH levels and ROS generation are affected by apigenin and whether these alterations could be associated with the mechanism of apoptosis induced by apigenin in HepG2 human hepatoma cells.

Our results indicate that catalase downregulation (mRNA expression levels and protein activity) may play an essential role in apigenin-induced apoptosis in HepG2 cells, since catalase added to the culture medium partially protected from apigenin-induced cell death, suggesting the direct involvement of H_2O_2 in the apoptotic mechanism induced by apigenin in HepG2 human hepatoma cells. Together, our results suggest that the effects of apigenin could contribute to ROS generation, GSH depletion and apoptotic cell death in HepG2 human hepatoma cells, opening new avenues for therapeutic strategies.

2. Materials and methods

2.1. Materials

DMEM high-glucose medium and fetal bovine serum (FBS) were obtained from Cultilab (Campinas, Brazil). Apigenin, crystal violet, NADPH, cytochrome c and DCF–DA were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Merck (São Paulo, SP, Brazil). All other reagents were commercial products of the highest available purity grade.

2.2. Apigenin solutions

Apigenin (100 mmol/L) was dissolved in DMSO and then further diluted in DMEM high-glucose medium (10–100 μmol/L). The apigenin stock solution was stored at −20 °C and warmed to 25 °C before use.

2.3. HepG2 cell culture

The human hepatocarcinoma HepG2 cell line (from the American Type Culture Collection – ATCC) was maintained in Dulbecco's modified Eagle's medium (DMEM high-glucose), supplemented with 10% fetal bovine serum (FBS) and 50 μg/mL gentamycin, at 37 °C, 5% CO_2 under controlled humidity.

2.4. Primary culture of mouse hepatocytes

Male mice from the Central Animal House of the Federal University of Paraná, which received a standard laboratory diet (Purina), were euthanized (with 4 mg/kg xylazine/0.6 mg/kg acepromazine/60 mg/kg ketamine) and manipulated, using aseptic techniques, under a laminar airflow. All recommendations of the Brazilian national regulations for scientific management of animals (No. 6638, 05/11/1979) were respected. The liver was transferred to Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS), washed three times with PBS, minced with scissors and then centrifuged at 2000*g* for 3 min. The material was incubated with 1 mg/mL collagenase (Sigma) for 3 min in a 37 °C incubator. After tissue digestion, the supernatant was centrifuged at 2000*g* for 3 min. The pellet was resuspended in high-glucose DMEM supplemented with 10% FBS and seeded onto P60 mm plates. This operation was repeated three more times, increasing the incubation period in the presence of collagenase. The culture was maintained in a humidified atmosphere in 5% CO_2 at 37 °C.

2.5. Cell viability assays

HepG2 cells were seeded at a density of 2.5×10^4 cells/well into 24-well culture plates. After overnight incubation, the cells were treated with varying concentrations of apigenin (0, 10, 25, 50, or 100 μmol/L) for 24, 48, 96, or 144 h. For catalase protection assays, the cells were seeded at a density of 1×10^4 cells/well into 96-well culture plates. After overnight incubation, the cells were simultaneously treated with 100 μmol/L apigenin and exogenous catalase (100, 250, 500, or 1000 U/mL), BHA or BHT (10, 50, or 100 μmol/L), or ascorbic acid (100, 250, or 500 μmol/L) for 24 h. To assess the viability of the hepatocyte cells, the cells were seeded at a density of 1×10^4 cells/well into 96-well culture plates. After overnight incubation, the cells were treated with 10, 25, 50, or 100 μmol/L apigenin for 24 or 48 h. Cell viability was evaluated through the crystal violet staining assay, as described by Kueng et al. (1989) [26], and absorbance was determined at 550 nm, using an Infinite® 200 TECAN microplate reader. Control experiments were performed with high-glucose DMEM containing 0.1% DMSO (v/v). The results were expressed as a percentage of viable cells in comparison to the control (100%).

2.6. Determination of cytochrome c release

Cells were seeded at a density of 1×10^6 cells onto a 60-mm plate. After overnight incubation, the cultures were treated with apigenin (100 μmol/L) for 24 h and then washed with phosphate-buffered saline (PBS) pH 7.2. Cells were harvested and permeabilized with 10 μmol/L digitonin for 30 min at 4 °C in a solution containing 10 mg/ml Tris–HCl (pH 7.0), 3 mg/mL ethylene glycol tetra-acetic acid (EGTA), and 50 μg/mL sucrose. The cells were then

centrifuged at 10,000*g* for 30 min. The supernatant was filtered through a 0.2 μm filter. Cytochrome c release was evaluated by the method of Appaix et al. (2000) [27]. The absorbance of clarified supernatants was recorded at 414 nm, using the medium as a blank, in a spectrophotometer (Shimadzu UV-2450). The values were calculated based on $\varepsilon = 100\ mmol^{-1}\ cm^{-1}$ of cytochrome c at 414 nm [27].

2.7. Cell-cycle analysis

Cells were seeded at a density of 1×10^6 cells onto a 60-mm plate. After overnight incubation, the cultures were treated with apigenin (100 μmol/L) for 24 h, and then the culture supernatants and the adhered cells were collected. The samples were centrifuged at 2000*g* for 5 min, and the cell pellet was resuspended in 0.5 mL of PBS and 4.5 mL of cold 70% EtOH, and stored at 4 °C. For flow-cytometry analysis, the cell pellet was again collected by centrifugation, and 0.3 mL of propidium iodide (PI) staining solution (50 μg/mL) was added to the samples. The samples were analyzed in a FC500 flow cytometer (Beckman Counter) and the results were calculated using the MXP software.

2.8. Optical microscopy analysis

HepG2 cells (2.5×10^4 cells/well) were seeded onto 24-well cell-culture plates containing glass coverslips. After overnight incubation, the cultures were treated with apigenin (100 μmol/L) for 24 or 48 h. After treatment, the coverslips were removed and the cells were immediately fixed in absolute ethanol and then stained with hematoxylin–eosin. The cells were evaluated by means of BEL® Photonics microscopy.

