Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Programa de Pós Graduação em Ciências Biológicas: Bioquímica

A IMPORTÂNCIA DA INTERAÇÃO ENTRE ESTRESSE OXIDATIVO, BIOGÊNESE DE MITOCÔNDRIAS E MITOFAGIA NA RESPOSTA DE CÉLULAS ESTRELADAS HEPÁTICAS AO RESVERATROL

LEO ANDERSON MEIRA MARTINS

Orientadora: Prof. Dra. Fátima Costa Rodrigues Guma

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito para obtenção do grau de Doutor em Bioquímica.

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“And in the end, the love you take is equal to the love you make.”
(Lennon & McCartney)

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PARTE I

I.1. RESUMO

A fibrose hepática é uma patologia que acompanha outras doenças crônicas do fígado como a cirrose e o hepatocarcinoma. As células estreladas hepáticas (HSC, do inglês *hepatic stellate cells*) compõem uma população celular heterogênea que se caracteriza por transitar entre dois fenótipos. As células com fenótipo quiescente possuem a capacidade de armazenar vitamina A em gotas lipídicas. Os insultos ao fígado desencadeiam uma resposta inflamatória que gera estímulos parácrinos e autócrinos mediados por citocinas e espécies reativas. Neste contexto, as HSC assumem um fenótipo ativado fibrogênico e tornam-se responsáveis pela cicatrização hepática. Danos crônicos ao fígado levam a uma deposição de matriz extracelular exagerada que configura o estado patológico da fibrose. O resveratrol (RSV – 3,4',5-tri-hidroxi-*trans*-estilbeno) é uma fitoalexina produzida por algumas espécies de plantas. Inúmeros efeitos benéficos à saúde são atribuídos ao RSV por causa do seu potencial antioxidante, antiinflamatório e pró-apoptótico. Estudos anteriores mostraram que tratamento da GRX, uma linhagem murina de HSC ativadas, com concentrações de RSV próximas as biodisponíveis (0,1 a 1 µM) resultou em parada do ciclo na fase S com consequente inibição de proliferação celular, um efeito associado à citotoxicidade e que pode favorecer a resolução da fibrose hepática. Neste estudo, por técnicas espectrofotométricas, foi demonstrado que tratamento da GRX por 24 horas com concentrações entre 0,1 a 50 µM de RSV promoveu um efeito pró-oxidante que causa uma citotoxicidade dependente da dose, bastante aumentada no grupo tratado com a concentração mais alta. Os efeitos citotóxicos atenuados encontrados nas células tratadas por 120 horas sugerem que a GRX pode se tornar resistente a estes efeitos. O potencial pró-oxidante do RSV foi o ponto de partida para investigar a possibilidade de que esta fitoalexina provocasse uma alteração no metabolismo mitocondrial da GRX. Para isso, os efeitos do RSV (1 a 50 µM) na função mitocondrial, na indução de morte mediada por estas organelas e na autofagia/mitofagia foram investigados por técnicas de espectrofotometria, de imunocitoquímica, de citometria de fluxo, de microscopia confocal e de microscopia eletrônica de transmissão em GRX tratadas por 24 e 120 horas. Foi demonstrado que todas as concentrações de RSV promovem apoptose por meio da ativação de caspases, alteram a dinâmica/função mitocondrial e induzem o aumento de autofagia/mitofagia na GRX. No entanto, o RSV provocou biogênese de mitocôndrias nos grupos tratados com 1 e 10 µM, enquanto que o tratamento com 50 µM causou dano celular evidente na GRX, sem induzir biogênese de mitocôndrias. Desta forma, é possível que a citotoxicidade "dose-dependente" do RSV, que causa a morte celular e dano oxidativo em 24 horas de tratamento, esteja relacionada com o desequilíbrio entre a indução concomitante de apoptose mediada por dano mitocondrial, autofagia/mitofagia e biogênese de mitocôndrias. Por fim, foi investigada a liberação de TNF-α, Interleucina-6 e Interleucina-10 pela GRX tratada por 24 e 120 horas com RSV (0,1 a 50 µM), considerando o papel antiinflamatório do RSV e o papel das HSC ativadas na sinalização autócrina que contribui para a modulação fenotípica destas células. Foi demonstrado que o tratamento da GRX com RSV por 24 e 120 horas induziu a redução da liberação de Interleucina-6; enquanto que a liberação de TNF-α e Interleucina-10 foi aumentada. Estes resultados confirmam um efeito antiinflamatório do RSV que

deve contribuir na prevenção da ativação ou da perpetuação do estado ativado das HSC por meio de sinalização autócrina. Ainda que a concentração do RSV seja importante para efetivamente induzir a morte das HSC ativadas, o tratamento com esta fitoalexina pode ser promissor para a resolução da fibrose hepática por diminuir a população de células ativadas e, possivelmente, prevenir a perpetuação do estado fenotípico ativado. Estudos avaliando indicadores de quiescência em células tratadas são ainda necessários para desvendar completamente os efeitos do RSV quanto às possibilidades de inibição da perpetuação ou reversão fenotípica das HSC ativadas.

Palavras-chaves: Células estreladas hepáticas; Biogênese de mitocôndrias; Função mitocondrial; GRX; Interleucina-6; Interleucina-10; Resveratrol; TNF-α.

