Antiproliferative effect of catechin in GRX cells

Cristina Machado Bragança de Moraes, Denizar Alberto da Silva Melo, Roberto Christ Vianna Santos, Shanna Bitencourt, Fernanda Cristina Mesquita, Fernanda dos Santos de Oliveira, Edgardo Rodrıguez-Carballo, Ramon Bartrons, Jose Luis Rosa, Francesc Pujol Ventura, and Jarbas Rodrigues de Oliveira

Abstract: The phenolic compounds present in cocoa seeds have been studied regarding health benefits, such as antioxidant and anti-inflammatory activities. Fibrosis is a wound healing response that occurs in almost all patients with chronic liver injury. A large number of cytokines and soluble intercellular mediators are related to changes in the behavior and phenotype of the hepatic stellate cell (HSC) that develop a fibrogenic and contractile phenotype leading to the development of fibrosis. The objective of this study was to assess the catechin effect in GRX liver cells in activities such as cell growth and inflammation. The GRX cells treatment with catechin induced a significant decrease in cell growth. This mechanism does not occur by apoptosis or even by autophagy because there were no alterations in expression of caspase 3 and PARP (apoptosis), and LC3 (autophagy). The expression of p27 and p53 proteins, regulators of the cell cycle, showed increased expression, while COX-2 and IL-6 mRNA showed a significant decrease in expression. This study shows that catechin decreases cell growth in GRX cells and, probably, this decrease does not occur by apoptosis or autophagy but through an anti-inflammatory effect and cell cycle arrest. Catechin also significantly decreased the production of TGF-β by GRX cells, showing a significant antifibrotic effect.

Key words: GRX cells, catechin, fibrosis, inflammation.

Résumé : Les composés phénoliques présents dans les graines de cacao ont été étudiés en fonction de leurs bienfaits sur la santé telles leurs activités anti-oxydantes et anti-inflammatoires. La fibrose est une réponse à la cicatrisation qui survient chez presque tous les patients atteints de maladies chroniques du foie. Un grand nombre de cytokines et de médiateurs intercellulaires solubles sont reliés à des changements de comportement et de phénotype des cellules stellaires hépatiques qui développent un phénotype fibrogène et contractile qui mène au développement de la fibrose. L'objectif de cette étude était d'évaluer l'effet de la catéchine sur les cellules hépatiques GRX, notamment sur la croissance et l'inflammation. Le traitement de cellules GRX avec de la catéchine induisait une diminution significative de la croissance. Ce mécanisme ne survenait pas par apoptpse ou même par autophagie car l'expression de la caspase 3 et de la PARP (apoptose), ou de la LC3 (autophagie) n'était pas affectée. L'expression de p27 et de p53, des régulateurs du cycle cellulaire, était augmentée, alors que l'expression de l'ARNm de COX-2 et de l'IL-6 était significativement diminuée. Cette étude montre que la catéchine diminue la croissance des cellules GRX et que, probablement, une telle diminution ne survient pas par apoptose ou par autophagie mais par l'intermédiaire d'un effet anti-inflammatoire et d'un arrêt du cycle cellulaire. La catéchine diminuait aussi significativement la production de TGF-β par les cellules GRX, montrant un effet antifibrotique important.

Mots-clés : cellules GRX, catéchine, fibrose, inflammation.

[Traduit par la Rédaction]

Introduction

Fibrosis is a wound healing response that occurs in almost all patients with chronic liver injury (Friedman 2008*a*). Although the mechanisms of acute injury activate fibrogenesis, the signs associated with chronic lesions caused by infections, drugs, metabolic disorders, alcohol abuse, nonalcoholic hepatitis, or immune attack are necessary to perpetuate fibro-

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C.M. Bragança de Moraes, D.A.S. Melo, R.C.V. Santos, S. Bitencourt, and F.C. Mesquita. Laboratório de Biofísica Celular e Inflamação, Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Avenida Ipiranga 6681, prédio 12, CEP: 90619-900 Porto Alegre, Rio Grande do Sul, Brazil.
F. Santos de Oliveira. Hospital de Clínicas de Porto Alegre, Research Center, Ramiro Barcelos 2.350 CEP 90035-90 Porto Alegre / RS.
E. Rodrıguez-Carballo, R. Bartrons, J.L. Rosa, and F.P. Ventura. Departament de Ciències Fisiològiques II, IDIBELL, Universitat de Barcelona, Campus de Bellvitge, Barcelona, Spain.
J. Rodrigues de Oliveira. Laboratório de Biofísica Celular e Inflamação, Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Avenida Ipiranga 6681, prédio 12, CEP: 90619-900 Porto Alegre, Rio Grande do Sul, Brazil; Hospital de Clínicas de Porto Alegre, Research Center, Ramiro Barcelos 2.350 CEP 90035-90 Porto Alegre / RS.

Corresponding author: Jarbas Rodrigues de Oliveira (e-mail: jarbas@pucrs.br).

Fig. 1. (A) Test of cell growth in the GRX cell line by MTT reduction after 6 days of incubation with cocoa extract at concentrations of 0.06 and 0.125 mg/mL, indomethacin (0.13 mmol/L), and a control group. a, cocoa extract (0.125 and 0.06 mg/mL) and indomethacin < control ($p < 0.001$); and b, 0.125 mg/mL cocoa extract and indomethacin < 0.06 mg/mL cocoa extract ($p < 0.001$). (B) Test of cell growth in the GRX cell line by MTT reduction after 6 days of incubation with catechin at concentrations 0.01 and 0.02 mg/mL, indomethacin (0.13 mmol/L) and a control group. a, catechin (0.02and 0.01 mg/mL) and indomethacin < control ($p < 0.001$); and b, indomethacin < 0.02 mg/mL catechin ($p < 0.05$). (C) Count of GRX cells by light microscopy after treatment with catechin at concentrations of 0.01 and 0.02 mg/mL and incubation times of 0, 24, 48, and 72 h. a, catechin (0.01and 0.02 mg/mL) < control ($p < 0.05$). Data are expressed as the mean ± SD of 3 independent experiments.

sis (Friedman 2008*a*; Krizhanovsky et al. 2008; Iredale 2008; Sarem et al. 2006).