2.9. Determination of GSSG and GSH levels

HepG2 cells were treated with apigenin (25, 50 and 100 μmol/L) or with buthionine sulfoximine (BSO) (50 μmol/L) for 24 h. To measure the levels of GSH and GSSG, we followed the protocols of Griffith (1980) [28] and Rahman et al. (2007) [29] with the following modifications: after treatment, the supernatant was removed and cells were washed with PBS. HCl was added (10 mmol/L) and cells were lysed by freezing and thawing process. After an equal volume of 5-sulfosalicylic acid was added, the samples were maintained on ice for 5 min and then centrifuged (10,000*g*/10 min, 4 °C). The supernatant was collected and separated it into two equal aliquots of 100 μL (for GSx and for GSSG). To measure GSSG, 2-vinylpyridine was added (2%, v/v), the solution was mixed and kept for 1 h in ice. Upon neutralization by the addition of triethanolamine (0.5 mmol/L), 50 μL of each sample were distributed for quantification of total GS per well of a 96-well plate and 60 μL for GSSG.

2.10. Measurement of intracellular ROS generation

Intracellular ROS generation was determined as described by Wan et al. (2003) [30]. Cells were seeded at a density of 1×10^4 cells/well in 96-well plates. After overnight incubation, the cultures were washed twice with PBS and incubated for 30 min with 50 μmol/L of 2′,7′-dichlorofluorescein diacetate (DCFH–DA) diluted in PBS. At the end of the incubation period, the cultures were again washed twice with PBS and treated with 100 μmol/L apigenin and 400 μmol/L H_2O_2 as a positive control. Immediately after the treatment period, the plates were read using an Infinite® 200 TECAN microplate reader at excitation and emission wavelengths of 485 and 525 nm, respectively. The level of cellular fluorescence, measured in relative fluorescence units (RFU), was converted as a percentage compared to control cells, which were arbitrarily assigned the value of 100%.

2.11. RNA isolation and first-strand cDNA synthesis

Cells were seeded at a density of 1×10^6 cells/well onto a 60 mm plate. After overnight incubation, the cultures were treated with apigenin (100 μmol/L) for 24 h. Total RNA was isolated using the spin Mini RNA isolation kit (Qiagen), according to the manufacturer's instructions, and stored at −80 °C. One microgram of total RNA was reverse-transcribed using oligo(dT) and Superscript III Reverse Transcriptase (Invitrogen). cDNA synthesis was carried out according to the manufacturer's instructions, and the resulting cDNA was stored at −20 °C.

2.12. Quantification of mRNA levels by real-time RT-PCR

Using cDNAs as the template, quantitative real-time PCR was carried out using the SYBR Green PCR Master Mix (Applied Biosystems) in a StepOnePlus™ Real-Time PCR Detection System (Applied Biosystems), according to the manufacturer's instructions, using specific oligonucleotide primers for human *CuZnSOD, MnSOD, CAT, Gred,* and *Gpx1* genes. A dissociation cycle was performed after each run to check for non-specific amplification or contamination. The mRNA expression levels were normalized using the geNorm 3.4 software [31], and the corresponding *HPRT*, *Tubulin,* and *GAPDH* housekeeping gene expression levels. Relative expression levels were estimated using the method described by Pfaffl (2001) [32]. Sets of specific primers were designed, using the Primer Express (Applied Biosystems) software and validated through BLAST and BLAT, and their sequences are shown in Table 1.

2.13. Enzymatic activity assays

After incubation with apigenin (100 μmol/L) for 24 h, HepG2 cultures were washed with PBS and the cells were collected, resuspended in PBS, and sonicated on ice (three times for 20 s). This suspension was centrifuged for 10 min at 13,000*g* (4 °C) in a microcentrifuge, and the supernatant was used for enzymatic activity assays. Antioxidant enzyme activities were determined with a spectrophotometer (Shimadzu UV-2450). Catalase (E.C. 1.11.1.6) was determined by following the decrease in absorbance at 240 nm, using hydrogen peroxide as the substrate [33]. Superoxide dismutase (SOD) (E.C. 1.15.1.1) was measured by monitoring the inhibition of epinephrine auto-oxidation at 480 nm [34]. Catalase activity was previously calculated as nmol of H_2O_2 consumed $min^{-1}\ mg^{-1}$ protein, and SOD activity as U/mg protein. For total

Table 1
Nucleotide sequences of PCR primers used for quantitative real-time RT-PCR.

Gene	Oligonucleotide primers
hCuZnSOO	F: 5′-GTGCAGGGCATCATCAATTTC-3′
	R: 5′-AATCCATGCAQGCCTTCAGT-3′
hZnSOD	F: 5′-GGACACTTACAAATTGCTGCTTGT-3′
	R: 5′-AGTAAGCGTGCTCCCACACAT-3′
hCAT	F: 5′-GATAGCCTTCGACCCAAGCA-3′
	R: 5′-ATGGCGGTGAGTGTCAGGAT-3′
hGSR	F: 5′-GTGGCACTTGCGTGAATGTT-3′
	R: 5′-GGAAAGCCATAATCAGCATGATC-3′
hGpxl	F: 5′-TGCTGGCCTCCCCTTACAG-3′
	R: 5′-GCACACATGGCGCAATTG-3′
hGAPDH	F: 5′-ACCCACTCCTCCACCTTTGA-3′
	R: 5′-CTGTTGCTGTAGCCAAATTCGT-3′
hTubuin	F: 5′-TCAACACCTTCTTCAGTGAAACG-3′
	R: 5′-AGTGGCAGTGCGAACTTCATC-3′
hHPRT	F: 5′-GAACGTCTTGCTCGAGATGTGA-3′
	R: 5′-TCCAGCAGGTCAGCAAAGAAT-3′

SOD, the control corresponds to one unit of SOD (one SOD unit corresponds to 50% inhibition of activity). Glutathione reductase (E.C. 1.6.4.2) activity was measured using the method of Sies et al. (1979) [35]. Glutathione reductase activity was calculated as *p*mol of NADPH consumed/min/mg protein. Enzyme activities were expressed as percentages of the control (100%).