I.2. ABSTRACT

Liver fibrosis is a disease that accompanies other hepatic chronic diseases such as cirrhosis and hepatocellular carcinoma. Hepatic stellate cells (HSC) are a heterogeneous cell population characterized by transiting between two phenotypes. Cells with a quiescent phenotype are able to store vitamin A into lipid droplets. Damage to the liver trigger an inflammatory response that generates paracrine and autocrine stimulation mediated by cytokines and reactive species. In this context, HSC assume an activated and fibrogenic phenotype responsive for hepatic wound-healing. Chronic insults to the liver lead to an excessive deposition of extracellular matrix that configures the pathological state of fibrosis. Resveratrol (RSV – 3,4',5-tri-hidroxi-*trans*-stilbeno) is a phytoalexin produced by some species of plants. Several beneficial effects are attributed to this molecule due to its antioxidant, antiproliferative and pro-apoptotic potential. Previous studies showed that treatment with bioavailable concentrations of RSV (0.1 to 1 µM) promoted an arrest cycle at the S phase in GRX, a murine activated HSC model, leading to cell proliferation inhibition, a cytotoxic effect that contributes to the liver fibrosis resolution. In this study, it was shown by spectrophotometric techniques that GRX treatment for 24 hours at concentrations between 0.1 to 50 µM of RSV promoted a fairly clear pro-oxidant effect that causes a dose-dependent cytotoxicity that was higher in the group treated with 50 µM. The attenuated cytotoxicity found after 120 hours of GRX treatment suggest that these cells became resistant to this effect. The pro-oxidant potential of RSV was the starting point for investigating the possibility that this phytoalexin would cause a change in the GRX mitochondrial metabolism. Thus, the effects of RSV (1 to 50 µM) on altering the mitochondrial function, on inducing mitochondrial-mediated cell death, and autophagy/mitofagia were investigated in GRX treated for 24 and 120 hours by spectrophotometric techniques, immunocytochemistry, flow cytometry, confocal microscopy, and transmission electron microscopy. All the RSV concentrations promote cell apoptosis through caspases activation, alter the mitochondrial dynamics and function, and induce an increase of autophagy/mitofagia. Curiously, only 1 and 10 µM of RSV induced mitochondrial biogenesis in GRX, while the highest concentration caused an evident cell damage without inducing mitochondrial biogenesis. Thus, it is possible that the "dose-dependent" cytotoxicity of RSV, which causes cell death and oxidative damage in 24 hours of treatment, is related to an imbalance between the concomitant induction of mitochondrial-mediated apoptosis, autophagy/mitofagia, and mitochondrial biogenesis. Finally, it was investigated the release of TNF-α, Interleukin-6 and Interleukin-10 by GRX treated for 24 and 120 hours with RSV (0.1 to 50 µM), considering the anti-inflammatory role of RSV and the autocrine signalling role of HSC that contributes to the perpetuation of its activated phenotype. It was demonstrated that GRX treatment with RSV for 24 and 120 hours reduced the release of Interleukin-6 in the culture medium; whereas the release of TNF-α and Interleukin-10 was increased. These results confirm the anti-inflammatory properties of RSV and may contribute to the prevention of HSC activation through autocrine signalling. Although RSV concentration is important to effectively induce activated HSC death, cells treatment with this phytoalexin may be promising for liver fibrosis resolution through decreasing the population of activated cells or through preventing the

perpetuation of activated state of HSC. Future studies evaluating the quiescence indicators of GRX under RSV treatment are still needed to fully unravel the effects of this phytoalexin on inhibiting the perpetuation of activated HSC or reversing its activated phenotype.

Keywords: GRX; Hepatic stellate cells; Interleukin-6; Interleukin-10; Mitochondrial biogenesis; Mitochondrial function; Resveratrol; TNF-α.

I.3. LISTA DE ABREVIATURAS

AO – *Acridine Orange*

DCF – 2'-7'-*diclorofluoresceína*

DRP1 – *Dynamin related protein 1*

ER – Espécie(s) reativa(s)

FIS1 –*Mitochondrial fission protein 1*

GRX – Linhagem celular representativa de HSC ativadas

HSC – *Hepatic stellate* cells, em português: células estreladas hepáticas

IL-6 – Interleucina-6

IL-10 – Interleucina-10

LC3-I/II – *Light chain protein 3-I/II*

LYSR – *Lysotracker Red DND-99*

MFN1/2 – *Mitofusin 1/2*

MTG – *MitoTracker™ Green FM*

MTO – *MitoTracker™ Orange CM-H2TM ROS*

ND1 – *NADH dehydrogenase subunit 1*

OPA1 – *Optic atrophy protein 1*

RSV – Resveratrol

SOD – Superoxido dismutase

TNF-α –*Tumour necrosis factor*-α, em português: fator de necrose tumoral-α

I.4. INTRODUÇÃO

I.4.1. A fibrose hepática e as células estreladas hepáticas

As doenças crônicas do fígado estão entre as maiores causas de mortalidade humana e figuram entre os maiores problemas de saúde no mundo. A fibrose hepática e, em último estágio, a cirrose representam as manifestações patológicas mais comuns. As maiores causas de fibrose e cirrose hepática incluem as infecções crônicas do fígado (hepatite), o abuso de álcool e a esteatose não alcoólica [1-3].

O fígado é um órgão que possui grande capacidade de regeneração em resposta a danos agudos. Este processo, que envolve a reposição de hepatócitos apoptóticos ou necróticos, está associado a uma resposta inflamatória controlada e a uma deposição limitada de matriz extracelular, que forma cicatrizes para separar a área danificada das áreas saudáveis. Quando os danos ao fígado são contínuos, ocorre um processo inflamatório crônico não controlado que resulta no acúmulo excessivo de tecido conjuntivo com conteúdo proteico alterado e rico em colágeno do tipo I. Neste contexto, o desequilíbrio entre a produção e a degradação de matriz extracelular também contribui para uma alteração da arquitetura normal do fígado, provocando a morte massiva de hepatócitos, situação que caracteriza a cirrose e a perda de função hepática [2, 4-5].

Atualmente, o tratamento de doenças crônicas do fígado ainda é limitado a duas possibilidades: a redução do estímulo de dano ou o transplante hepático.Infelizmente, cerca de 40% dos pacientes com diagnóstico de fibrose e cirrose hepática não apresentam os sintomas destas doenças, que incluem inchaço abdominal, hipertensão portal, encefalopatia e icterícia. Nestes casos,

quando as complicações decorrentes da patologia surgem, a deterioração hepática pode ser irreversível [5-6]. Por outro lado, as pesquisas mais recentes que buscam a compreensão destas patologias hepáticas sugerem uma possível capacidade de recuperação a partir de qualquer grau de fibrogênese hepática, incluindo a fibrose associada ao estado de cirrose [7].

As células estreladas hepáticas (HSC, do inglês *hepatic stellate cells*), descritas inicialmente pelo anatomista alemão Karl Wilhelm Von Kupffer no século 19, tem sido destacadas nos últimos 30 anos como um tipo de célula mesenquimal consideravelmente versátil, que desempenha um papel crucial na resposta a danos e na manutenção da homeostasia hepática[8]. Estas células são pericitos localizados no espaço de Disse, uma região situada entre os hepatócitos e as células endoteliais que estruturam os sinusóides hepáticos [Figura 1]. As HSC possuem a capacidade de armazenar retinol (vitamina A) em gotas lipídicas citoplasmáticas, o que caracteriza seu fenótipo quiescente. O paradigma da ativação destas células e sua modulação fenotípica para miofibroblastos proliferativos estão amplamente envolvidos no processo fibrogênico hepático. De fato, a capacidade das HSC em transitar entre o fenótipo quiescente e ativado, dependendo da necessidade de resposta regenerativa hepática, faz com que estas células constituam uma população heterogênea que difere na sua capacidade de armazenar gotas lipídicas, na expressão e organização do citoesqueleto e no potencial para produção de matriz extracelular [9-10].