Hepatic stellate cell (HSC) activation is the main step that leads to hepatic fibrosis. This activation involves changes in liver-like fibrogenesis, proliferation, contractility, chemotaxis, matrix degradation, and cytokine release. These cytokines include profibrotic transforming growth factor (TGF-β), a central mediator of fibrotic response. Fibrosis is potentially a reversible process in early stages (Friedman 2008*b*).

The GRX cell line is representative of HSC in mice (Guimarães et al. 2006; Vicente at al. 1998; Souza et al. 2008). They might express the phenotype of activated myofibroblasts in response to in vitro treatment with profibrogenic cytokines or express quiescent lipocytes if treated with retinol, retinoic acid, or drugs that modify lipid metabolism such as indomethacin (a nonsteroidal, nonselective anti-inflammatory) (Souza et al. 2008). In the quiescent state, there are phenotypic changes, the reorganization of the actin cytoskeleton, an accumulation of fat droplets in the cytoplasm, and changes such as reduction of secretory activity of collagen (Guimarães et al. 2006). Thus, the GRX cell line is considered an in vitro model of HSC.

The phenolic compounds present in cocoa seeds, 60% of these phenolic compounds corresponding to catechin, have been studied regarding health benefits, such as high antioxidant activity (Zeeb et al. 2000; Bharrhan et al. 2011; Salah et al. 1995; Steinberg et al. 2003; Wan et al. 2001) and the reduction of low density lipoproteins (LDL) concentrations in plasma. Studies have proved that the cocoa procyanidins are capable of reducing platelet activation, help in maintaining cardiovascular health, and modulate the immune system (Steinberg et al. 2003; Wan et al. 2001; Ursini and Sevanian 2002; Auger et al. 2004).

Therefore, the objective of this study was to assess the catechin effect in GRX liver cells on cell growth.

Materials and methods

Materials

Cocoa seeds (*Theobroma cacao* L.) of the variety Forastero were from CEPLAC/CEPEC (the Executive Plan of Cocoa Farming and the Cocoa Research Centre) located in Ilhéus/Bahia, Brazil. Catechin was purchased from Sigma.

Cocoa extract

The cocoa seeds were first crushed and degreased in a Soxhlet apparatus using cyclohexane as a solvent, they were then submitted to the extraction apparatus for the extraction of phenolic compounds, using 70% methanol (MeOH) as solvent. The extracts were dried in a rotary evaporator.

Cell culture

GRX cells were obtained from a Cell Bank at the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. The cells were maintained in Dulbeco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal calf serum, 1% penicillin/streptomycin, pH 7.4, and kept in a humidified incubator with 5% CO_2 at 37 °C.

Cell growth

MTT assay

To assess cell growth and (or) cell number, GRX cells were treated with cocoa at concentrations of 0.06 and 0.125 mg/mL and catechin at concentrations of 0.01 and 0.02 mg/mL for a 6 day incubation period. Indomethacin (0.13 mmol/L) was used as a control for the inhibition of cell growth.

The cell viability was assessed by testing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) in 96-well plates. Briefly, a MTT stock solution was prepared at a concentration of 5.0 mg/mL. In the experiment, this concentration was diluted 1:10, so the final concentration in the wells was 0.5 mg/mL. Subsequently, the plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for 4 h. Then, they were removed and the supernatants were discarded and isopropanol added to dissolve the blue crystals formed by MTT. After 5 min, the plates were placed for reading in a Hyperion MicroReader, using a wavelength of 540 nm with reference at 650 nm. All experiments were performed in triplicate.