2.14. Protein determination

Total protein concentration was performed by the Bradford method (1976). [36], calibrated with bovine serum albumin.

2.15. Statistical analysis

Statistical analysis was carried out by analysis of variance (one-way ANOVA) followed by a Tukey test or Kruskal–Wallis/Dunn's Multiple comparison test for comparison of the means, using the Graph Pad Prism 5 program. Values for means ± SD were used. For all comparisons, differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Apigenin induces inhibition of cell growth, cytochrome c release, and DNA fragmentation in HepG2 cells

In order to assess the effects of apigenin in the human hepatoma model, HepG2 cells were exposed to different concentrations of apigenin (10–100 μmol/L) for 24, 48, 96, or 144 h, and then cellular viability was determined by violet crystal staining. Addition of apigenin decreased cell viability in a dose- and time-dependent manner, as depicted in Fig. 1A. Treatment for 24 h with the lowest dose (10 μmol/L) significantly decreased cell viability (16.7%), and produced an approximately 40% reduction in cell viability when these cells were treated with the highest dose (100 μmol/L). Apigenin (100 μmol/L) treatment of HepG2 cells for 6 days caused more than 80% reduction in cell viability.

In order to better understand the mechanism of cell death induced by apigenin, HepG2 cells were treated with apigenin (100 μmol/L) for 24 h and then analyzed for DNA fragmentation and cytochrome c release. A significant increase (>2-fold) in cytochrome c release to the cytoplasm was observed after apigenin treatment (Fig. 1B). In addition, apigenin also induced DNA fragmentation, as assessed by determining the hypodiploid DNA content after staining with propidium iodide, with a concomitant decrease in the percentage of cells in the G1 phase, compared to untreated control cultures (Fig. 1C).

3.2. Morphological analysis

The HepG2 cells exposed to 100 μmol/L of apigenin for 24 and 48 h showed discreet morphological changes, evidenced by the lost of cellular limits and alterations in cell volume (Fig. 2C and Fig. 2D). Control cultures treated with vehicle (0.1% DMSO) did not have an apparent effect compared to untreated controls.

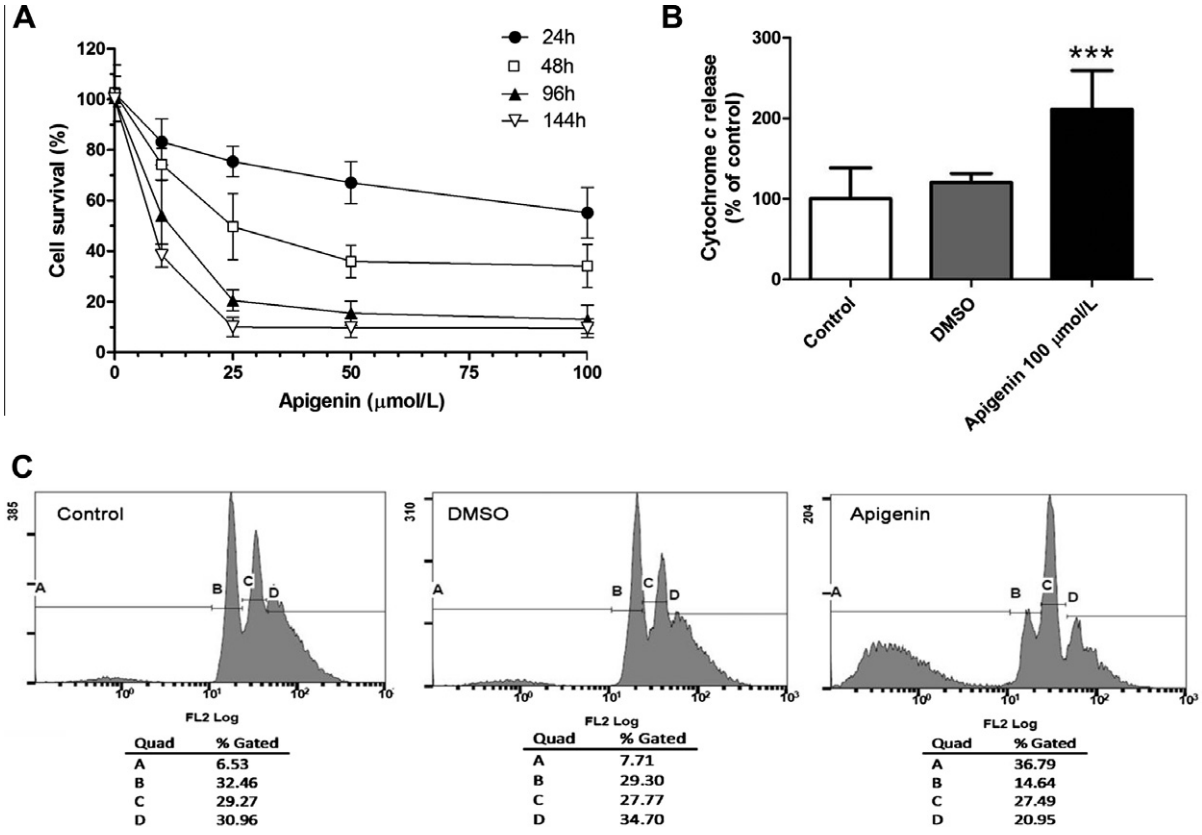

Fig. 1. Effects of apigenin on cell viability, cytochrome c release, and DNA fragmentation. (A) Viability of the HepG2 cell line after apigenin treatment. Values represent the mean ± SD of percentage of viable cells in comparison to the control (data represent three independent experiments, each in triplicate). Significant differences ($p < 0.05$) were found in the percentage of cell death at all doses tested. (B) Release of cytochrome c from mitochondria, caused by apigenin treatment (100 μmol/L). Data are shown as a percentage relative to control (100%) of five independent experiments, each in triplicate. Control (100%) corresponds to 1.12 ± 0.24 nmol of cytochrome c released. min^{-1} mg^{-1} protein. $^{***}p < 0.001$. (C) Flow cytometry analysis of PI-stained HepG2 cells treated with apigenin (100 μmol/L). The *x*-axis represents relative fluorescence intensity and the *y*-axis represents cell number. The sub-G1 peak, designated as A, was defined by cells undergoing DNA fragmentation. The G1, S/G2, and M phases were designated B, C, and D, respectively. Data represent three independent experiments, each in triplicate.