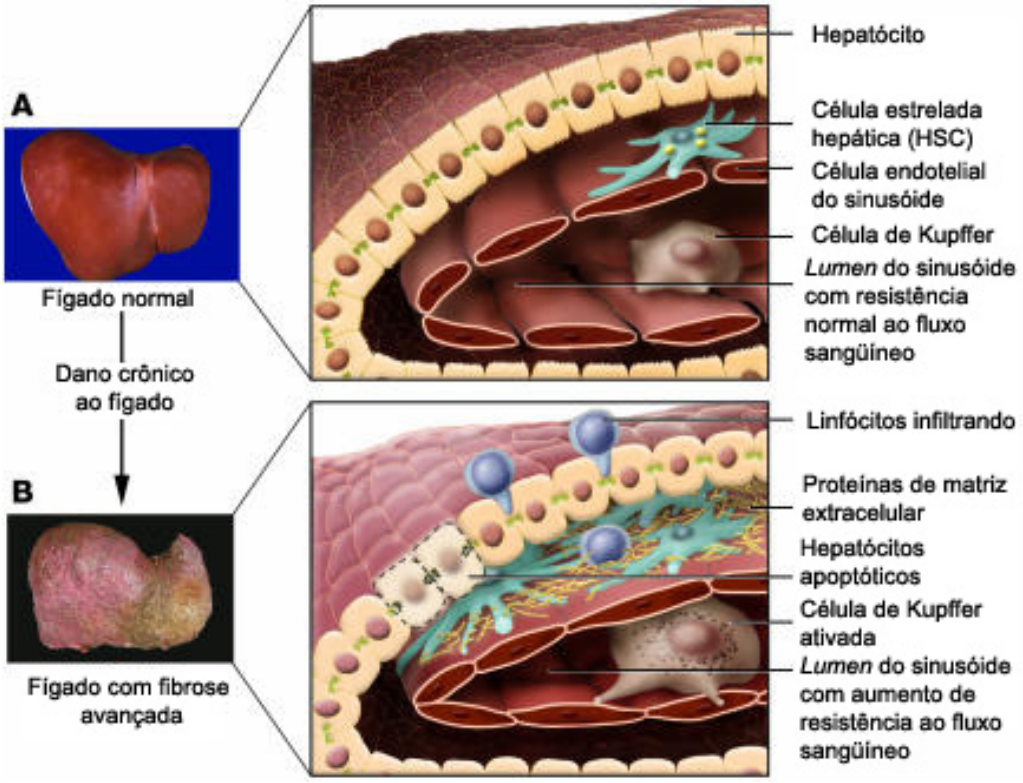

Figura 1. As Células Estreladas Hepáticas se localizam no espaço de Disse, entre os hepatócitos e as células endoteliais dos sinusóides. A ativação das HSC contribui amplamente para o processo fibrogênico hepático (Traduzida de Bataller, R.& Brenner, D.A., 2005).

O estudo da fibrose hepática envolve o entendimento dos mecanismos que levam à ativação e à proliferação descontrolada das HSC. A modulação fenotípica dessas células consiste de duas fases definidas: a iniciação e a perpetuação [Figura 2]. A fase de iniciação corresponde às primeiras mudanças na expressão gênica e no fenótipo das HSC. Estas alterações são respostas a estímulos parácrinos dos tipos celulares vizinhos, que incluem as células de Kupffer, os hepatócitos lesionados, as células endoteliais, as plaquetas e os leucócitos. De fato, a ativação e a infiltração das células de Kupffer e demais leucócitos despontam como um dos principais eventos que contribuem para a ativação das HSC através da ação de diversas citocinas pró-inflamatórias. A ação de produtos

de lipoperoxidação em consequência de estresse oxidativo também exerce um papel importante no processo de ativação das HSC [4, 8, 11].