Cell count

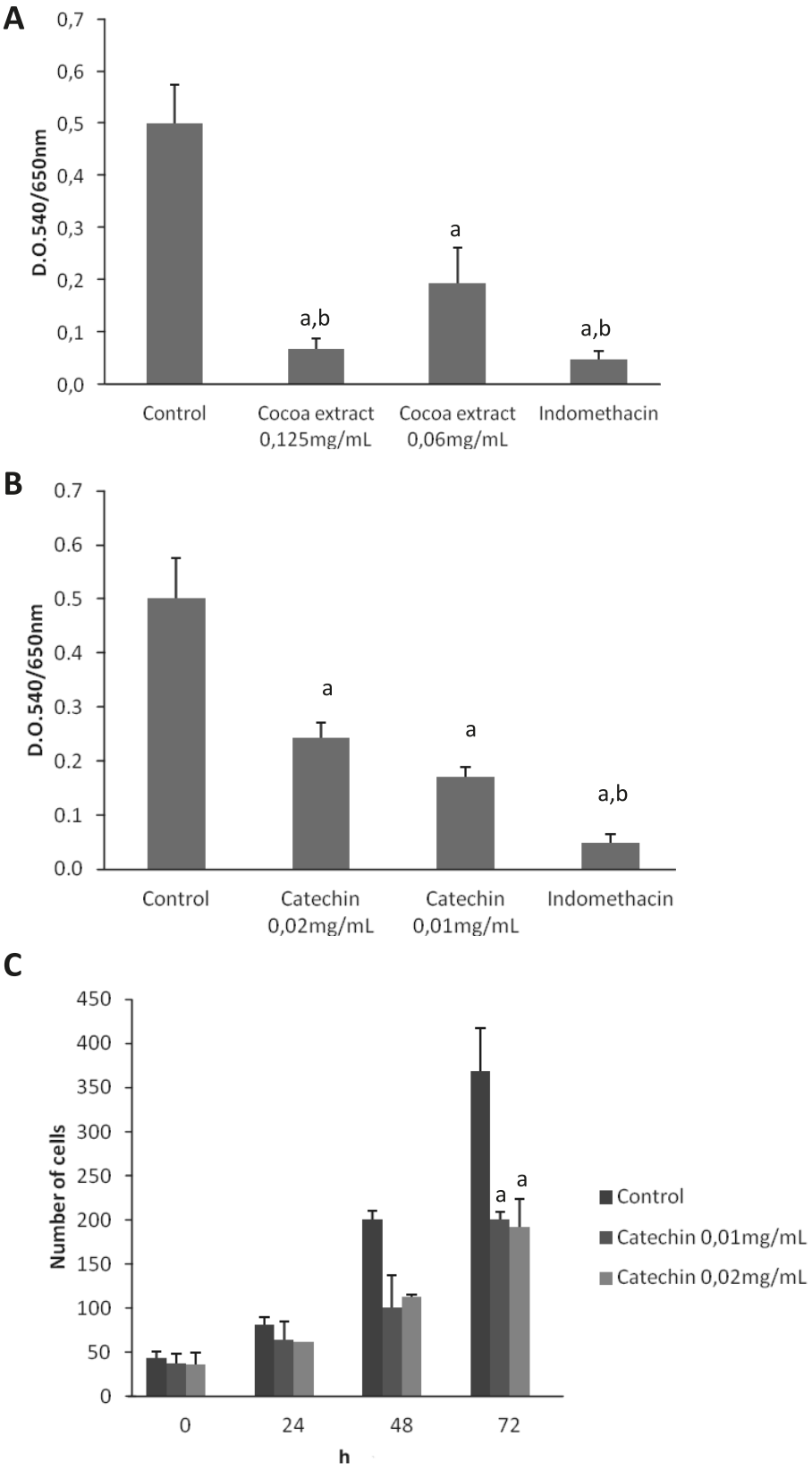
Cell growth was also evaluated by counting GRX cells treated with catechin at concentrations of 0.01 and 0.02 mg/mL in an inverted microscope (400× magnification) at incubation times of 0, 24, 48, and 72 h.

Cell death by release of lactate dehydrogenase

To evaluate the cytotoxicity of catechins in GRX cells, we used the determination of lactate dehydrogenase (LDH) in supernatants of cultures, compared with the control group. The LDH activity was measured by a colorimetric assay. For the control of cell lysis, 5% Tween was used.

Protein analysis

For the assessment of apoptosis and autophagy, cells were plated on 100 mm × 20 mm plates, treated with catechin at concentrations of 0.01and 0.02 mg/mL, and incubated for 72 h. Apoptosis and autophagy were evaluated by protein expression of caspase 3 and poly-ADP ribose polymerase (PARP) for apoptosis and LC3 for autophagy. Protein expression of other proteins like p27, pp38, p53 (regulators of the cell