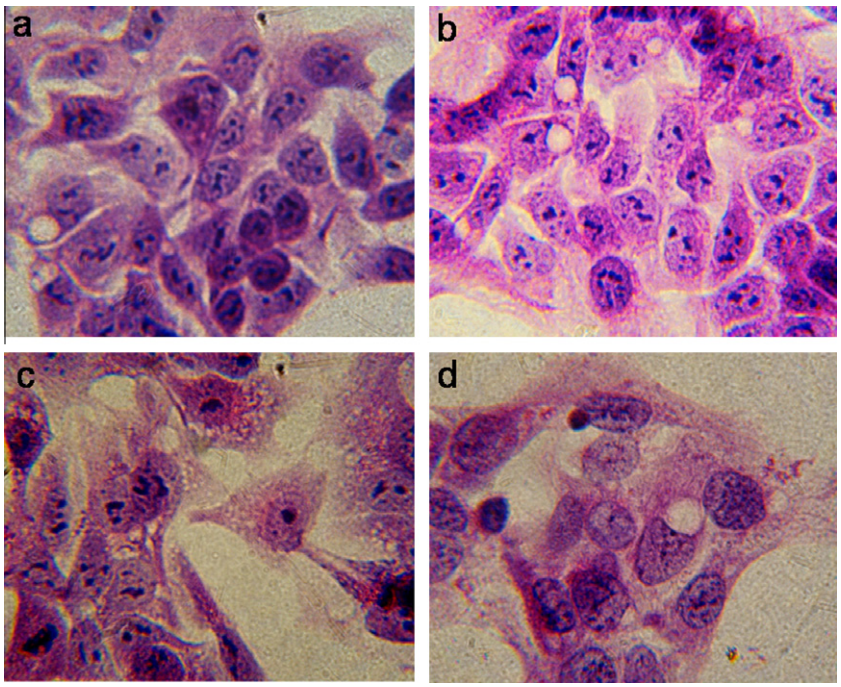

Fig. 2. Morphological assessment of HepG2 cells after treatment with apigenin (100 μmol/L) for 24 and 48 h. Optical microscopy analysis (230×). (a) Control 24 h; (b) Control 48 h; (c) Apigenin (100 μmol/L) treatment for 24 h. (d) Apigenin (100 μmol./L) treatment for 48 h.

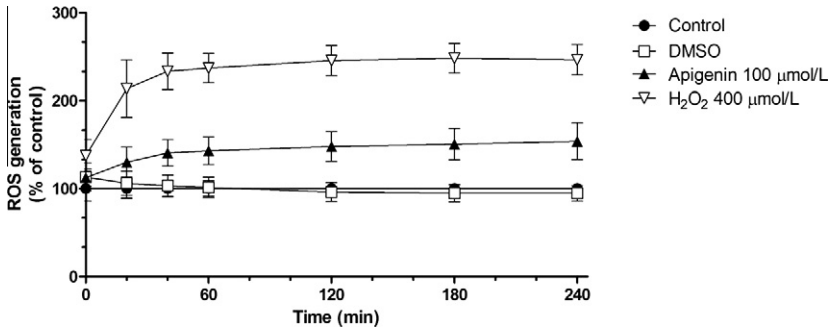

Fig. 3. ROS generation after apigenin treatment of HepG2 cells. HepG2 cells were preloaded with DCFH–DA substrate in PBS and treated with apigenin (100 μmol/L). The fluorescence was read at the indicated time periods. The experiments represent the mean ± SD of fluorescence percentage with respect to the control, of three independent experiments, each in triplicate.

3.3. Effects of apigenin on ROS generation and expression, and activity of antioxidant enzymes

HepG2 cells were treated with apigenin (100 μmol/L), and ROS generation was measured using fluorometric analysis. Treatment with H_2O_2 (400 μmol/L) was used as a positive control. The apigenin treatment led to enhanced generation of ROS, as a function of time, up to 60 min of treatment, after which the effect reached a plateau (Fig. 3).

The expression levels of CAT, CuZnSOD, MnSOD, GPx1, and Gred by qRT-PCR in HepG2 cells were examined upon treatment with apigenin (100 μmol/L) for 24 h. Apigenin-treated HepG2 cells displayed significantly lower CAT mRNA levels ($p < 0.001$) (Fig. 4A). The mRNA expression levels of MnSOD, CuZnSOD, GPx1, and Gred were not altered under these conditions (Fig. 4A). The relative mRNA expression data were normalized using the geNorm 3.4 software [31], and the corresponding housekeeping genes *HPRT*, *Tubulin*, and *GAPDH*.

In addition, the effects of apigenin on the activity of antioxidant enzymes were evaluated. The results (Fig. 4B) showed that only catalase activity was significantly inhibited, by approximately 20% ($p < 0.001$), in HepG2 cells treated with apigenin (100 μmol/L for 24 h).

3.4. Catalase protection of HepG2 cell death

In order to determine whether downregulation of catalase plays an essential role in the mechanism of apigenin-induced apoptosis, cells were treated simultaneously with apigenin (100 μmol/L) and different concentrations of exogenous catalase for 24 h. Cell viability was evaluated using the crystal violet staining assay. The results (Fig. 5A) demonstrated that addition of exogenous catalase (500 U/mL and 1000 U/mL) resulted in a significant reduction of the effect of apigenin on HepG2 cell death, by approximately 20% ($p < 0.01$) and 30% ($p < 0.001$), respectively.

