



# Resveratrol increases the activation markers and changes the release of inflammatory cytokines of hepatic stellate cells

Cleverson Moraes de Oliveira<sup>1</sup> · Leo Anderson Meira Martins<sup>1,2</sup> · Arieli Cruz de Sousa<sup>1</sup> · Ketlen da Silveira Moraes<sup>1</sup> · Bruna Pasqualotto Costa<sup>3</sup> · Moema Queiroz Vieira<sup>1</sup> · Bárbara Paranhos Coelho<sup>1</sup> · Radovan Borojevic<sup>4</sup> · Jarbas Rodrigues de Oliveira<sup>3</sup> · Fátima Costa Rodrigues Guma<sup>1,5</sup>

Received: 26 June 2020 / Accepted: 7 October 2020 / Published online: 19 October 2020  
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## Abstract

The phytoalexin Resveratrol (3,5,4'-trihydroxystilbene; RSV) has been related to numerous beneficial effects on health by its cytoprotection and chemoprevention activities. Liver fibrosis is characterized by the extracellular matrix accumulation after hepatic injury and can lead to cirrhosis. Hepatic stellate cells (HSC) play a crucial role during fibrogenesis and liver wound healing by changing their quiescent phenotype to an activated phenotype for protecting healthy areas from damaged areas. Strategies on promoting the activated HSC death, the quiescence return or the cellular activation stimuli decrease play an important role on reducing liver fibrosis. Here, we evaluated the RSV effects on some markers of activation in GRX, an HSC model. We further evaluated the RSV influence in the ability of GRX on releasing inflammatory mediators. RSV at 1 and 10  $\mu\text{M}$  did not alter the protein content of  $\alpha$ -SMA, collagen I and GFAP; but 50  $\mu\text{M}$  increased the content of these activation-related proteins. Also, RSV did not change the myofibroblast-like morphology of GRX. Interestingly, RSV at 10 and 50  $\mu\text{M}$  decreased the GRX migration and collagen-I gel contraction. Finally, we showed that RSV triggered the increase in the TNF- $\alpha$  and IL-10 content in culture media of GRX while the opposite occurred for the IL-6 content. Altogether, these results suggested that RSV did not decrease the activation state of GRX and oppositely, triggered a pro-activation effect at the 50  $\mu\text{M}$  concentration. However, despite the increase of TNF- $\alpha$  in culture media, these results on IL-6 and IL-10 secretion were in accordance with the anti-inflammatory role of RSV in our model.

**Keywords** Hepatic stellate cells · Liver fibrosis · Liver wound healing · Resveratrol

## Introduction

Resveratrol (3,5,4'-trihydroxystilbene; RSV) is a phytoalexin produced by several plant species, such as peanuts and grapes, in response to pathogenic infection and environmental stresses. This molecule is present at relevant concentrations in red wine and has been related to numerous beneficial effects on health by its cytoprotection and chemoprevention activities, which has been largely associated with its anti-inflammatory and anti-oxidant effects. Paradoxically, RSV can also exert cytotoxicity through inducing cell death and cell growth inhibition, two positive effects for treating several pathological conditions such as cancer [1, 2].

Liver fibrosis is a dynamic process characterized by the accumulation of extracellular matrix resulting from hepatic injury, including those caused by a viral infection, alcoholic liver disease (ALD), non-alcoholic steatohepatitis (NASH) and hepatic steatosis. It is a consensus that liver fibrogenesis

✉ Cleverson Moraes de Oliveira  
cleverson.oliveira@ufrgs.br

<sup>1</sup> Departamento de Bioquímica, ICBS, Universidade Federal Do Rio Grande Do Sul (UFRGS), Rua Ramiro Barcelos, 2600-Anexo I, Porto Alegre, RS, CEP 90035-003, Brazil

<sup>2</sup> Departamento de Fisiologia, ICBS, Universidade Federal Do Rio Grande Do Sul, Rua Sarmento Leite, Porto Alegre, RS, CEP 500, Brazil

<sup>3</sup> Laboratório de Pesquisa Em Biofísica Celular E Inflamação, Pontifícia Universidade Católica Do Rio Grande Do Sul (PUCRS), Porto Alegre, RS, Brazil

<sup>4</sup> Centro de Medicina Regenerativa, Faculdade de Medicina de Petrópolis, Petrópolis, RJ, Brazil

<sup>5</sup> Centro de Microscopia E Microanálise (CMM), Universidade Federal Do Rio Grande Do Sul, Av. Bento Gonçalves, 9500 - Prédio 43.177 - B1 1Campus do Vale, Porto Alegre, RS, CEP 91501-970, Brazil

can be reversed if the cause of injury is ceased. However, the unchecked chronic liver injury and fibrogenesis can lead to cirrhosis, which compromises the hepatic architecture leading to abnormal blood flow and eventually to portal hypertension. In addition, liver cirrhosis may lead to the onset of complications, such as hepatic encephalopathy and variceal haemorrhage, which may increase the chances of hepatocellular carcinoma (HCC) development and, thus, the mortality risk [3, 4].

Hepatic stellate cells (HSC) are known to store lipid droplets enriched in retinyl ester in their cytoplasm, a condition that characterizes their quiescent phenotype. As one of their physiological features, HSC can differentiate into an activated phenotype in response to paracrine stimulation from damaged hepatocytes after liver injury. At this condition, HSC loses their lipid droplets and becomes fibrogenic myofibroblast-like cells, playing an important role in liver wound healing through protecting healthy areas from damaged areas. Nonetheless, continuous damage to the liver results in a chronic inflammatory response in which hepatic environment may not recover its homeostatic balance. In this context, increased production/activity of cytokines may be critical for both autocrine and paracrine perpetuation of HSC activation, which contributes to the excessive extracellular matrix accumulation that leads to liver fibrosis. In this way, strategies that promote the activated HSC death, the quiescence return or the cellular activation stimuli decrease play an important role on treating chronic liver injuries, focusing in the liver fibrosis reduction [5–7].