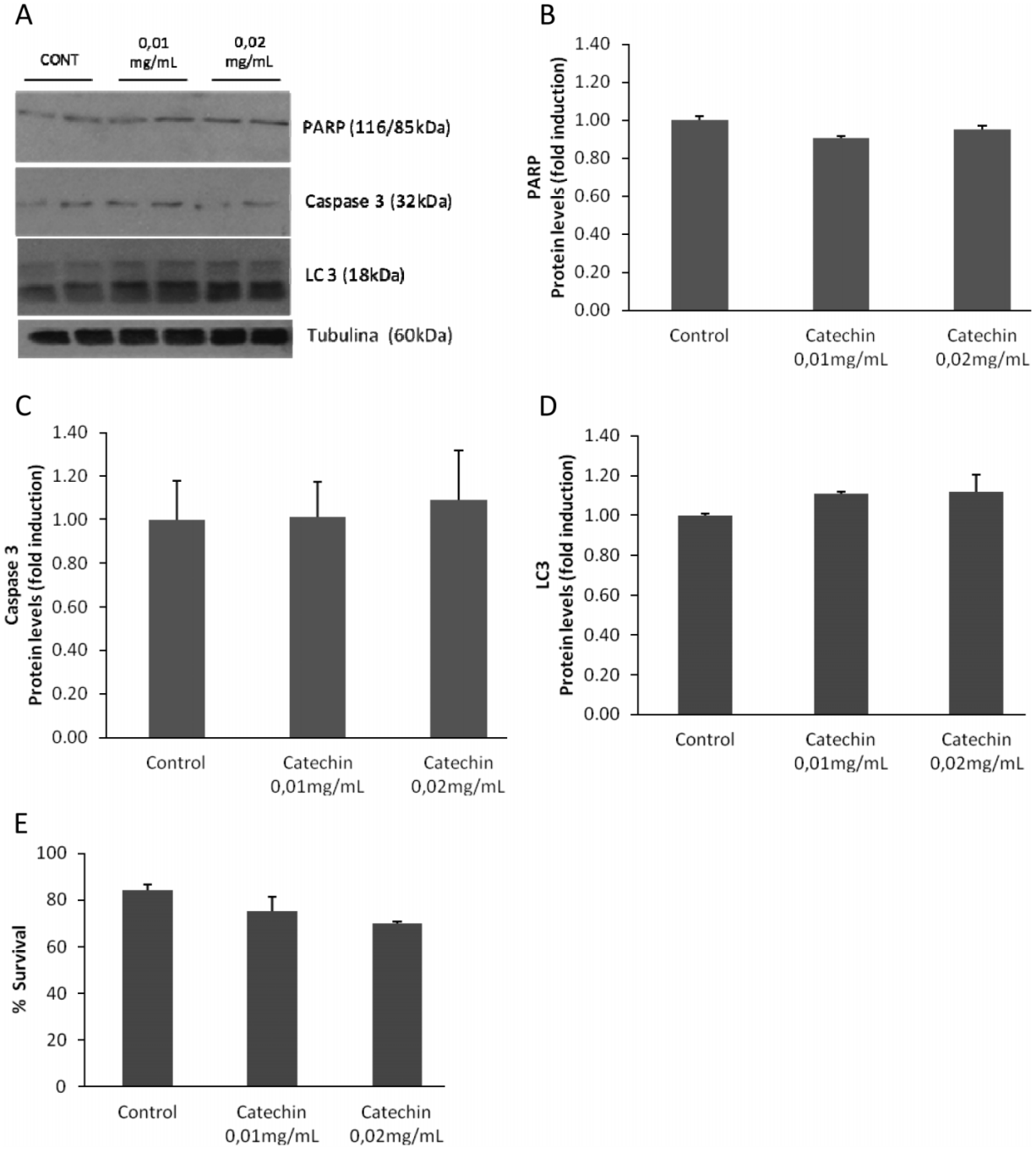


cycle), pS6k (proliferative protein and protein synthesis), pAkt-thr308 (PI3K/AKT/mTor pathway), and pERK1 (MAPK pathway) was performed to elucidate the catechin mechanism. The phosphorylation of these proteins was evaluated by Western blot analysis.

Western blot analysis

Cells were washed twice in cold PBS and lysed with 50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.2% Igepal, 10% glycerol supplemented with protease, and phosphatase inhibitors. Protein extracts were resolved on 10% SDS–

Fig. 2. (A) Expression of proteins poly-ADP ribose polymerase (PARP), caspase 3, and LC3 in GRX cells treated with catechin at concentrations of 0.01 and 0.02 mg/mL after 72 h of incubation by Western blot analysis. The tubulin was used as a normalizer of protein expression. (B) Protein levels (fold induction) of the expression of PARP in GRX cells treated with 0.01 and 0.02 mg/mL catechin ($p > 0.05$). (C) Protein levels (fold induction) of the expression of caspase 3 in GRX cells treated with 0.01 and 0.02 mg/mL catechin ($p > 0.05$). (D) Protein levels (fold induction) of the expression of LC3 in GRX cells treated with 0.01 and 0.02 mg/mL catechin ($p > 0.05$). (E) Percent survival by the release of lactate dehydrogenase (LDH) of GRX cells treated with catechin at concentrations of 0.01 and 0.02 mg/mL after 6 days of incubation and a control group ($p > 0.05$). Data are expressed as the mean ± SD of 3 independent experiments.

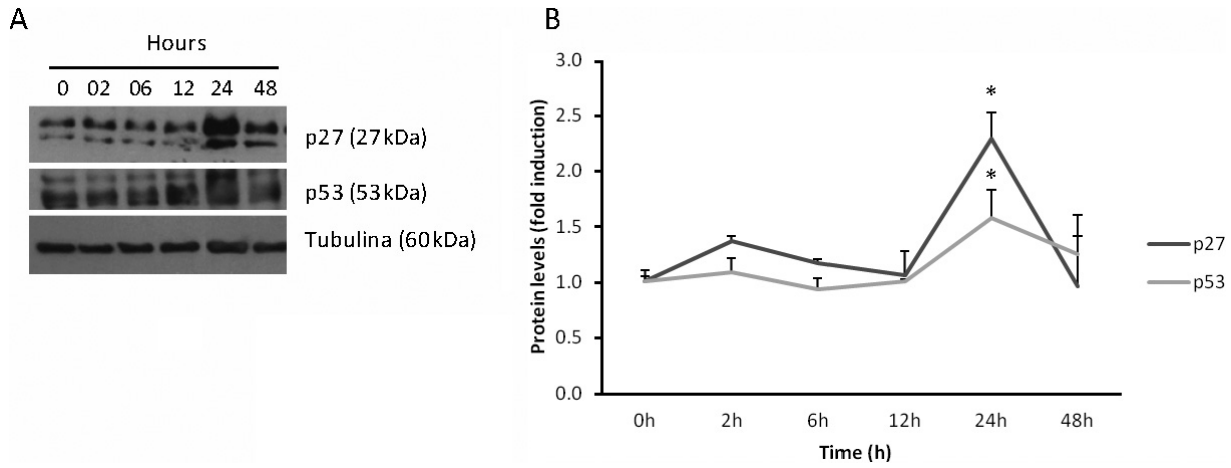


polyacrylamide gels, transferred to nitrocellulose membranes (Millipore), and subjected to Western blotting using the antibodies indicated below at a 1:1000 dilution. Immunocomplexes were visualized with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (1:10 000) followed by incubation with enhanced chemiluminescence (ECL) Western blot reagent (GE Healthcare).

For the development of proteins, a kit containing ECL Western blotting detection reagents (GE Healthcare) was used in accordance with the manufacturer's instructions . The membranes were then exposed to medical X-ray film (Kodak).

The quantification of bands on Western blots was made using ImageJ 1.41 software.