Interestingly, when HepG2 cells were co-treated with apigenin (100 μmol/L) in the presence or in the absence of other antioxidant compounds without specificities for H_2O_2 decomposition, such as BHA, BHT, and ascorbic acid, no significant reduction in the percentage of cell death was observed (Fig. 5B).

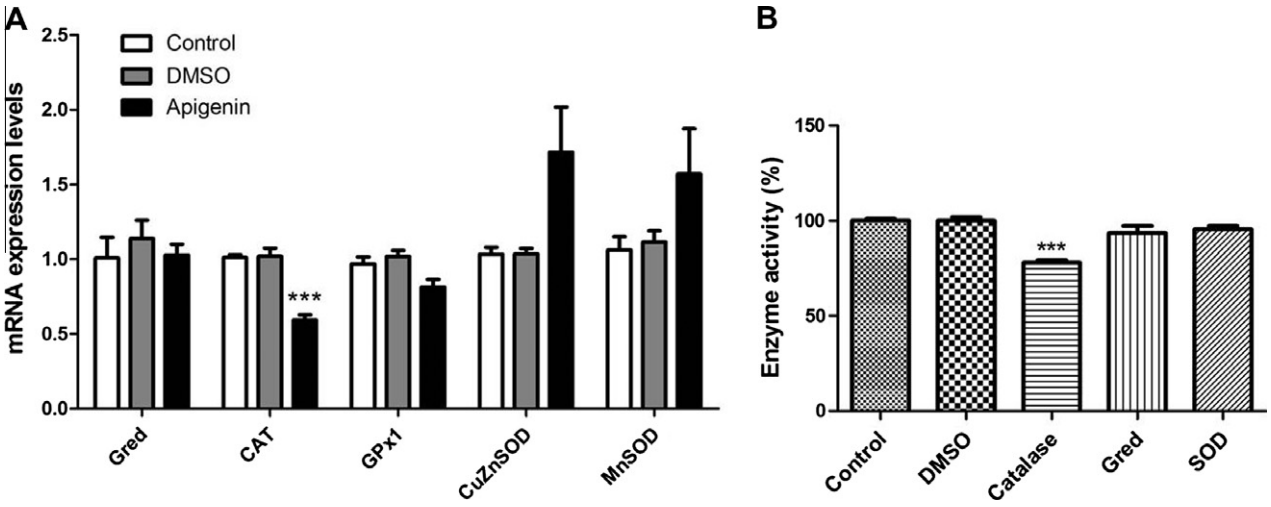

Fig. 4. Effects of apigenin on mRNA expression levels and activity of antioxidant enzymes in HepG2 cells. (A) Quantification of transcript levels of antioxidant enzymes. The data represent the mean ± SD of three independent experiments, each in triplicate. (B) Effects of apigenin (100 μmol/L) on activity of antioxidant enzymes. Data are presented as a percentage relative to control (100%) of three independent experiments, each performed in triplicate. For catalase data, control (100%) corresponds to 0.853 nmol of H_2O_2 consumed (μg protein min^{-1}). For total SOD, control (100%) corresponds to 1 unit of SOD (1 SOD unit corresponds to 50% inhibition of activity). For the Gred data, control (100%) corresponds to 0.14 nmol of NADPH consumed (μg protein.min^{-1}). $^{*}p < 0.001$ and $^{***}p < 0.001$.

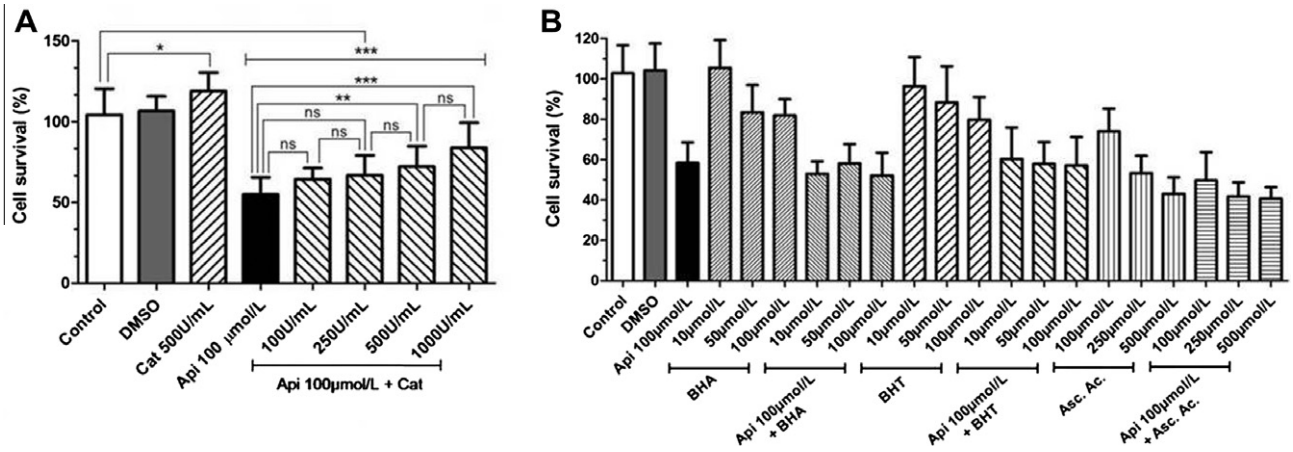

Fig. 5. Effects of co-treatment with apigenin and antioxidant compounds on viability of HepG2 cells. (A) Cells were co-treated with apigenin (100 μmol/L) and different exogenous catalase concentrations for 24 h. (B) Co-treatment with apigenin (100 μmol/L) and BHA or BHT or ascorbic acid at different concentrations for 24 h. Values represent the mean ± SD of percentage of viable cells in comparison to untreated control (data represent three independent experiments, each in triplicate). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus control.