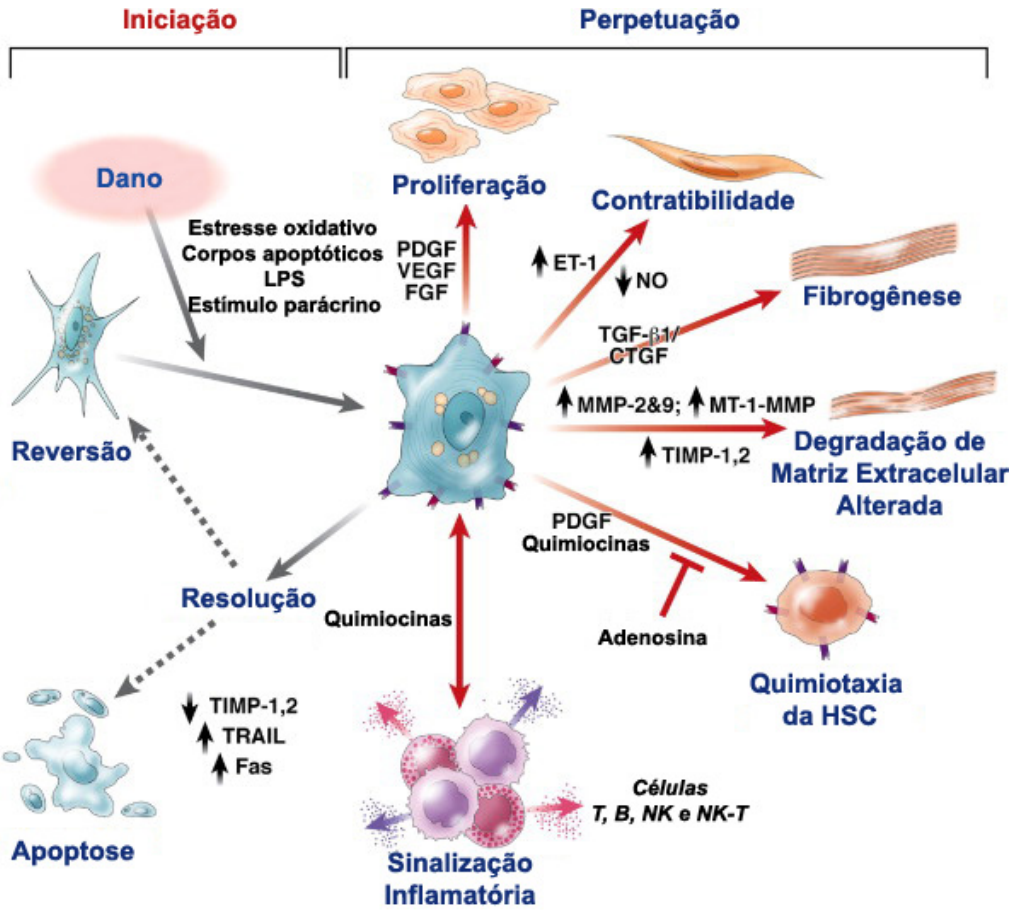

Figura 2. Vias de ativação da Célula Estrelada Hepática (HSC). Os aspectos envolvidos na ativação das HSC são distintos nas fases de iniciação e de perpetuação. As estratégias que buscam o tratamento da fibrose hepática envolvem a indução de apoptose das HSC ativadas ou sua reversão ao fenótipo quiescente, reduzindo o estado inflamatório do fígado e a fibrogênese (Traduzida de Friedman, S.L., 2008).

A fase de perpetuação da ativação se caracteriza pela aquisição e manutenção das novas funções celulares das HSC, que incluem: aumento da proliferação celular, da contratibilidade citoplasmática, da quimiotaxia celular, da liberação de mediadores inflamatórios e da capacidade fibrogênica. Nesta fase,

além da sinalização parácrina, uma sinalização autócrina também contribui para o aumento da produção e da resposta a moléculas inflamatórias, sustentando muitas das novas funções das HSC. Nesse sentido, diversas citocinas podem desempenhar um papel pró-fibrogênico (entre elas, o Fator de Crescimento Tumoral-β) ou anti-fibrogênico (entre elas, a Interleucina-10). O equilíbrio na ação dessas citocinas pode determinar a duração da resposta ao dano por parte das HSC ativadas [8, 12].

O destino das HSC quando a fibrose e/ou a cirrose hepática se estabelecem ainda não é claro e não há um tratamento padrão para estas doenças. No entanto, é fato que as HSC são um dos alvos potenciais para o tratamento destas patologias antes da falência hepática. Por este motivo, as melhores estratégias a serem planejadas são as que buscam a neutralização da resposta proliferativa, fibrogênica e contráctil das HSC, ou as que estimulam a apoptose de células ativadas, a degradação de matriz extracelular, a modulação das citocinas envolvidas e a redução de estresse oxidativo [10, 13].

I.4.2 Resveratrol (3,4',5-tri-hidroxi-*trans*-estilbeno)

Os estudos sobre o potencial benéfico do Resveratrol (RSV) associados ao seu consumo na dieta tem crescido muito nos últimos anos. Tal interesse surgiu devido a uma situação ocorrida na Europa do século XIX, denominada como "paradoxo francês". A este fenômeno foram relacionadas as contradições existentes entre a alimentação da população francesa, rica em lipídios saturados, e os baixos índices de mortalidade por aterosclerose coronariana, uma doença que alcançava altos índices nos demais países do continente europeu. A alta

concentração de RSV no vinho tinto, muito consumido pelos franceses, gerou a especulação de que este fosse o responsável pelo baixo índice de doenças cardíacas [14-15].

O RSV é uma fitoalexina produzida por aproximadamente 31 gêneros de plantas – incluindo uvas, amendoins e ameixas – em resposta às adversidades ambientais e às infecções patogênicas como, por exemplo, ataque de fungos. Trata-se de um composto polifenólico derivado da fenilalanina, que contém dois anéis aromáticos com hidroxilas reativas na sua estrutura e que pode se apresentar sob duas formas isoméricas: *cis*- e *trans*-resveratrol [Figura 3] [16]. A forma *trans*-resveratrol é mais comum por ser relativamente estável se protegida da luz e da alteração de pH [17]. A forma *cis*-resveratrol pode ser resultado de uma conversão foto-isomérica ou de alterações de pH [18]. Por ser uma fitoalexina, o conteúdo de RSV presente nas diferentes fontes pode variar amplamente dependendo de fatores que podem gerar mais ou menos estresse, tais como: ambiente em que a planta é cultivada, exposições a patógenos e, no caso dos vinhos, método de produção [16]. Muitos estudos sobre RSV são direcionados aos vinhos tintos e apontam a presença das duas formas isoméricas dessa molécula nessa bebida [19].

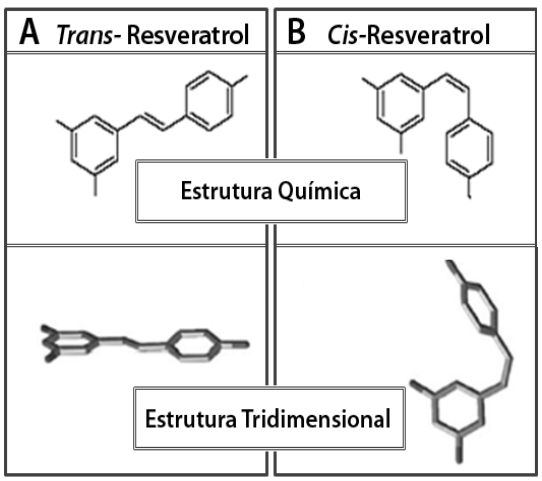

Figura 3. Estrutura molecular química e tridimensional do (A) *Trans*-Resveratrol e do seu isômero (B) *Cis*-Resveratrol.