Fig. 3. (A) Time course (0, 2, 6, 12, 24, and 48 h) of the expression of proteins p27 and p53 in GRX cells treated with catechin at a concentration of 0.02 mg/mL by Western blot analysis. Tubulin was used to normalize protein expression. (B) Protein levels (fold induction) of the expression of p27 and p53 by Western blot analysis. *, 24 h > 0 h ($p < 0.05$). Data are expressed as the mean ± SD of 3 independent experiments.



Antibodies

The primary antibodies used were caspase 3 (PharMingen), PARP (Santa Cruz), LC3 (T. Yashimori), p27 (PharMingen), pp38 (Cell Signaling), p53 (Labvision), pS6k (Cell Signaling), pAkt-thr308 (Labvision), ERK1 (Cell Signaling), α-tubulin (Sigma), and the secondary antibodies used were α-mouse IgG and α-rabbit IgG (GE Healthcare). All antibodies were used at a dilution of 1:1000.

TGF-β quantification

TGF-β (R&D Systems) was measured in GRX cells conditioned medium using a commercially available ELISA kit. Optical density was measured using an ELISA plate reader at a wavelength of 450 nm. TGF-β levels were expressed as picograms per millilitre.

Anti-inflammatory activity

The anti-inflammatory activity of catechin was assessed in GRX cells using catechin at concentrations 0.01 and 0.02 mg/mL incubated for 24 h and the mRNA quantification of IL-6 and COX-2 by real time – PCR (RT–PCR).

RT–qPCR analysis

Total RNA was isolated from GRX cells using the Ultraspec RNA Isolation System (Biotecx), and 5 mg of total RNA was reverse transcribed using a high-capacity cDNA RT kit (Applied Biosystems, Inc., Foster City, Calif.) with random primers. Quantitative PCRs were carried out using an ABI Prism 7900 HT Fast Real-Time PCR System and a *Taq*man 5′-nuclease probe method or using customized TLDA arrays (Applied Biosystems). All transcripts were normalized to GAPDH.

Statistical analysis

Data were analyzed by ANOVA followed by a Tukey's post-test at a significance level of $p < 0.05$. The statistical program used was SPSS 15.0.

Results

To study the effect of cocoa on GRX cell number or cell growth, cells were incubated with cocoa extract at concentrations of 0.06 and 0.125 mg/mL for 6 days, Cocoa extract decreased cells numbers when compared with the control group ($p < 0.001$) (Fig. 1A).

By treating GRX cells with catechin at concentrations of 0.01 and 0.02 mg/mL for a 6 day incubation period, a significant decrease in cell number at both concentrations of catechin were observed when compared with the control group ($p < 0.001$) (Fig. 1B).

To confirm the results of the MTT assay, GRX cells were incubated with catechin at concentrations of 0.01 and 0.02 mg/mL for 0, 24, 48, and 72 h and then counted under an inverted microscope. Catechin significantly decreased the cell number at all concentrations evaluated at the time of 72 h (Fig. 1C).

Searching for possible mechanisms for the reduction in cell number, the expression of proteins involved in processes of programmed cell death, apoptosis (caspase 3 and PARP), and autophagy (LC3) was assessed by Western blot analysis. There was no fragmentation of caspase 3 (only 1 32 kDa band appeared) or PARP (no 24 and 89 kDa fragments were observed); therefore, both are inactivated. There was no increased expression of LC3 in cells treated with catechin at the times assessed (Fig. 2A–2D and 2E). To assess the possible death by necrosis, the LDH release by cells treated with catechin was also evaluated, and there was no difference between the control group and treated groups (Fig. 2E).

To evaluate the possible action of catechin on the cell cycle, the expression of p27 and p53 proteins, regulators of the cell cycle, were also assessed by Western blotting (Fig. 3), which showed increased expression at 24 h ($p < 0.05$).

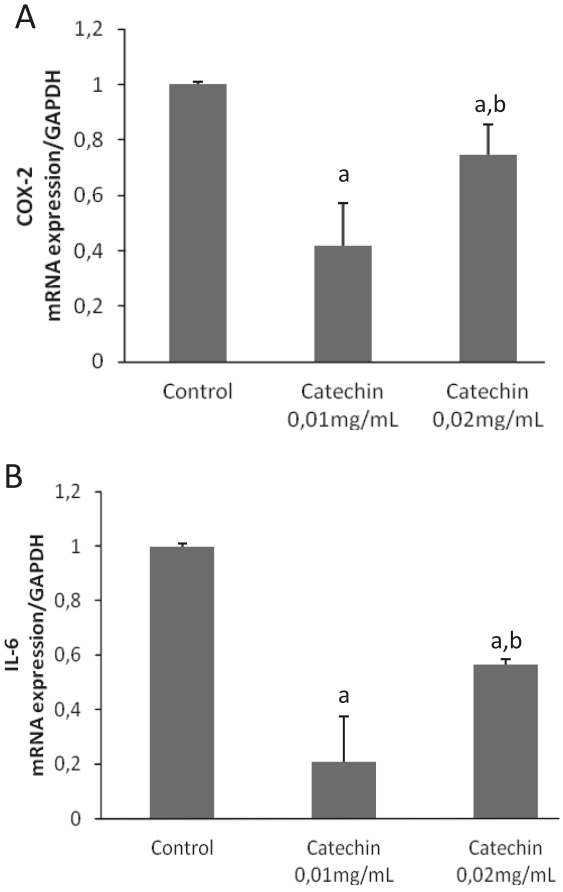
Stellate cells, when activated, increase the synthesis of pro-inflammatory mediators. Due to the close relationship among pro-inflammatory mediators, cell proliferation, and liver fibrosis, one of the mechanisms assessed was the expression of COX-2 mRNA by RT–PCR, which showed a significant decrease in its expression after treatment with catechin at the doses tested ($p < 0.001$). Although, when comparing the concentrations tested, 0.01 mg/mL catechin showed a greater capacity to inhibit COX-2 mRNA when compared with

0.02 mg/mL ($p < 0.05$) (Fig. 4A). In addition to this result, IL-6 was assessed, and it presented a decrease in mRNA expression in cells treated with catechin ($p < 0.001$). Comparing the tested concentrations, 0.01 mg/mL catechin was more effective in reducing the expression of IL-6 in these cells ($p < 0.05$) (Fig. 4B).