Along the past years, our research group has been studying the effects of RSV treatment in the murine cell line GRX, an activated HSC model [8]. GRX cells have been an excellent tool for studying the extrinsic and intrinsic factors that could trigger or prevent liver fibrosis since these cells can be induced to display the HSC quiescent-like or a more activated-like phenotype [9–13]. We already found that RSV treatment (0.1–50  $\mu\text{M}$ ) compromised the GRX cell viability through inhibiting cell cycle at the S-phase, impaired mitochondria and induced apoptosis, especially in the cell group that received the highest dose, where the cell population was drastically reduced. However, it was interesting that these effects were attenuated by the concomitant induction of mitochondrial biogenesis and autophagy, two survival mechanism against cellular environmental toxicity, which culminated in the GRX resistance to the cytotoxic effects of RSV [14–16]. Further, we found that 0.1  $\mu\text{M}$  of RSV was not able to restore the GRX capacity of storing lipid droplets. On the contrary, our results suggested that RSV could play a SIRT1-mediated lipolysis in GRX stimulated to store lipid droplets by Retinol treatment or by PPAR $\gamma$  super expression [17].

RSV treatment showed positive effects on compromising viability or reducing the number of activated HSC especially

at the highest concentration (50  $\mu\text{M}$ ). On the other hand, RSV compromised the ability of GRX on storing lipid droplets, a characteristic of quiescent HSC. Here, we seek for evaluating the effects of RSV towards HSC activation by measuring some molecular markers and cell migration after wound induction in GRX cell culture. We further evaluated the effects of RSV in the HSC ability on releasing TNF- $\alpha$ , IL-6 and IL-10 in the culture media, considering the importance of these cytokines during liver fibrogenesis.

## Material and methods

### Cell culture

The GRX cell line was obtained from the Cell Bank of Rio de Janeiro (HUCFF, UFRJ, RJ). For most experiments,  $3 \times 10^4/\text{cm}^2$  cells were seeded in 24-well culture plates (Nunc, Roskilde, Denmark). For cell migration evaluation,  $1.5 \times 10^4/\text{cm}^2$  cells were seeded in 96-well culture plates (Nunc, Roskilde, Denmark). During culture, cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 5% foetal bovine serum (Cultilab, Campinas, SP, Brazil) and 2 g/L HEPES buffer (pH 7.4) in a humidified atmosphere containing 5%  $\text{CO}_2$  at 37 °C.

### Resveratrol treatment

Resveratrol (Sigma Inc., St. Louis, MO, USA) was dissolved in 20  $\mu\text{L}$  of ethanol (Merck, Darmstadt, Germany) to a stock concentration of 100 mM and sequentially diluted in DMEM to a final concentration of 1, 10 and 50  $\mu\text{M}$  just before use. After reaching confluence, cells were treated for 24 h. Vehicle-treated cells were considered experimental control.

### Analysis of HSC activation markers by flow cytometry

The cellular protein content for glial fibrillary acidic protein (GFAP), collagen I and smooth muscle actin- $\alpha$  (SMA- $\alpha$ ) in GRX treated with RSV was measured by flow cytometry. Briefly, after 24-h treatment, cells were harvest by trypsin/EDTA (Sigma Inc.) and fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 15 min. Sequentially, cells were overnight incubated with the primary antibodies (GFAP, n.34001, from Cell Signalling, Danvers, MA, USA; collagen I, n.8784, from Santa Cruz Biotechnology, Dallas, TX, USA; SMA- $\alpha$ , n.A5228, from Sigma Inc.) diluted in PBS with 5% of albumin (1:500). Then, cells were incubated with adequate secondary antibodies (1:1000, diluted in PBS with 5% of albumin) for 2 h at room temperature: GFAP and SMA- $\alpha$ -labelled cells were exposed to anti-mouse

AlexaFluor 488 (n.A11001, from Invitrogen) while collagen I-labelled cells were exposed to anti-goat AlexaFluor 647 (n.A21447, from Invitrogen). Cells incubated only with respective fluorescence secondary antibodies were used as negative controls. A total of 20,000 events were acquired by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) at FL-1 (green fluorescence) and FL-4 (red fluorescence) channels. All data analyses were performed with FCS Express 4 software (De Novo, Software, Ontario, Canada). Results were expressed as fluorescence units [Control = 1].

### Analysis of HSC morphology by confocal microscopy

For analysing the cytoskeleton morphology, GRX was stained with tetramethyl-rhodamine isothiocyanate–phalloidin (Invitrogen), which specifically binds to F-actin with high affinity. Briefly, cells were cultured under coverslip, fixed in 4% paraformaldehyde for 15 min at 4 °C and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Filamentous actin was stained in accordance to the manufacturer's instructions. Images were collected using Olympus FV1000 laser-scanning confocal microscope. Ten single confocal sections of 0.7 μm were taken parallel to the bottom plates (*xy* sections) with a ×60 (numeric aperture 1.35) oil-immersion objective (Olympus, U plan-super apochromat, UPLSAPO60XO). Images from six random fields were acquired and deconvolved using the interactive 3D plugin of ImageJ software (<https://rsb.info.nih.gov/ij>).

### Analysis of HSC-induced contraction of collagen I gel

In order to evaluate the RSV effects in the HSC ability of contracting ECM, a characteristic of activated cells, a ready-to-use storable gel of collagen I was prepared after extracting it from rat tail tendon as previously described [18]. Animals were obtained from the Center for Experimental Biological Models at *Pontifícia Universidade Católica do Rio Grande do Sul* (PUCRS) and kept in a controlled temperature environment ( $24 \pm 2$  °C), light/dark cycle of 12 h, with free access to water and food. The experimental protocol was approved by the Ethics Research Committee of PUCRS.

Collagen I gels (constituted by 125 μl of 4 × DMEM and 125 μl of 4 mg/mL Rat Tail Tendon extracted collagen I) were impregnated with  $10^5$  cells resuspended in 250 μl of PBS and added into a 24-well plate for polymerising at 37 °C during 1 h. Then, collagen I gels and impregnated cells were detached and suspended in culture (control) and treatment (1, 10 and 50 μM of RSV) media. Images were acquired in a gel documenter (L-Pix, Locus, Cotia, SP, Brazil) after 24 h of treatment, and the surface area for each gel was determined as percentage of well area using ImageJ

software (<https://rsb.info.nih.gov/ij/>), a public domain Java image processing software. Cell treatment with *N*-acetylcysteine (NAC) at 400 μg/mL was used as a positive control [19]. Results were expressed as area of gel, considering control as 100%.