Ainda não há um entendimento completo acerca das ações moleculares do RSV. A literatura atual, que inclui muitos estudos *in vivo* e *in vitro*, aponta diversos alvos moleculares – que incluem citocinas, fatores de transcrição e proteínas do ciclo celular – pelos quais o uso do RSV pode resultar em efeitos variados. Além deste fato, há um consenso de que o potencial benéfico do RSV à saúde também depende da sua absorção, biodisponibilidade e metabolismo celular. Por esse motivo, a ação desta fitoalexina pode depender da concentração e do tempo em que é administrada. Da mesma forma, modelos variados de estudo podem responder de maneira diferente ao tratamento com RSV [14, 17, 20]. Em células normais, por exemplo, os efeitos benéficos do RSV são principalmente atribuídos ao seu potencial quimiopreventivo, que é acompanhado de uma ação antiinflamatória, anti-apoptótica e antioxidante [17, 21]. Nesse sentido, já foi mostrado que o tratamento prévio com esta fitoalexina é capaz de induzir neuroproteção em tecidos nervosos colocados em condições adversas,como a privação ao oxigênio e à glicose [22]; cardioproteção, prevenindo danos ao tecido cardíaco em virtude de isquemias [21, 23]; e proteção às células β pancreáticas, prevenindo o desenvolvimento da diabetes [24]. Por outro lado, o RSV pode exercer um efeito citotóxico, induzindo a parada de ciclo celular (inibição de crescimento) e a morte por apoptose ou necrose, que geralmente é acompanhada de uma ação pró-oxidante [25]. Esses efeitos são igualmente considerados benéficos no tratamento de diversas patologias, como, por exemplo, o câncer [17, 26].

Considerando que o estado de fibrose hepática requer todos os elementos que se relacionam com a presença de mediadores inflamatórios ou de espécies reativas de oxigênio, que a redução destes estímulos ou a indução de apoptose

de HSC ativadas é essencial para resolução desta doença [8, 27], os efeitos antiinflamatórios, antioxidantes e pró-apoptóticos do RSV [17, 21] são, sem dúvidas, interessantes para o estudo em modelos animais que representem este estado patológico ou em linhagens celulares que representem HSC ativadas, como a GRX. Da mesma maneira, é relevante pensar que o consumo ou o uso do RSV pode ser uma ferramenta importante para a prevenção ou para o tratamento da fibrose hepática, uma vez que essa fitoalexina é uma molécula natural facilmente obtida na alimentação [20].

I.4.3 GRX, uma linhagem de células estreladas hepáticas ativadas

A linhagem GRX foi estabelecida a partir de granulomas produzidos em fígados de camundongos C3H/HeN, induzidos por infecção através de penetração subcutânea com *Schistossoma mansoni*. A cultura celular primária a partir deste granuloma gerou, entre outras, esta linhagem imortalizada similar às células estreladas hepáticas (HSC) ativadas [28]. Desta forma, a GRX possui as características morfológicas e bioquímicas de um miofibroblasto, apresentando uma capacidade de secretar matriz extracelular e uma taxa de proliferação alta, de forma similar às células conjuntivas encontrada em abundância no tecido fibrótico hepático [29-30].

Ainda que sejam células que tiveram a ativação estimulada, resultando em um modelo que mimetiza o estado patológico da fibrose hepática, a GRX consiste em uma população heterogênea em que grande parte das células se encontra em um estado intermediário de ativação. Por este motivo, estas células podem ser induzidas não apenas a retornarem ao seu fenótipo quiescente como podem ser

estimuladas a expressarem um fenótipo mais ativado [Figura 4]. De fato, estudos anteriores usando as células GRX como modelo já demonstraram que os tratamentos com retinoides, indometacina, β-caroteno e capsaicina podem estimular o acúmulo de gotas lipídicas, a diminuição da proliferação e da produção de matrix extracelular na GRX [31-33]. Por outro lado, já foi também demonstrado que, na presença de mediadores inflamatórios ou que induzam estresse oxidativo, a GRX responde se transformando em uma célula miofibroblástica mais ativada [29, 34].

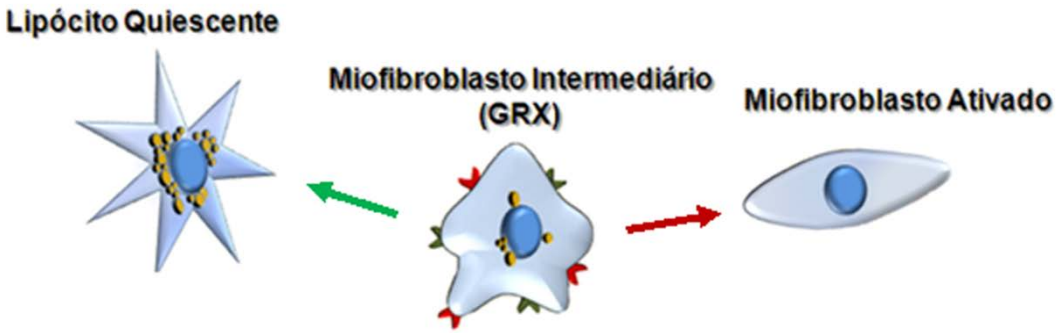

Figura 4 Ativação e modulação fenotípica das células GRX, linhagem representativas das células estreladas hepáticas (HSC) ativadas. O tratamento destas células com retinóides, por exemplo, induzem a expressão do fenótipo quiescente (seta verde). Por outro lado, o tratamento da GRX com mediadores inflamatórios, por exemplo, induzem a expressão de um fenótipo mais ativado (seta vermelha).

O estudo do uso do RSV na GRX já apresentou resultados que podem ser considerados promissores quanto à resolução da fibrose hepática: o tratamento com concentrações entre 0,1 e 1 μM de RSV foi capaz de inibir o crescimento destas células por induzir a parada do ciclo celular na fase S [35]. No entanto, o tratamento com estas mesmas concentrações de RSV não foi capaz de restaurar a capacidade deste modelo de HSC em armazenar gotas lipídicas, fato que foi

atribuído à indução de um desequilíbrio que favoreceu a transcrição da SIRT1 em relação à transcrição do PPARγ nas células tratadas [36]. Neste sentido, é importante ressaltar que a SIRT 1, uma desacetilase envolvida em diversos eventos celulares relacionados com a longevidade e com a resposta metabólica à restrição calórica, é conhecida por ser ativada pelo RSV [37-38]. Por outro lado, o PPARγ é um fator de transcrição relacionado com a adipogênese cuja expressão está associada com o acúmulo de gotas lipídicas por HSC quiescentes [36, 39].

Até o presente momento, pode-se concluir que o tratamento da GRX com RSV favorece a resolução da fibrose por provocar parada no ciclo celular. No entanto, o uso desta fitoalexina parece não induzir a reversão da GRX para seu fenótipo quiescente. Neste sentido, um estudo que busque elucidar ou amplificar o significado destes resultados quanto à resolução da fibrose hepática passa pelo entendimento do metabolismo e da biologia celular da GRX em resposta ao tratamento com RSV.