The MAPK and PI3K/AKT/mTor signaling pathways are closely related with survival in human cancer cells. It is now well established that MAPK and PI3K/AKT/mTor are 2 of the most predominant oncogenic routes, and they are intimately linked together. Therefore, we evaluated phosphorylation of proteins (pS6K (protein synthesis), pERK1 (MAPK pathway and inflammatory protein), pp38 (MAPK pathway and antiproliferative protein), and pAkt-thr308 (PI3K/AKT/mTor pathway)) that could explain the antiproliferative effect of catechin on GRX cells in time course of 0, 2, 6, 12, 24, and 48 h. In this experiment, we used catechin at a concentration of 0.02 mg/mL. There was no significant difference among the times assessed in the phosphorylation of proteins pp38, ERK1, and pAkt-thr308, although pS6K showed increased phosphorylation at 48 h of incubation ($p < 0.05$) (Fig. 5).

When the production of TGF-β by GRX cells was evaluated, a significant decrease in TGF-β concentration was observed after treatment with catechin at the doses tested ($p < 0.001$) (Fig. 6).

Fig. 4. (A) Expression of the COX-2 protein by RT–PCR in GRX cells treated with catechin at concentrations of 0.01 and 0.02 mg/mL after 24 h of incubation. a, catechin (0.01 and 0.02 mg/mL) < control ($p < 0.001$); and b, 0.02 mg/mL catechin > 0.01 mg/mL catechin ($p < 0.05$). (B) Expression of the IL-6 protein by RT–PCR in GRX cells treated with catechin at concentrations of 0.01 and 0.02 mg/mL after 24 h of incubation. a, catechin (0.01 and 0.02 mg/mL) < control ($p < 0.001$); and b, 0.02 mg/mL catechin > 0.01 mg/mL catechin ($p < 0.005$). The mRNAs indicated were measured by RT–qPCR, normalized to GAPDH, and plotted as relative expression to untreated cells. Data are expressed as the mean ± SD of 3 independent experiments.



Discussion

Several evidences have shown the benefits of phenolic compounds present in foods such as cocoa in the treatment of cancer, neurodegenerative diseases, atherosclerosis, among others (Steinberg et al. 2003). Catechin is one of the main phenolic compounds in cocoa seeds, the raw material of the chocolate, one of the most popular foods in the world. However, the physiological mechanisms have to be better clarified.

In this study, there was an investigation of the activity of catechin on the growth of GRX cells. The aim was to deepen the research to clarify the mechanisms by which catechin may influence the parameters of cell proliferation and inflammation.

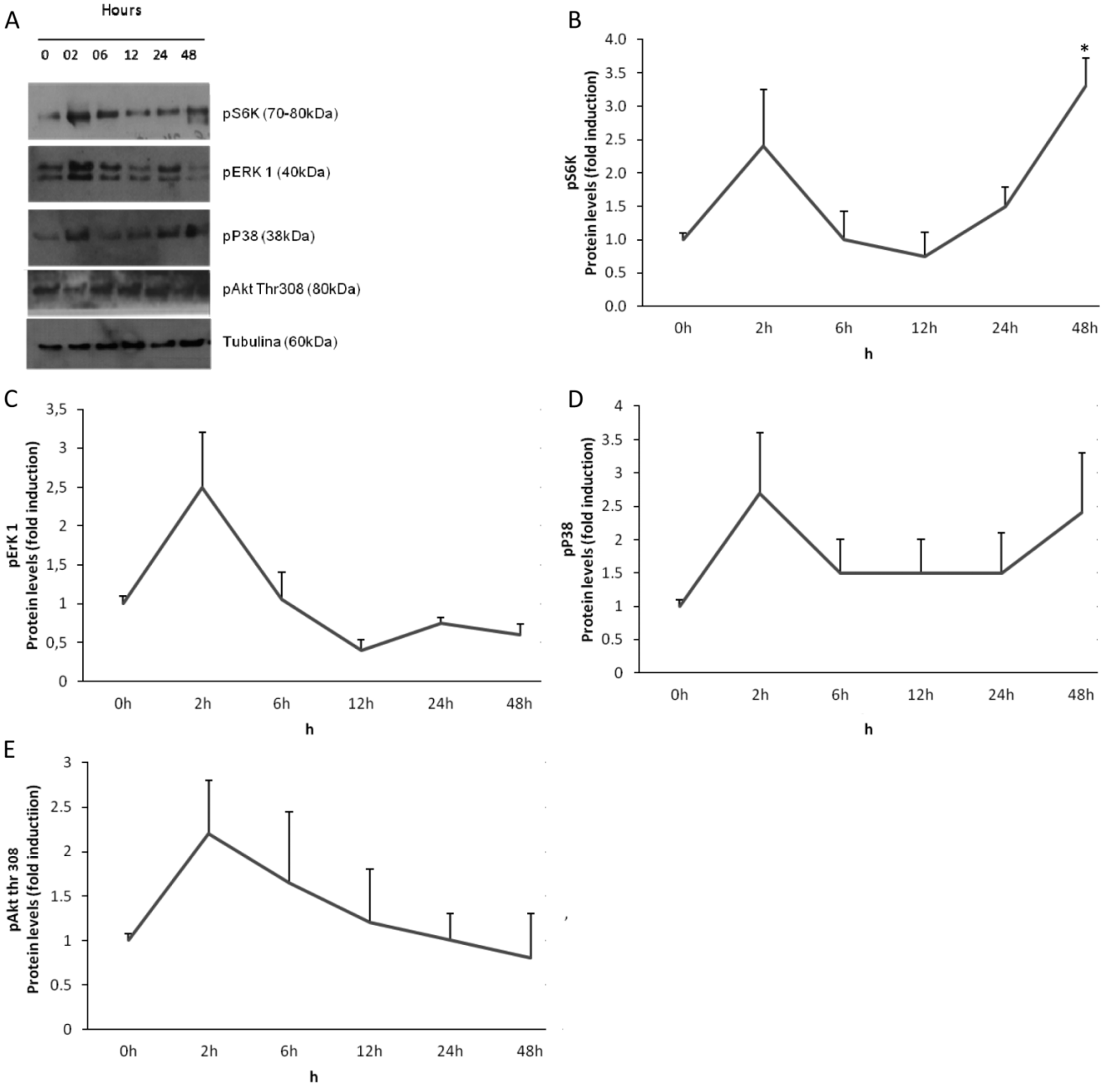
The GRX cell line represents an interesting and efficient study tool (Guimarães et al. 2006; Guma et al. 2001), which when maintained under standard conditions of culture, it presents a myofibroblastic phenotype, similar to the activated HSCs. HSCs when activated express a myofibroblastic phenotype and when in the quiescent state, they present a lipocyte phenotype. When expressing the myofibroblastic phenotype, they are primarily involved with the production of components of the extracellular matrix and control of liver connective tissue homeostasis. The increase in the cell number and activation of myofibroblasts is associated with liver fibrosis and cirrhosis (Vicente et al. 1998).