### Analysis of HSC migration by in vitro scratch assay

The effect of RSV in the migration capacity of GRX was evaluated by the in vitro scratch assay as previously described [20]. Briefly, after 24-h treatment, a circular gap was created with a 200-μL pipette tip at cell-confluent monolayer. Then, 24 images for each group were acquired in the SpectraMax i3 Multi-Mode Platform (Molecular Devices, Sunnyvale, CA, USA) at 0, 6, 12 and 24 h. Wound area at the aforementioned times was measured using ImageJ software. Results were expressed as the percentage of cell migration which represents the wound area reduction (wound closure).

### Analysis of HSC inflammatory release by ELISA assay

After 24-h treatment, the interleukine-6 and interleukine-10 concentrations were quantified in cell culture media using Quantikine ELISA Kit and protocol (R&D Systems, Minneapolis, MN, USA). Tumour necrosis factor-α concentration in the culture medium was determined using Sigma ELISA Kit (Sigma Inc.), accordingly to the manufacturer's protocol. Optical density was collected in a microplate fluorimeter reader (M5, Molecular Devices, USA). For allowing a more precise estimate for the RSV effects on cytokine releasing by GRX, cell quantity had to be considered. Results were then normalized by protein content [21] at the end of RSV treatments and were expressed as pg/μg.

### Statistical analysis

Data were expressed as mean ± standard deviation of the mean. Experiments were repeated at least three times ( $n=3$ ). One-way ANOVA was used to analyse the effect of RSV treatment, and Tukey *post-hoc* was performed. Results were considered statistically different when the *p* values were less than 0.05.

## Results

### Resveratrol induces an increase in the protein markers of activation in HSC

HSC have well-known molecular markers for activation and myofibroblast differentiation and among them, the increase in the protein content of GFAP, collagen I and SMA-α [3, 5, 22–24]. Thus, we sought to evaluate the content of these

proteins in GRX by flow cytometry. Treatment with 1 and 10  $\mu\text{M}$  of RSV did not alter the intracellular GFAP, collagen I and SMA- $\alpha$  protein quantity; however, 50  $\mu\text{M}$  of RSV triggered an increase in the protein content of these molecular markers of activation in GRX (Fig. 1).

### **Resveratrol does not alter HSC cytoskeleton morphology, but the highest concentration decreases cells ability of contracting collagen I gel**

The increase of cell contractibility, characterized by the F-Actin cytoskeleton rearrangement in stress fibres, is an important feature of activated HSC [25, 26]. Thus, RSV-treated cells were stained with tetramethyl-rhodamine isothiocyanate–phalloidin for evaluating cell cytoskeleton. No significant changes were observed since all groups were presented with cells with stress fibres and a myofibroblastic-like morphology with elongated cytoplasm (Fig. 2a). However, the collagen I gels containing cells treated with 50  $\mu\text{M}$  of RSV were significantly less contracted (Fig. 2b).

### **Resveratrol induces a decrease in the HSC migration**

The increase in the cell migration ability is also a feature of activated HSC [27]. Thus, we also evaluate GRX capacity on migrating after creating a circular gap in cultured cell at monolayer. Interestingly, after 6 and 12 h of wound creation, cells treated with 10 and 50  $\mu\text{M}$  of RSV presented a decreased rate of migration. At 24 h after wound creation, only cells treated with 50  $\mu\text{M}$  of RSV remained with a decreased rate of migration (Fig. 3).

### **Resveratrol alters the HSC capacity of releasing tumour necrosis factor- $\alpha$ , interleukin-6 and interleukin-10 in cellular medium**

Numerous cytokines, which may be pro- or anti-fibrogenic, have been shown to play a major role in wound-healing response during liver diseases [3, 5–7]. Therefore, the largely discussed anti-inflammatory property RSV [1, 28–30] may interfere on cytokines signalling among cultured HSC. Thus, we evaluated the RSV effects in the GRX ability on releasing TNF- $\alpha$ , IL-6 and IL-10; three important cytokines that are involved in liver fibrogenesis [5, 22, 31, 32]. All concentrations of RSV were able to increase the release of TNF- $\alpha$  in culture medium by GRX (Fig. 3a) while treatment with 10 and 50  $\mu\text{M}$  triggered a similar effect for IL-10 releasing (Fig. 3c). Oppositely, all concentrations of RSV triggered the decrease on IL-6 releasing by GRX in culture media (Fig. 3b).

## **Discussion**

The phytoalexin resveratrol (RSV) has attracted a lot of researchers' attention for being a nutraceutical compound with a large pharmacological potential for clinical treating of many diseases. In this regard, the potential health-promoting properties of RSV have been associated to its pleiotropic-like effects, which are a consequence of its interaction with a large number of signalling pathways that covers a broad range of pathologies including cancer, metabolic syndrome, cardiovascular diseases, neurodegenerative disorders, ageing and inflammation [1, 28, 33–35].

Understanding liver fibrosis focuses primarily on events that lead to activation and proliferation of HSC, which consists of two major phases: initiation and perpetuation. Under liver inflammatory condition, the paracrine stimuli from neighbouring cells—namely injured hepatocytes, endothelial cells, Kupffer cells and platelets—initiate HSC activation. The pathways for perpetuating the activated HSC phenotype include the acquisition of new functions such as proliferation, release of pro-inflammatory cytokines, matrix rearrangement and fibrogenesis. The HSC activation may substantially contribute for the maintenance of liver cirrhosis that can culminate in hepatocarcinoma or liver failure. Thus, the search for treating chronic liver disease, including advanced cirrhosis, shall be focusing in the liver fibrosis regression by controlling the fibrotic activity of activated HSC through inducing these cells to quiescence or apoptosis [3–7].