I.4.4 Metabolismo mitocondrial e autofagia na sobrevivência celular

É consenso que as reações de oxirredução (que definem o estado redox celular) desempenham um papel importante para a sobrevivência celular. As mitocôndrias são organelas responsáveis por muitos processos catabólicos, cumprindo uma função fundamental no metabolismo energético celular [40-41].

As mitocôndrias se caracterizam por possuírem duas membranas bi-lipídicas as circundando (uma interna e outra externa) e por possuírem DNA próprio, fato que possibilita um aumento no número de organelas conforme a demanda energética celular. Além disto, estas organelas são muito dinâmicas e podem

alterar sua morfologia e distribuição na célula em resposta a eventos de fissão e fusão [41-45] [Figura 5].

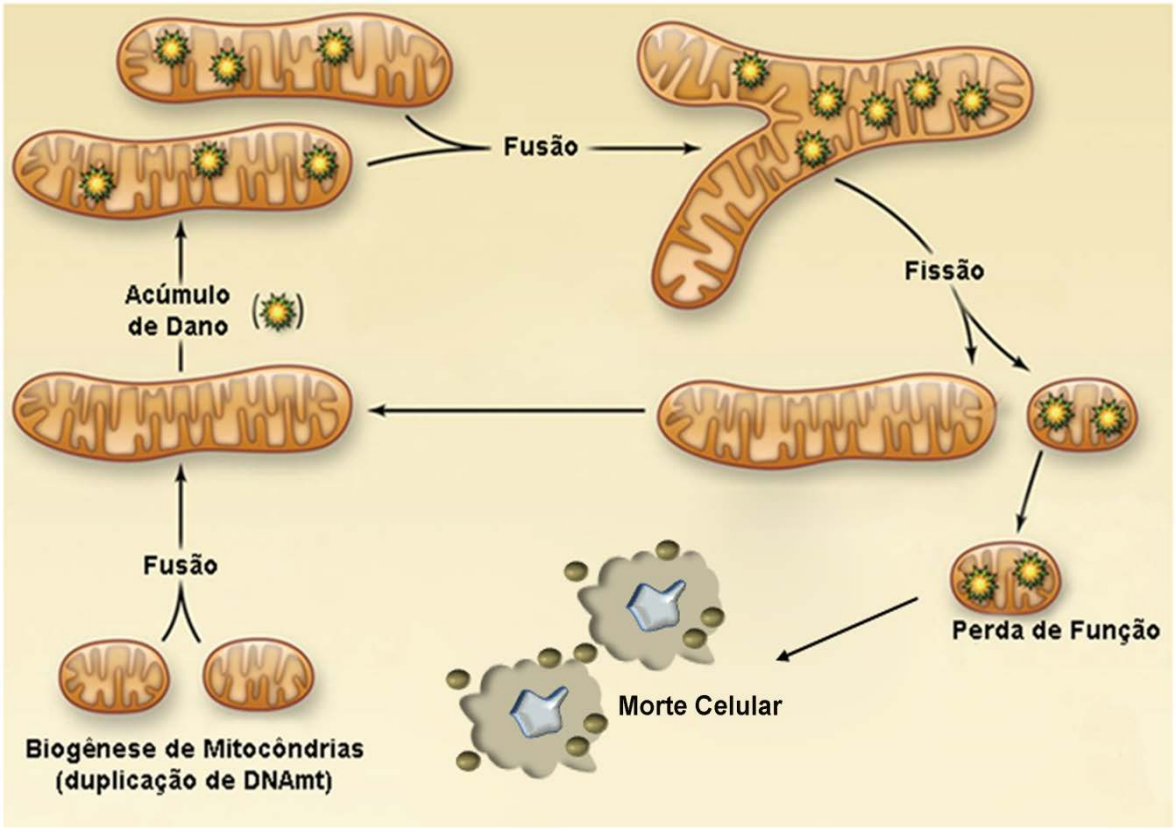

Figura 5 Biogênese, fissão e fusão mitocondrial. Por conter DNA próprio, as mitocôndrias podem aumentar sua quantidade de acordo com a demanda energética. Além disso, eventos de fusão e de fissão interferem na morfologia e na distribuição destas organelas nas células. Mitocôndrias danificadas podem mediar vias de sinalização de morte celular (Adaptada de Kluge, M.A. *et al.*, 2013).

Qualquer alteração da cadeia respiratória mitocondrial pode favorecer a produção de espécies reativas de oxigênio que danificam a estrutura de membranas da organela, alterando a sua arquitetura. Nesta situação, as mitocôndrias perdem sua função no metabolismo energético e se tornam mediadoras das principais vias de sinalizações que induzem morte celular programada (como a apoptose intrínseca/extrínseca e a necrose regulada) através da liberação de proteínas presentes no espaço intermembranas como,

por exemplo, o citocromo c [41, 46-49]. Nesse sentido, é importante ressaltar que o desequilíbrio no estado redox das células, em razão da alteração de função mitocondrial, pode causar danos às demais membranas celulares, incluindo membranas nucleares. A perda da integridade estrutural das membranas nucleares é um processo que favorece o dano oxidativo ao DNA, contribuindo para a indução da sinalização que conduz à morte celular [50-51].

A autofagia é um fenômeno que envolve uma sinalização proteica complexa e é caracterizada por um movimento coordenado de membranas intracelulares, através das quais as células conseguem sequestrar materiais citoplasmáticos danificados ou desnecessários em estruturas denominadas autofagossomos. Estas estruturas são “entregues” aos lisossomos, onde são degradadas e recicladas. Trata-se de um mecanismo celular extremamente conservado que é frequentemente referido como um tipo de morte celular. No entanto, a autofagia é crucial para a resposta celular a situações de estresse, e deve contribuir para a adaptação e sobrevivência das células em condições adversas como a privação de nutrientes, o estresse oxidativo e a citotoxicidade. Neste sentido, a autofagia de mitocôndrias, referida como mitofagia, é importante para manter o número de organelas adequado à demanda energética celular e para remover mitocôndrias danificadas que possam mediar a sinalização de morte [26, 42, 46, 52-57] [Figura 6].