In the present study, cocoa extract and catechin decreased thenumber of GRX cells at the concentrations tested. In this sense, we sought to investigate the possible cellular mechanisms involved in this effect.

Friedman (2008*b*) states that the reduction of cell growth in stellate cells depends on reducing the inflammatory process, the immune response, the inhibition of cell activation, induction apoptosis, and (or) autophagy. Caspases are a series of proteases activated upon cleavage at aspartate residues during the earliest stages of apoptosis. Activated caspases can cleave the enzyme PARP. PARPs are DNA repair enzymes that are activated by DNA strand breaks. Cleavage of PARP by caspase 3 into 24 and 89 kDa fragments inactivates the PARP enzyme. In our study, we showed that catechin decreases the cell number and the mechanism does not occur by apoptosis because there was no breakdown of caspase 3 and PARP or autophagy, because there was no increase in expression of LC3. The decrease of cell numbers was also not due to cell lysis, because no increase was detected in LDH release in cells treated with catechin when compared with the control group. Therefore, these results show that the decrease in cells number was caused by decreased cell proliferation.

To search for mechanisms that would justify a reduction in cell growth, the expression of protein p53 was assessed. The

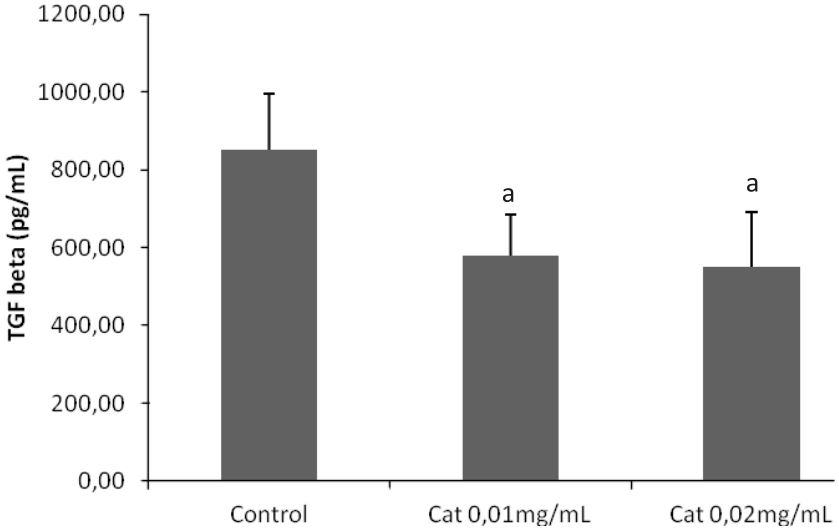
Fig. 5. (A) Time course (0, 2, 6, 12, 24, and 48 h) of the expression of proteins pS6k, pERK1, pp38, and pAkt-thr308 in GRX cells treated with catechin at a concentration of 0.02 mg/mL for Western blot analysis. Tubulin was used as a normalizer of protein expression. (B) Protein levels (fold induction) of the expression of pS6k in GRX cells treated with 0.02 mg/mL catechin. *, 48 h > 0 h ($p < 0.05$). (C) Protein levels (fold induction) of the expression of ERK1 in GRX cells treated with 0.02 mg/mL catechin ($p > 0.05$). (D) Protein levels (fold induction) of the expression of pp38 in GRX cells treated with 0.02 mg/mL catechin ($p > 0.05$). (E) Protein levels (fold induction) of the expression of pAkt-thr308 in GRX cells treated with 0.02 mg/mL catechin ($p > 0.05$). Data are expressed as the mean ± SD of 3 independent experiments.