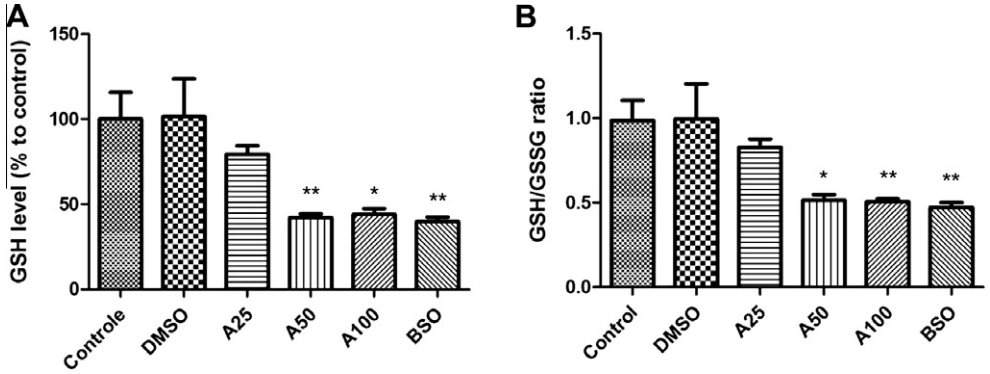

Fig. 6. GSH levels and GSH/GSSG ratio in apigenin-treated HepG2 cells. Cells were treated with 0 (untreated), 25, 50 and 100 μM apigenin for 24 h. Glutathione depletion with GSH neosynthesis inhibitor buthionine sulfoximine (BSO) was used as a control. (A) GSH depletion analysis in apigenin-treated HepG2 cells. Data are shown as a percentage relative to control (100%) of three independent experiments, each in triplicate. Values are expressed as mean ± SD. (B) Apigenin reduces the GSH/GSSG ratio in HepG2 cells. Data represent three independent experiments, each in triplicate. Values are expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

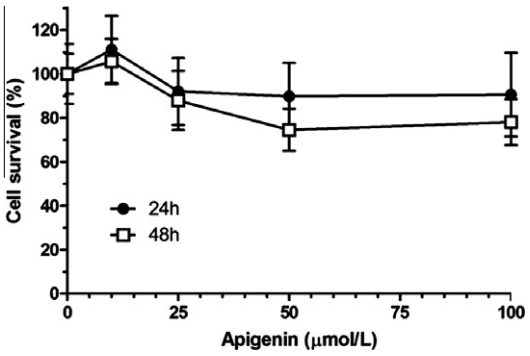

Fig. 7. Cell viability of mouse hepatocytes after apigenin treatment. Cell viability assessed by crystal violet staining after 24 and 48 h of treatment with apigenin, as indicated. Values represent the mean ± SD of percentage of cell viability with respect to the untreated control. Data represent two independent experiments, each in triplicate.

3.5. GSSG and GSH determinations

HepG2 cells treated with apigenin (50 and 100 μmol/L) for 24 h displayed reduced GSH levels (Fig. 6A). Pharmacological glutathione depletion with GSH neosynthesis inhibitor buthionine sulfoximine (BSO) was used as a control. The GSH levels were reduced by 48% and 46% relative to the control condition in the presence of 50 and 100 μmol/L of apigenin, respectively ($p < 0.05$). We also observed a significant decrease (approximately 50%) in the GSH/GSSG ratio in HepG2 cells after apigenin treatment (50 and 100 μmol/L, for 24 h) ($p < 0.05$) (Fig. 6B).

3.6. Differential sensitivity of human hepatoma cells versus primary mouse hepatocytes to the growth-inhibitory effects of apigenin

Agents that are capable of inducing selective apoptosis of cancer cells are receiving considerable attention, in the effort to develop novel approaches to prevent cancer. In order to evaluate the growth-inhibitory effects of apigenin in normal versus tumor cells, primary monolayer cultures of adult mouse hepatocytes were treated with different concentrations of apigenin for 24 and 48 h. Our results showed (Fig. 7) that the growth of primary mouse hepatocytes was not inhibited by the apigenin treatment at lower levels, when compared with the effects observed for the HepG2 cell line (Fig. 1A).

4. Discussion

Cell apoptosis is initiated by extracellular and intracellular signals via two main pathways, namely, the death receptor- and the mitochondrial-mediated pathways; in both of which ROS play an important role. The extrinsic apoptosis pathway is mediated by death receptors in which ligand-receptor binding initiates protein–protein interactions at the cell membrane, activating initiator caspases. Recent evidence suggests a possible direct role for ROS in mediating death receptor activation and apoptotic induction through ROS-induced receptor clustering and the formation of lipid-raft-derived signaling platforms [25]. The stimulation of the intrinsic mitochondrial apoptotic pathway by ROS and mitochondrial DNA damage promotes outer membrane permeabilization and mitochondria-to-cytosol translocation of cytochrome c, AIF or Smac/Diablo, which triggers caspase-dependent or caspase-independent cytosolic signaling events [37,38]. Mitochondrial permeabilization, permeability transition pore processes, and the release of apoptogenic factors are observed. However, JNK activation by ROS may induce both extrinsic or intrinsic apoptotic signaling [39]. ROS-mediated apoptotic signaling is associated with decreased cellular GSH levels and loss of the cellular redox balance [40]. Decreased cell GSH can be generated by ROS-induced GSH oxidation or GSH export from cells. It was demonstrated that GSH loss due to decreased *de novo* GSH synthesis triggered redox activation of protein kinase C and through GSH efflux, induced JNK-dependent apoptosis [41,42]. GSH oxidation is a major contributor to cell apoptosis mediated by oxidants through activation of mitochondrial apoptotic signaling. It has been observed that post-oxidant recovery of the cellular GSH/GSSG redox status did not influence the apoptotic outcome, indicating that oxidant-induced apoptotic initiation occurred within an early and narrow window of the GSH/GSSG redox shift [43].