A morte celular programada mediada por mitocôndrias danificadas e a autofagia são processos fisiológicos extremamente regulados que podem ser acionados por vias comuns, acarretando em respostas combinadas ou em duas respostas independentes. Desta forma, a relação entre a dinâmica mitocondrial, a apoptose e a autofagia é fundamental para definir o destino das células quanto a

sua sobrevivência ou morte [53-55]. Sobre esta questão, o aumento de espécies reativas de oxigênio e a diminuição da qualidade do DNA mitocondrial podem interferir nos eventos de fissão e fusão, fato que altera a dinâmica destas organelas, promovendo uma regulação negativa ou positiva de morte celular. De fato, a fusão mitocondrial facilita a troca de biomoléculas entre as mitocôndrias e o reparo das organelas por permitir a complementação de componentes danificados com componentes de organelas saudáveis. Por outro lado, a fissão mitocondrial tanto facilita na remoção de organelas danificadas pelos autofagossomos como pode ser um evento inicial para apoptose por promover ruptura da membrana externa mitocondrial [44, 58].

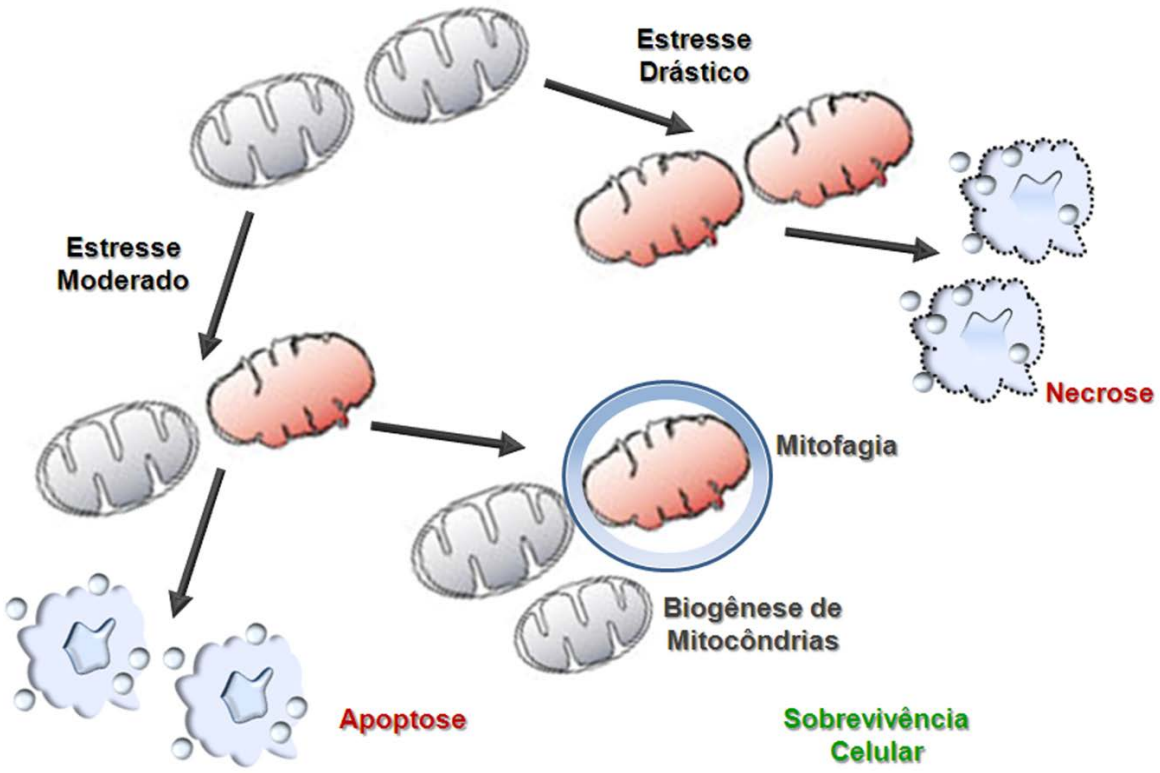

Figura 6 Apoptose e Mitofagia. Mitocôndrias danificadas medeiam a sinalização das principais vias de morte celular que, dependendo do nível de estresse, pode ser apoptose ou necrose. A mitofagia remove mitocôndrias danificadas e, junto com a biogênese de novas mitocôndrias, contribui para a sobrevivência celular.

Por fim, é importante ressaltar que somente a mitofagia de organelas danificadas não é suficiente para garantir a sobrevivência celular; a biogênese de mitocôndrias é um processo fundamental também para garantir a reposição destas organelas e a manutenção do metabolismo celular. De fato, a indução de apoptose, autofagia e de biogênese de mitocôndrias pode ser concomitante e estes eventos podem interferir um no outro para determinar não apenas o estado redox intracelular, mas a morte ou a sobrevivência das células diante de situações de estresse como as provocadas pelo tratamento com moléculas bioativas [59].

I.5. OBJETIVOS

Apesar de ser tratada como uma molécula antioxidante, durante os últimos anos, o RSV tem sido apontado como uma molécula bioativa capaz de alterar o estado redox intracelular. Além deste fato, os efeitos desta fitoalexina na indução de apoptose também são amplamente discutidos. Este efeito citotóxico pode ser acompanhado de um aumento intracelular de espécies reativas. Por outro lado, também já foi discutido que o RSV pode induzir autofagia e biogênese de mitocôndrias, efeitos relacionados com a sobrevivência celular e a redução de estresse oxidativo [25-26, 60-64]. Sem dúvidas, a compreensão acerca da possibilidade de uma interação entre a alteração do estado redox intracelular, indução de apoptose por um eventual dano às mitocôndrias, a autofagia/mitofagia e a biogênese de mitocôndrias mediadas por RSV, que acompanhem a parada do ciclo celular observada no trabalho anterior [35], é importante para o entendimento da fisiologia das HSC ativadas, representadas pela GRX.