Recent studies have demonstrated the preventive and therapeutic role of RSV for many liver disorders. Among these beneficial effects, RSV was able (1) to provide liver protection against chemical, cholestatic and alcohol injury; (2) to improve glucose metabolism and lipid profile, thus decreasing liver fibrosis and steatosis; (3) to increase the survival period after liver transplantation; and (4) to decrease fat deposition, necrosis and apoptosis in hepatocytes after liver ischemia–reperfusion (I/R) injury. When focusing on HSC metabolism, previous studies had shown the RSV effects on promoting the reduction of  $\alpha$ -SMA protein content [28, 29]. In light of the huge number of studies pointing the beneficial effects of RSV for treating numerous pathologies including liver diseases, our research group has been studying the effects of this phytoalexin in GRX cell line, seeking for evaluating its treatment effects focusing on activated HSC.

RSV was indeed cytotoxic to GRX, but these effects seemed to be dose-dependent, being attenuated along time of cell treatment. Furthermore, this phytoalexin was not able to restore the capacity of GRX cells on storing lipid droplets. Oppositely, RSV treatment promoted lipolysis in quiescent-like cells [14–17], which is an event that

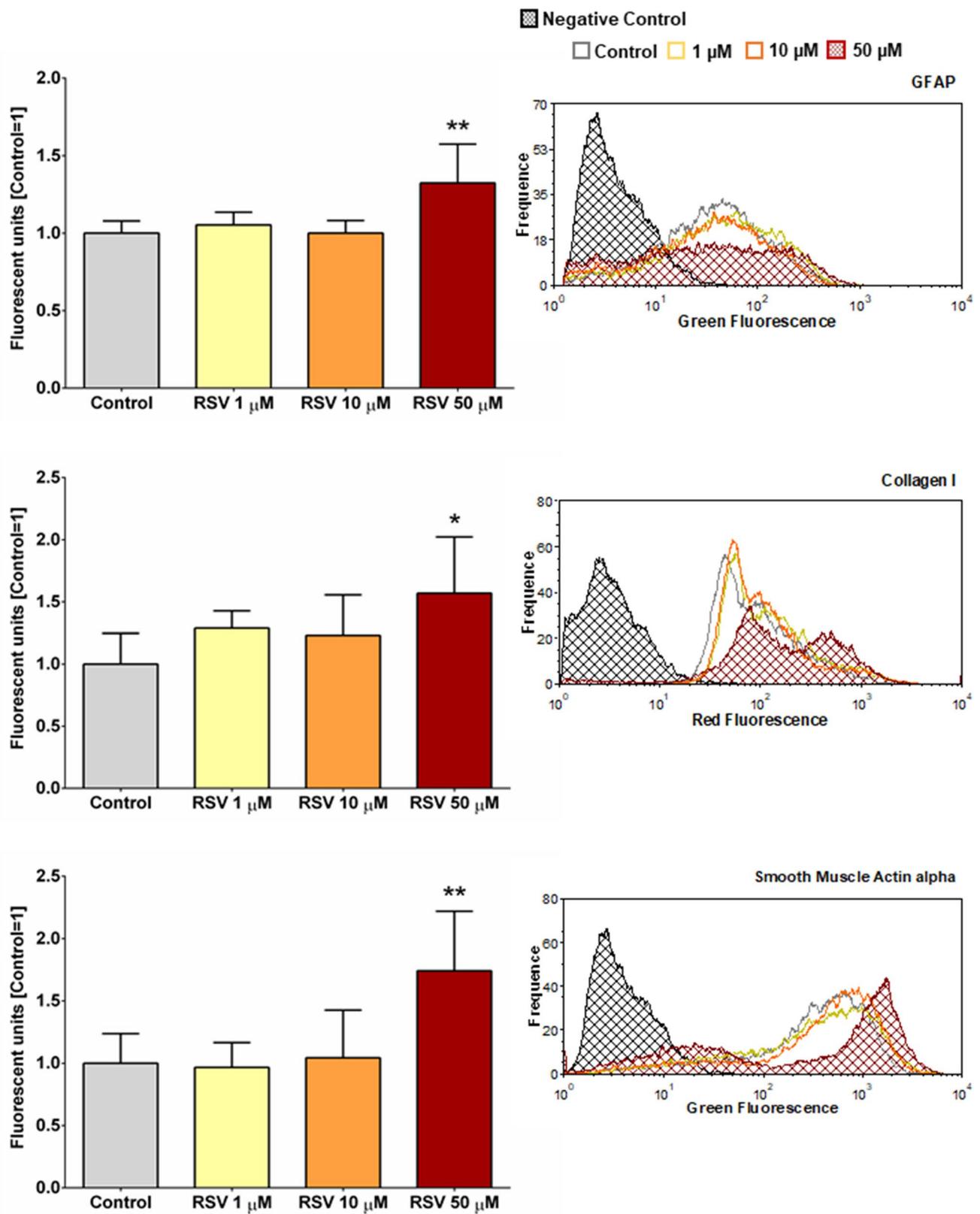
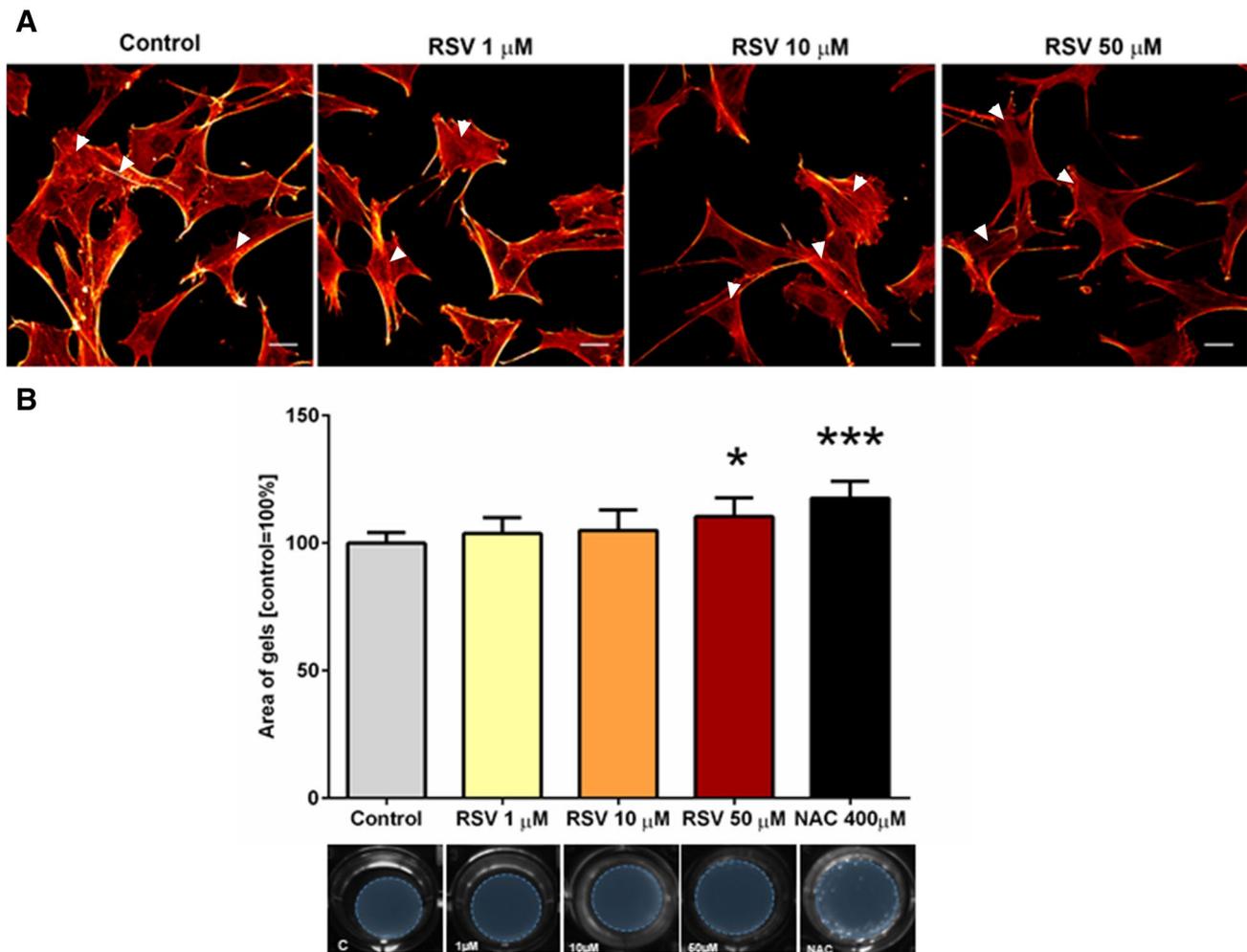