p53 protein suppresses cell growth not only by inducing apoptosis, but also by causing cell cycle arrest. If such arrest is not possible, the cell goes through apoptosis, and under severe stress, p53 increases reactive oxygen species production and cell death, whereas low levels of p53 induce a decrease in intracellular reactive oxygen species levels. Indeed, basal p53 has an antioxidant function. For example, p53 induces upregulation of glutathione peroxidase (Guimarães et al. 2006). In this study, an increase of p53 was observed and, consequently, could provoke cell cycle arrest. We also evaluated the expression of p27, another inhibitor of the cell cycle. According to Lu et al. (2005), the cell cycle is controlled by cyclins and cyclin-dependent kinases. The p27 protein is an inhibitor of cyclin-dependent kinases and is, therefore, involved in cell cycle arrest. An increased expression of this protein was observed, showing for the first time that catechin can decrease cell growth by decreasing the cell cycle by significantly increasing the expression of p53 and p27.

GRX cell proliferation is also related to the inflammatory process. In this sense, we investigated the expression of IL-6

Fig. 6. TGF-β concentration in the supernatant of GRX cell cultures treated with 0.01 and 0.02 mg/mL catechin after 24 h of incubation. a, catechin (0.01and 0.02 mg/mL) < control ($p < 0.001$). Data are expressed as the mean ± SD of 3 independent experiments.



mRNA, which according to Huang and Zhang (2003) is a pro-inflammatory cytokine involved in cell proliferation and synthesis of the extracellular matrix. In our study, this interleukin had its expression reduced, which is suggested to be related to decreased proliferation of these cells, since the pro-inflammatory phenotype of stellate cells is closely related to the development of fibrosis. According to Thirunavukkarasu et al. (2006), the activated HSCs secrete IL-6, a key cytokine in the acute phase response in the context of liver inflammation. COX-2 is an enzyme that catalyzes the first step of the synthesis of prostanoides, related to the pathogenesis of the inflammatory route, being induced in most tissues by inflammatory cytokines and growth factors in response to inflammation or tissue damage. In our study, there was reduced expression of COX-2 mRNA. In GRX cells, the fibrotic form needs a constant inflammatory state (Cicconetti et al. 2004). In this sense, it is possible to infer that catechin reduced the pro-inflammatory phenotype inflammatory state of GRX cells by decreasing the expression of COX-2.

The studies of Guo et al. (2002) and Tamura et al. (2003) describe that there is a direct connection between signaling ERK1/2 (extracellular signal-regulated kinases) with the induction of the COX-2 gene in human endometrial stromal cells and intestinal epithelial cells, demonstrating that signaling ERK1/2 is an important pathway in the regulation of COX-2. The overexpression of COX-2 is described in several cell lines when in intense inflammation, especially in cancer cells. Some studies have shown that the inhibition of COX-2 may be an important therapeutic target for controlling tumor growth and development of metastases (Kurland et al. 2007; Tamura et al. 2003). ERK1/2, also known as a classical MAPK, plays a fundamental role in survival, proliferation, and apoptosis. In addition, the ERK1/2 signaling pathway is preferentially activated (phosphorylated) in response to growth factors and phorbol ester and regulates cell proliferation and differentiation. Nevertheless, our results show no significant decrease in the expression of pERK1, so ERK1 is not involved in the process.

It is important to highlight that pp38 (MAPK pathway) did not present an increase in its phosphorylation, although this protein, when phosphorylated, is antiproliferative. It is therefore suggested that this was due to the high antioxidant activity of catechin reported in several studies. Gelain et al. (2011) reported that pp38 is activated by oxidizing agents, which were inhibited by catechins, preventing the increased phosphorylation of this protein.

AKT signaling pathways are closely related with survival in human cancer cells. The PI3K/AKT/mTor signaling pathway is probably the best characterized and most prominent pathway with regard to the antiapoptotic signals in cell survival (Shah et al. 2000). Akt is downstream of phosphoinositide 3-kinase and is activated by phosphorylation in response to insulin, insulin-like growth factor (IGF), or various cytokines that mediate the progrowth effects of these signals. Akt activity drives many anabolic pathways including protein synthesis by activating initiation and elongation factors of the translational machinery (Shah et al. 2000). Akt is also known to oppose apoptosis by phosphorylating and inactivating several proapoptotic factors such as GSK3β, BAD, fork-head family members, and pro-caspase-9 (Seol 2008). Nevertheless, our results show no alterations in the expression of pAkt-thr308, so it is not involved in the process

TGF-β is the dominant stimulus to extracellular matrix production by stellate cells. A role for TGF-β in perpetuating rather than initiating stellate cell activation has been established by examining the behavior of stellate cells in TGF-β knockout mice with acute liver injury. These animals show markedly reduced collagen accumulation in response to liver injury as expected. TGF-β is increased in experimental and human hepatic fibrosis. There are many sources of this cytokine; however, autocrine expression is the most important transcriptional upregulation, demonstrated in culture-activated stellate cells. In our experiments, catechin significantly decreased the production of TGF-β by GRX cells, showing a significant antifibrotic effect.

Conclusions

This study shows the antiproliferative activity of cocoa extract and catechin. It was shown that catechin decreases cell

growth in GRX cells and, probably, such a decrease does not occur by apoptosis or autophagy but through an anti-inflammatory effect and cell cycle arrest. Catechin significantly reduced the synthesis of TGF-β by GRX cells, showing a potencial use in the control of liver fibrosis.

Acknowledgments

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