Previous reports have demonstrated a dose-dependent inhibition of cell viability and induction of apoptosis by apigenin in several human cancer cell lines, such as the cervical carcinoma HeLa cells [12], leukemia HL-60 cells [11], breast cancer cell lines [10], prostate epithelial cells [8], prostatic adenocarcinoma LNCaP cells [20], lung cancer A549 cells [13], and HepG2 hepatoma cells [14,23,15]. In addition, the role of ROS as a mediator of the apoptosis induced by apigenin has been established [22]. However, the signaling pathways whereby apigenin leads to ROS generation and execution of the apoptosis program are still not fully characterized.

To further elucidate the mechanisms of apigenin-induced cell death in HepG2 cells, we initially evaluated morphological alterations induced by this flavone and biochemical markers of apoptosis. The results indicated that apigenin induced morphological changes (Fig. 2), suggesting cell suffering, however, to clarify the morphological changes induced by apigenin, further experiments such as electron microscopy are required. In addition, our results showed that apigenin treatment leads to a decreased percentage of viable cells (Fig. 1A), cytochrome c release from mitochondria (Fig. 1B), and DNA fragmentation (Fig. 1C), consistent with the apoptotic process. DCF assays also confirmed apigenin-induced ROS generation (Fig. 3). These data are in agreement with those of Choi et al. (2007) [15], who observed increased levels of intracellular ROS in apigenin-treated HepG2 cells. The cytochrome c release and DNA fragmentation promoted by apigenin in HepG2 cells, observed in this study, are in agreement with data reported by different authors upon treatment of other cell lines with apigenin [11,8,13]. Together, our results provide evidence that apigenin treatment promotes the apoptotic pathway in HepG2 cells in a dose- and time-dependent manner.

It is recognized that intracellular generation of H_2O_2 is an important mediator of apoptosis. Different enzymes such as peroxidases, catalase, and superoxide dismutase are involved in H_2O_2 modulation. Interestingly, it has been reported that overexpression of human catalase in HepG2 cells promotes a protective effect against cytotoxicity or apoptosis induced by oxidative stress [44].

The effects of apigenin on the expression and activity of antioxidant enzymes that modulate H_2O_2 levels, such as catalase (CAT), superoxide dismutase (CuZnSOD and MnSOD), glutathione peroxidase (GPx), and glutathione reductase (Gred), in HepG2 cells had not been previously described. Here, we analyzed the effects of apigenin on these enzymes. In addition, we correlated the effects of apigenin on H_2O_2 modulation with HepG2 cell proliferation.

Our results demonstrated that apigenin treatment promotes downregulation of catalase mRNA expression levels and reduction of its activity (Fig. 4A and B), suggesting that increased H_2O_2 levels could be involved in the HepG2 cell death induced by apigenin.

In order to validate our hypothesis, we evaluated the effect of concomitant treatment of HepG2 cells with both apigenin and exogenous catalase. In the presence of exogenous catalase, we observed protection from apigenin-induced cell death in a dose-dependent manner (Fig. 5A). These results showed that catalase

downregulation (mRNA expression levels and activity) plays an essential role in apigenin-induced apoptosis in HepG2 cells, suggesting the direct involvement of H_2O_2 in the apoptotic mechanism induced by apigenin in these cells. Supporting this hypothesis, concomitant treatment with apigenin and other antioxidant compounds (which are able to promote scavenging of free radicals, but do not act directly on H_2O_2) had no effect on HepG2 cell death (Fig. 5B).

Several recent reports have suggested a role for ROS in apoptosis induced by drugs, including certain chemopreventive agents, through the engagement of downstream proteins involved in the execution of apoptosis [45–48]. Previous reports demonstrated that apigenin-induced apoptosis in different cell lines (including HepG2 cells) occurs through an increase in p53 and p21 protein levels, suggesting a p53-dependent apoptosis pathway [14,12]. In agreement with our results, a recent study using the prostate cancer 22Rv1 cell line found that addition of H_2O_2 or apigenin increased ROS generation, with concomitant increases in p53 and p21 protein expression, which correlated with increased apoptosis. These effects were alleviated by pretreatment of cells with exogenous catalase [22]. In addition, these authors observed that apigenin promotes decreased levels of Bcl-XL and Bcl-2 and increased Bax, triggering caspase activation; and, also, that treatment with the Z-VAD-FMK and DEVD-CHO caspase inhibitors partially rescued these cells from apigenin-induced apoptosis [22]. These data support our hypothesis that increased intracellular H_2O_2, induced by apigenin treatment, may be responsible for triggering the apoptotic pathway. We suggest that these effects of apigenin in p53 and other cell-cycle checkpoints, can be attributed at least in part to accumulation of ROS, due to direct effects of apigenin on some antioxidant enzymes, especially those involved in the control of H_2O_2 levels.

Wang et al. (1999) observed that apoptosis induced by flavonoids (including apigenin) is stimulated by the release of cytochrome c to the cytosol, by procaspase-9 processing as well as through a caspase-3 dependent mechanism in leukemia HL-60 cells [11].

Direct ROS effects on caspase activation have been documented. Redox-active catalytic site cysteines of caspases are prone to oxidation, nitrosation, or glutathiolation [49]. Thus, H_2O_2 derived from endogenous and exogenous sources has been shown to induce reversible inactivation of caspase 3 and 8 through oxidation of their catalytic site cysteines [50]. For caspase-9, H_2O_2-induced enzyme inactivation was specifically mediated through iron-catalyzed oxidation of the catalytic site cysteine in procaspase-9 [51]. Mitochondrial procaspase-9 activation occurred during the preapoptotic phase before cytochrome c release, suggesting that this mechanism could amplify the proapoptotic effect of cytochrome c [52].

Together, our results show that catalase downregulation (mRNA expression levels and activity) plays an essential role in apigenin-induced apoptosis in HepG2 cells, since the addition of this enzyme to the culture medium partially protected from apigenin-induced cell death, suggesting the direct involvement of H_2O_2 in the apoptotic mechanism induced by apigenin in HepG2 hepatoma cells.