Fig. 1 RSV at 1 and 10 μM concentrations did not alter the protein content of GFAP, collagen I, and SMA-α; however, the highest concentration, 50 μM, triggered an increase in these molecular markers of activation in GRX ( $n = 3$ , mean ± SDM,  $p < 0.05$ )



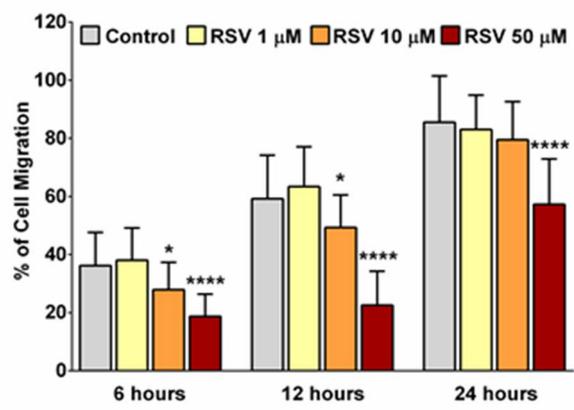
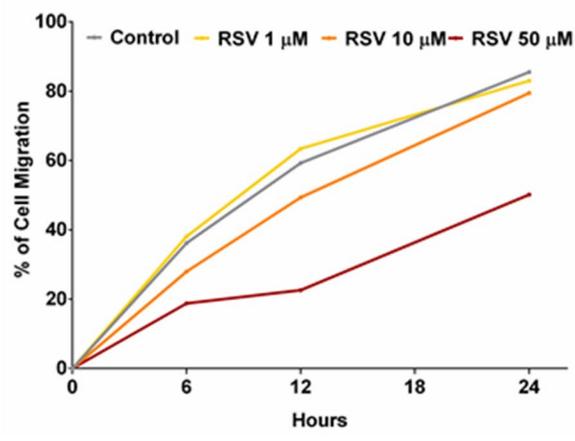
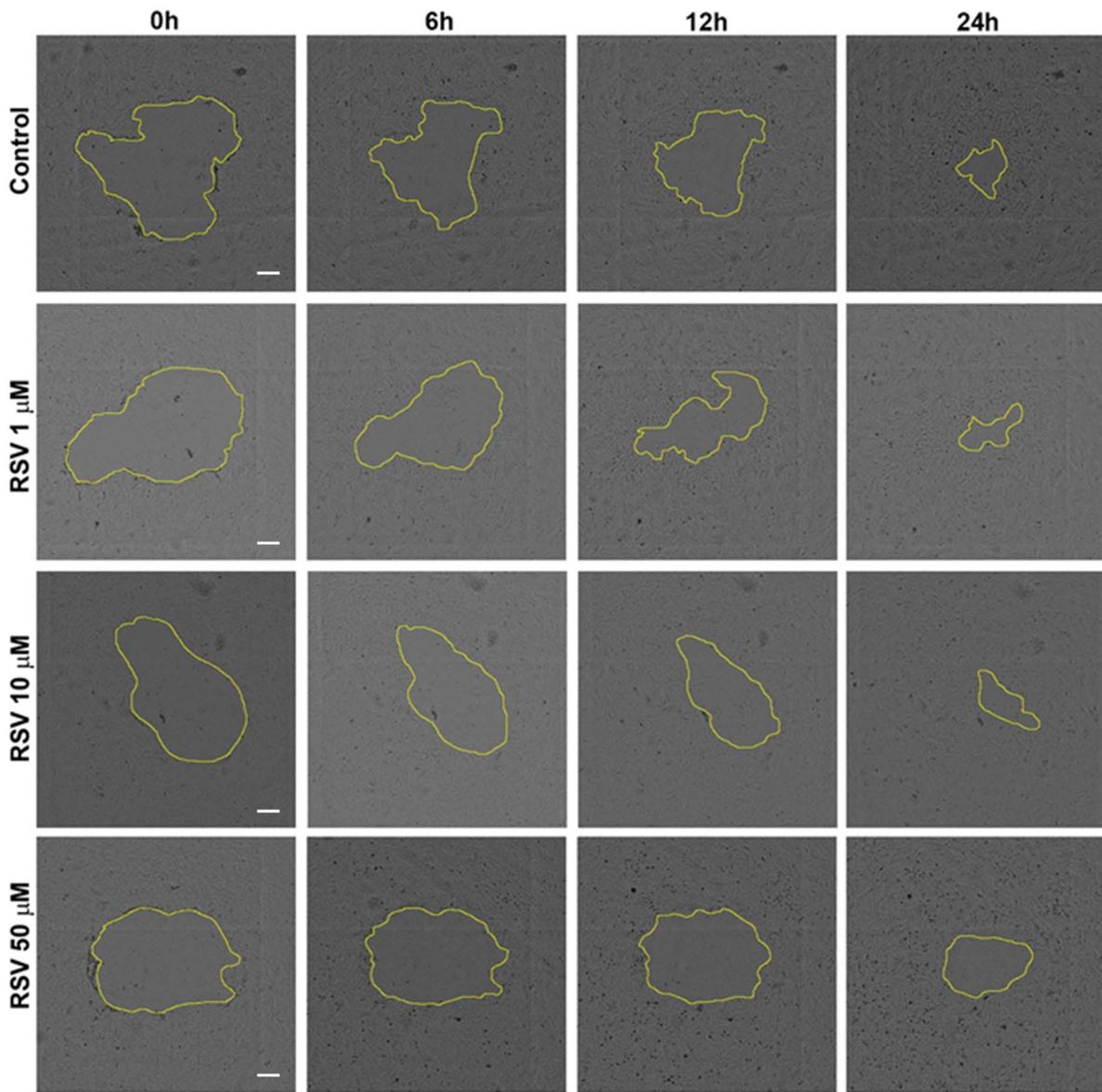
**Fig. 2 a** No significant changes were observed in GRX cytoskeleton since all-treated groups presented cells with stress fibres (white arrows) and a myofibroblastic-like morphology with elongated cytoplasm (images were pseudo-coloured in Red Hot by ImageJ; Scale

bar: 10  $\mu\text{m}$ ); **b** Collagen I gels containing cells treated with RSV at 50  $\mu\text{M}$  were significantly less contracted. *N*-acetylcysteine (400  $\mu\text{g}/\text{mL}$ ) was used as a positive control

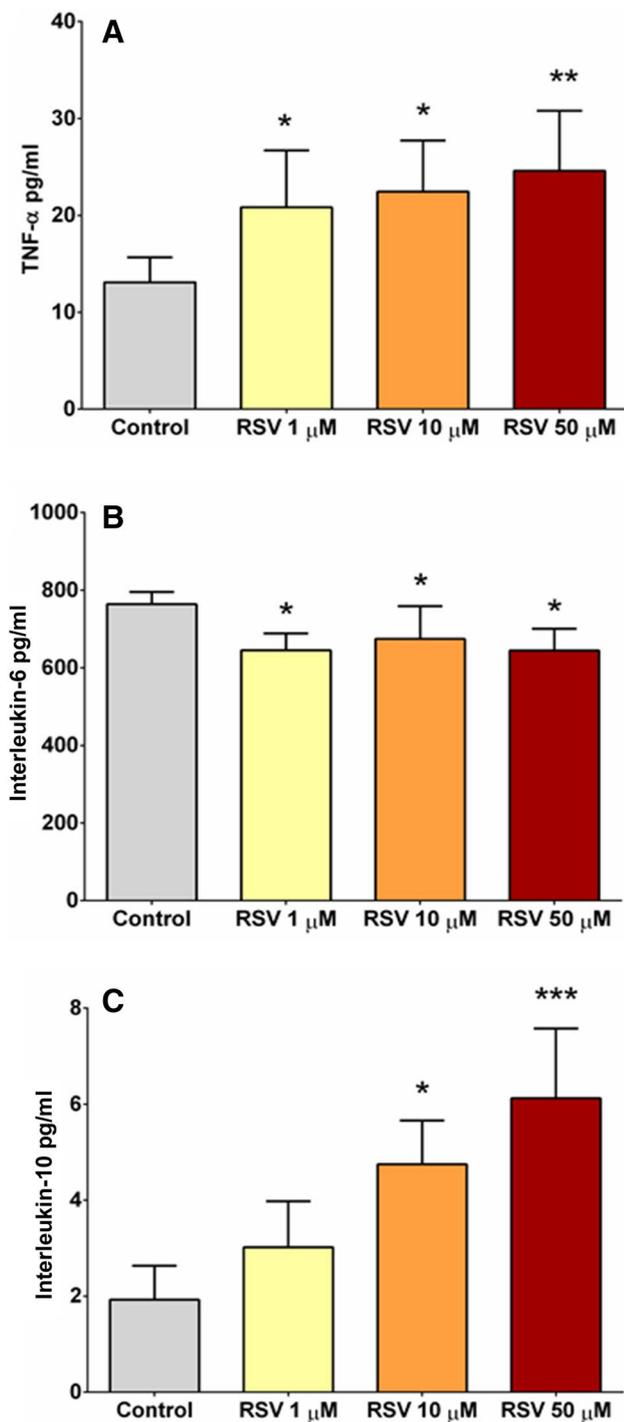
characterizes the early steps of HSC activation [3–5]. Thus, we sought to first evaluate some parameters of HSC activation in response to a 24-h treatment with RSV at 1, 10 and 50  $\mu\text{M}$ . We found that RSV at lowest concentrations (1 and 10  $\mu\text{M}$ ) did not alter the protein content of  $\alpha$ -SMA, collagen I and GFAP. However, treatment with 50  $\mu\text{M}$  of RSV induced an increase of content for these activation-related proteins. The increase of contractibility in activated HSC is an important feature that contributes to the intrahepatic resistance and portal hypertension, which is responsible for the morbidity in liver cirrhosis. In this situation, activated HSC displays morphological changes in their cytoskeleton characterized by the presence of F-actin stress fibre [25, 26]. In this way, RSV was not able to change the myofibroblast-like morphology of GRX, which remained displaying elongated cytoplasm characterized by the presence of stress fibres. Altogether, these