Recently, it has been observed that the mRNA expression levels of antioxidant enzymes are regulated by the Nrf2–Keap1 complex [53]. Nrf2, a ubiquitous protein, is a member of the subfamily of transcription factors [54]. Nrf2 is sequestered in cytoplasm by virtue of its binding to the Kelch-like ECH-associated protein, Keap1, with Nrf2 being led to ubiquitination and proteosomal degradation. However, when reactive oxygen species and reactive nitrogen species are generated, and exogenous Nrf2 inducers such as chemopreventive agents are used, the Nrf2–Keap1 complex disassociates, and Nrf2 translocates to the nucleus, where it activates the transcription of detoxifying enzyme genes by biding to ARE (antioxidant response element).

Flavonoids have recently been demonstrated to favor both Nrf2 nuclear translocation and eventually AREs activation in several cell lines [53]. Nrf2 activation results in transcriptional expression of some protective enzymes, including copper/zinc superoxide dismutase (CuZnSOD), manganese (mitochondrial) superoxide dismutase (MnSOD), glutathione *S*-transferase (GST) and catalase [24,55].

Here, we show that HepG2 cells treated with apigenin display reduced intracellular GSH levels and GSH/GSSG ratio (Fig. 6A and B). Our data are in agreement with recent previously report data in which apigenin treatment induced intracellular GSH depletion and ROS accumulation in prostate cancer cells [22]. The catalytic and the regulatory subunits of γ-glutamylcysteine ligase, the enzyme that catalyzes the rate-limiting step in the biosynthesis of glutathione, are regulated by Nrf2 [56]. The cystine/glutamate membrane transporter, which is important for glutathione synthesis, is an Nrf2 target [57,58]. Glutathione is used by glutathione peroxidases to reduce hydrogen peroxide to water, and glutathione reductase is Nrf2-dependent [59]. Intracellular loss of GSH is an early hallmark in the progression to cell death in response to different apoptotic stimuli [60,61].

Together, our results show that exposure of HepG2 cells to growth-suppressive concentrations of apigenin resulted in ROS generation, as indicated by increased DCF oxidation (Fig. 3), accompanied by a significant decrease in catalase mRNA expression levels and activity (Fig. 4A and B) and intracellular GSH depletion (Fig. 5).

Three main mechanisms may contribute to Nrf2-mediated ARE activation by flavonoids [62], namely: (i) Nrf2 nuclear accumulation as a consequence of its phosphorylation, through activation of upstream kinases such as Akt and MAPKs; (ii) pro-oxidant action of flavonoids, resulting in oxidized cysteine thiols in Keap1; and (iii) redox-cycling reactions leading to the formation of semiquinone radicals and *O*-quinine, which affects Keap1 activity. However, in human HepG2 cells, quercetin enhanced ARE activity by upregulating Nrf2 expression, preventing its ubiquitination and proteasomal degradation, and accelerating Keap1 degradation in a proteasome-independent manner [63].

Recently it has been observed that luteolin (3′,4′,5,7-tetrahydroxyflavone), is a potent Nrf2 inhibitor (reduces mRNA and protein levels), and promoted GSH depletion of reduced glutathione in non-small-cell lung cancer A549 cells [64].

In some lung cancer cell lines, GSH depletion has been found to enhance Bax protein translocation to the mitochondrial membrane, thus sensitizing cells to apoptosis [65]. Apoptotic cell death of Mahlavu cells via an oxidative stress-mediated caspase-dependent mechanism involves an initial overproduction of reactive oxygen species (ROS), followed by a severe depletion of intracellular GSH contents, which correlated with the severe drops in the mitochondrial transmembrane potential and the activation of caspases 3/7, resulting in the DNA fragmentation [66]. In addition, Ji et al. [67] showed the effects of andrographolide (ANDRO), an active compound isolated from a traditional medicinal herb, on the death of apoptotic hepatoma cells. These authors observed that ANDRO induced the activation of the ASK1–SEK1/MKK4–JNK-c-Jun signal cascade, initiating the apoptosis, and suggested that there is a crosstalk between JNK activation and cellular GSH homeostasis [67]. They also observed that the depletion of cellular GSH can potentiate the anti-cancer effects of ANDRO in hepatoma cells [67].

GSH depletion induced by apigenin in HepG2 cells may be caused by increased ROS promoted by the pro-oxidant action of apigenin. Increased ROS promotion by apigenin may be due to: (i) oxidation of its hydroxyl groups; (ii) mitochondrial membrane alterations; (iii) altered oxidases activity; and (iv) reduced catalase mRNA expression and activity. Therefore, it seems reasonable to

suggest that apigenin affects the enzyme activities or protein functions by direct interaction with the protein.

In addition, it is well established that cancer cells display a persistently higher oxidative stress level compared to normal cells. This effect may render cancer cells more susceptible to being killed by anticancer drugs that promote increased ROS levels, such as some phytochemicals [46]. Moreover, Nrf2 favors the potential of cancer cells for chemoresistance in certain cancers [68,69].

Agents capable of inducing selective apoptosis of cancer cells are receiving considerable attention in the effort to develop novel cancer-preventive approaches. In the present study, we also demonstrated that the human hepatoma HepG2 cell line is more sensitive to apigenin-mediated cell death than are primary cultures of mouse hepatocytes. Apigenin-treated HepG2 cells showed reduction in cell viability in a dose- and time-dependent manner, reaching 45% and 65% reduction in the presence of 100 μmol/L apigenin after 24 and 48 h of treatment, respectively (Fig. 1A). In contrast, we observed a lower reduction in cell viability (∼20%) when primary-culture mouse hepatocytes were maintained under the same experimental conditions (Fig. 7).

In agreement with our data, similar selective growth-inhibitory effects were also observed for human A431 epidermoid carcinoma cells, compared to normal human epidermal keratinocytes, and for human prostate adenocarcinoma, compared to normal human prostate epithelial cells [7]. The selective effect of apigenin in tumor versus normal cells suggests that apigenin is a promising chemopreventive and/or chemotherapeutic agent against liver cancer.

Conflict of interest

The authors declare that they have no conflicts of interest.

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