results suggested that RSV treatment did not decrease the activation state of GRX and, oppositely, triggered a pro-activation effect at the 50  $\mu\text{M}$  concentration.

Another remarkable feature of activated HSC is their ability to migrate towards damaged areas after liver injury, a chemotactic effect that is important to wound healing and hepatic tissue remodelling. However, an increased migration of activated HSC could exacerbate the fibrotic progression, worsening organ dysfunction [3, 27]. Cells treated with 10  $\mu\text{M}$  of RSV presented a decreased rate of cell migration after 6 and 12 h from wounding while this effect was observed in cells treated with 50  $\mu\text{M}$  of RSV at all times of wounding evaluation. In addition, it was also notable that 50  $\mu\text{M}$  of RSV apparently decreased cells ability of contracting collagen I gels. At first sight, all results regarding the RSV effects in GRX activation, contraction ability and migration were surprising and seemed to be contradictory,



**Fig. 3** Cells treated with 10 μM of RSV presented a decreased rate of cell migration after 6 and 12 h from wounding while this effect was observed in cells treated with 50 μM of RSV at all times of wounding evaluation (*n* = 3, mean ± SDM, *p* < 0.05, Scale bar: 200 μm)

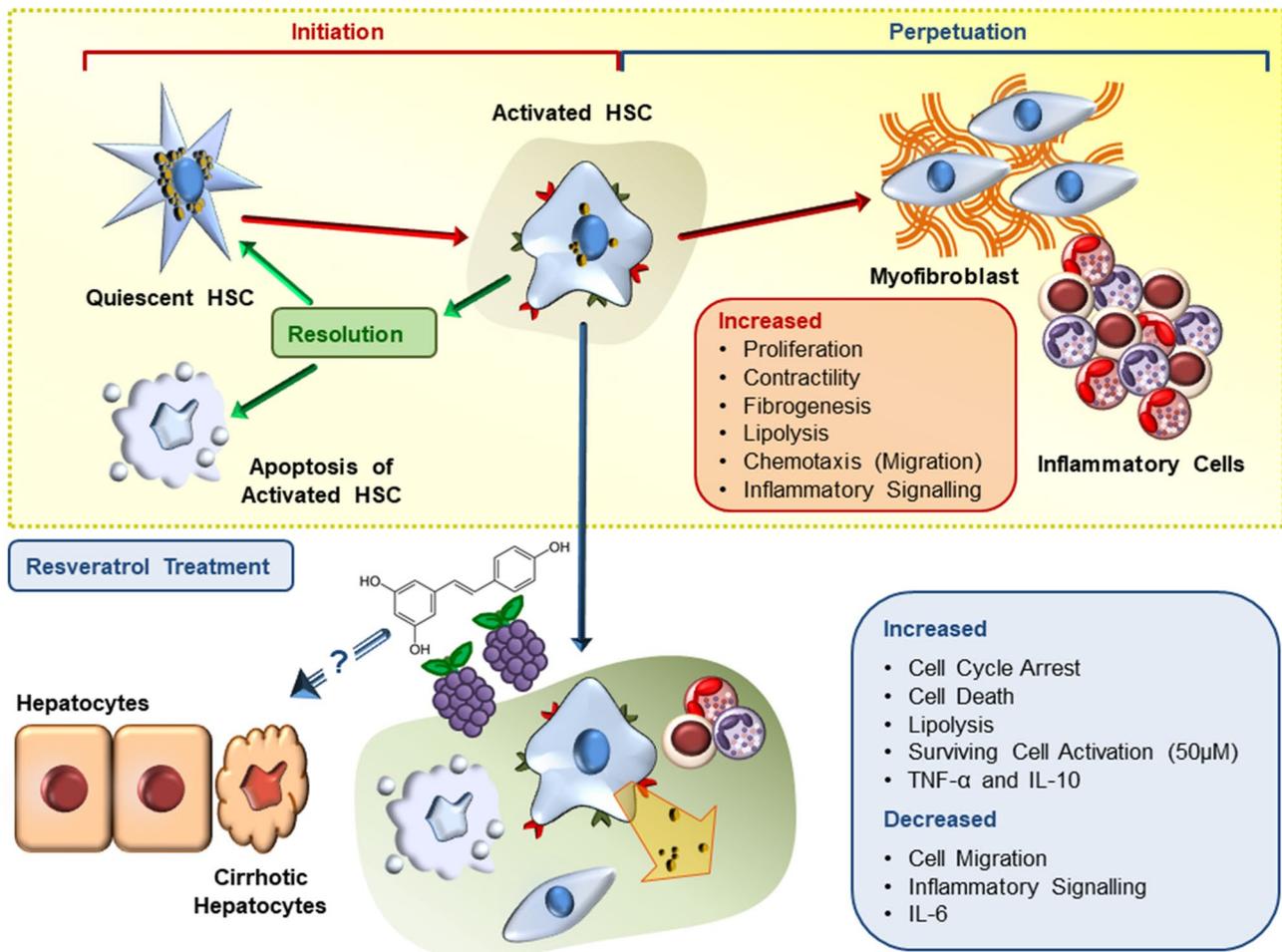


**Fig. 4** **a** All concentrations of RSV triggered the release of TNF- $\alpha$  in culture medium by GRX; **b** An opposite effect was observed regarding the release of IL-6; **c** RSV at 10 and 50  $\mu$ M concentrations triggered the release of IL-10 in culture medium by GRX ( $n=3$ , mean  $\pm$  SDM,  $p < 0.05$ )

especially on those cells that were treated with the highest concentration. However, it is necessary to consider that RSV was cytotoxic to our model [14–16]. Once the HSC is activated in response to stress mediators [3–5], and here RSV appears to display a stressor behaviour, it seems to be a plausible hypothesis that GRX cells respond to the RSV cytotoxicity through remaining at their activation state or, in the scenario by which this phytoalexin is remarkably toxic, through increasing their activation state. Furthermore, cytotoxicity and low cell migration use to be correlated events [36–38], which can make sense to explain the RSV effects on impairing GRX migration regardless the fact of these cells remaining at an activated—or more activated—phenotype. In the same way, the reduction of cell population due to the cytotoxicity of RSV at 50  $\mu$ M after 24-h treatment, as previously demonstrated [14], may be related to the smaller contraction of collagen I gel in this group.

Most of the new cellular functions of activated HSC are indeed sustained by an autocrine loop characterized by the enhancement of cell response to several mediators through both the upregulation of their membrane receptors and the enhancement of intracellular signalling [3–5]. Also, HSC-mediated inflammatory signalling may influence the function of hepatocytes and sinusoidal cells, and may favour the repair of injured tissue through promoting the restoration of hepatic homeostasis [3–5, 22]. Thus, we assume that the largely discussed anti-inflammatory property of RSV [1, 2, 28] would interfere on cytokines signalling mediated by HSC. Here, we showed that RSV was able to increase the release of TNF- $\alpha$  and IL-10 by GRX in culture media while the opposite occurred for the IL-6 releasing. Considering these results and since our model represents a restricted population of liver cells, the presence of TNF- $\alpha$  and IL-10 in culture media could influence the GRX itself metabolism.

Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) are important pro-inflammatory and pro-fibrogenic mediators that participate in the HSC activation during initiation phase. Curiously, some studies have also suggested that TNF- $\alpha$  can reduce liver fibrogenesis by inducing activated HSC to synthesize less collagen I during their perpetuation phase. Also, there are evidences that TNF- $\alpha$  mediates a pro-apoptotic pathway in which the mitochondrial potential decreases while reactive oxygen species (ROS) and caspase cascade act as downstream mediators, and this scenario is in accordance to the RSV effects on GRX, which were found in our previous studies. Similarly, IL-6 is thought to exert beneficial effects during liver chronic diseases through playing an important role for inducing the hepatocytes regeneration.



**Fig. 5** Treatment of chronic liver disease, including advanced cirrhosis, shall be focused in the liver fibrosis regression by controlling the fibrotic activity of activated HSC through inducing these cells to quiescence or apoptosis. Our previous findings showed that RSV treatment induced cell cycle arrest, cell death, and lipolysis in GRX cell line, which is an activated HSC model. Here we found that RSV

at 1–10  $\mu$ M did not decrease in HSC activation state; oppositely, the highest concentration induced an increase of activation markers (GFAP, collagen I, and SMA- $\alpha$ ). However, RSV treatment decreased activated HSC migration and triggered an anti-inflammatory effect. Further studies are needed to elucidate the RSV effect on other liver cells, especially healthy or cirrhotic-injured hepatocytes

These facts reveal that both TNF- $\alpha$  and IL-6 have, indeed, a pleiotropic function during the HSC activation process [32, 39–43]. Interleukin-10 (IL-10) has been regarded as one of the most important anti-inflammatory cytokines even in the presence of higher levels of pro-inflammatory cytokines [1, 2, 29, 34, 44]. Also, IL-10 may act on preventing an excessive liver fibrogenesis or an inappropriate inflammatory response through inducing activated HSC to apoptosis [45–48]. Altogether, considering that GRX is an activated HSC model, these results on the TNF- $\alpha$ , IL-6 and IL-10 media content may indicate an important role of RSV in our model, which may be helpful for controlling HSC activity during liver fibrosis (Fig. 4).

Several studies have demonstrated that RSV can exert contradictory effects depending on its concentration, time of treatment or model of study. Indeed, RSV studies in cell

culture models have demonstrated that low concentrations of this molecule increase proliferation while high concentrations impair cell growth [1, 2, 28, 49]. Until now, we found several relevant effects on treating activated HSC with RSV (Fig. 5). However, it is relevant to point that, during liver fibrosis associated to cirrhosis, at the same time by which is expected to control the proliferation of activated HSC or to induce apoptosis or quiescence of activated HSC, the hepatocyte regeneration or survival against damage stimuli must be sought [3, 6, 7, 50]. The RSV-mediated HSC release of TNF- $\alpha$  and IL-10, and the decrease of IL-6 release, may influence not only HSC itself but also hepatocytes. Thus, it is undoubtedly relevant to consider the effects of RSV for other liver cells on considering this phytoalexin for treating liver fibrosis during chronic hepatic diseases and cirrhosis. In this way, more studies focusing on the HSC relationship with

other liver cells, especially hepatocytes, shall be conduct for a better understanding of the RSV effects to liver as whole.

**Acknowledgements** C.M. de Oliveira is the recipient of a fellowship from CAPES (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*). This work was also supported by CNPq (*Conselho Nacional de Desenvolvimento Científico e Tecnológico—Brazil*), FAPERGS (*Fundação de Amparo à Pesquisa do Rio Grande do Sul*, PqG n°1009169) and PROPESQ-UFRGS. All authors would like to thank to Dr. Elena Bernard for the helpful comments in this study.

**Author contributions** Wrote the paper: CMO and LAMM. Establishment of GRX cell line: RB. Collected and analysed data: CMO, LAMM, ASC, KSM, BruPC, MQV, BarPC. Supervision and Contribution to the text writing: JRO and FCRG.

## Compliance with ethical standards

**Conflict of interest** Authors declare no conflict of interest.

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