Além deste fato, as HSC são conhecidas por também serem mediadoras da sinalização inflamatória hepática que pode ter um efeito autócrino, contribuindo para a perpetuação do seu fenótipo ativado. Neste sentido, o potencial antiinflamatório do RSV pode interferir nesta capacidade da GRX. Portanto, os objetivos da presente tese foram avaliar os efeitos do resveratrol (em concentrações que variaram entre 0,1 e 50 µM) em células GRX tratadas por 24 horas ou 120 horas, observando os seguintes parâmetros:

a) O estado redox intracelular, a proliferação e a viabilidade da GRX.
b) O metabolismo mitocondrial destas células, uma vez que estas organelas podem alterar o estado redox intracelular e podem ser mediadoras de vias de sinalizações clássicas que levam as células à morte.

c) Os mecanismos de autofagia/mitofagia destas células, uma vez que este evento fisiológico pode cooperar na sobrevivência celular diante de situações de estresse ou de alteração do estado redox intracelular.
d) A sinalização autócrina nas células, uma vez que este é um evento fisiológico importante na manutenção do fenótipo ativado das Células Estreladas Hepáticas.

PARTE II

II.1Resveratrol induces pro-oxidant effects and time-dependent resistance to cytotoxicity in activated hepatic stellate cells

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ORIGINAL PAPER

Resveratrol Induces Pro-oxidant Effects and Time-Dependent Resistance to Cytotoxicity in Activated Hepatic Stellate Cells

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Abstract Resveratrol (RSV) is known for its antioxidant properties; however, this compound has been proposed to have cytotoxic and pro-oxidant effects depending on its concentration and time of exposure. We previously reported the cell cycle arrest effect of low doses of RSV in GRX cells, an activated hepatic stellate cell model. Here, we evaluated the effects of RSV treatment (0.1–50 μM) for 24 and 120 h on GRX viability and oxidative status. Only treatment with 50 μM of RSV reduced the amount of live cells. However, even low doses of RSV induced an increased reactive species production at both treatment times. While being diminished within 24 h, RSV induced an increase in the SOD activity in 120 h. The cellular damage was substantially increased at 24 h in the 50 μM RSV-treated group, as indicated by the high lipoperoxidation, which may be related to the significant cell death and low proliferation. Paradoxically, this cellular damage and lipoperoxidation were considerably reduced in this group after 120 h of treatment while the surviving cells proliferated. In conclusion, RSV induced a dose-dependent pro-oxidant effect in GRX cells. The highest RSV dose induced oxidative-related damage, drastically reducing cell viability; but this cytotoxicity seems to be attenuated during 120 h of treatment.

Keywords Cell viability · Catalase · Hepatic stellate cells · Lipoperoxidation · Liver fibrosis · Resveratrol · Superoxide dismutase · Oxidative stress

Abbreviations

CAT	Catalase
ECM	Extracellular matrix
HSC	Hepatic stellate cells
ROS	Reactive oxygen species
RSV	Resveratrol
SOD	Superoxide dismutase

Introduction

Resveratrol (3,5,4′,-trihydroxystilbene; RSV) is a phytoalexin that responds to environmental stresses and pathogenic infections, produced by approximately 31 plant genera, including plums, peanuts, and grapes [1]. This polyphenolic compound has long been thought to be associated with the beneficial effects of red wines and the prevention of pathologies due to the nature of its molecular targets, which include cytokines, transcription factors, cell cycle proteins, and proteins associated with metastasis. Such characteristics endow RSV with important biological properties such as chemopreventive, antiproliferative, and antioxidant activities [2]. However, many studies also suggest that RSV exerts an opposite effect, acting as a pro-oxidant agent depending on its concentration, time of exposure, and cell type [3]. This dualistic behaviour

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characterises RSV as an active redox molecule [4]. Indeed, in different cell types, such as a fibroblast cell line and tumour human cells, RSV was found to exert its cytotoxic action at higher doses [5]. Likewise, it was already discussed that RSV induces cell cycle arrest [6, 7] and stimulates the reactive oxygen species (ROS)-activated mitochondrial pathway leading to apoptosis [8].

Hepatic stellate cells (HSC) are known to play an important role in the liver fibrogenic process. In the resting state, these cells maintain a quiescent or lipocytic phenotype characterised by the presence and storage of retinol droplets in the cytoplasm. The activation of HSC is associated with continuous damage to the liver due to virus infection, metabolic disorders, or alcohol abuse, and results mostly from paracrine stimulation due changes in the surrounding extracellular matrix (ECM). The oxidative stress as well as exposure to lipid peroxides and the products of damaged hepatocytes is a determining factor for the activation state of HSC, which ultimately leads to a chronic inflammatory process. In this context, these cells develop a myofibroblastic phenotype in which they lose their lipid droplets, proliferate more rapidly, and become the main ECM producers contributing to liver fibrosis [9, 10].

Much of our understanding on the cell and molecular mechanisms of liver fibrosis has been gained through animal models and from in vitro studies employing culture model of activated HSC [7]. The GRX cell line was established through liver fibre granulomas induced by *Schistosoma mansoni* experimental infection, and this cell line is the first HSC model [11, 12]. In standard cultures, GRX cells express an activated phenotype that is an intermediate of the lipocytic and myofibroblastic phenotypes [13]. GRX cells can be induced in vitro to express a lipocytic phenotype by treatment with retinoids, indomethacin, β-carotene, or capsaicin, which stimulate lipid storage and cause a decrease in cell proliferation and ECM production [14–16]. On the other hand, GRX cells can be differentiated into an activated myofibroblastic phenotype when exposed to cytokines, growth factors, or oxidative stress conditions [17].

Clearance of activated stellate cells by apoptosis remains an appealing target as an antifibrotic therapy in chronic liver diseases, using a physiological mechanism of endogenous pathway of fibrosis regression [10]. Besides the evidences that RSV induces ROS-mediated apoptosis, our earlier studies showed that even GRX treatment with low doses of RSV (0.1–1 μM) caused cell cycle arrest in the S phase [7]. In this regard, the major aim of the present study was to investigate if the cell cycle arresting effect of RSV on GRX cells found in our previous work is accompanied by ROS production. As RSV can trigger different responses depending on its dose or time of exposure, we chose to evaluate the effects of crescent concentrations (1–50 μM) on GRX cells under treatment for 24 and 120 h. Our results revealed that RSV exerts dose-dependent prooxidant effects in GRX under 24 and 120 h of treatment. This find may be related to the GRX cell cycle arrest at low doses. Furthermore, we found that 50 μM of RSV provoked oxidative cytotoxicity, which seems to be attenuated during 120 h of treatment.

Materials and Methods

Cell Culture

The GRX cell line was obtained from the Cell Bank of Rio de Janeiro (HUCFF, UFRJ, RJ). The cells were seeded (10^5/ml or 3×10^4/ml, respectively) on 6-well culture plates (for the TBARS assay) and 24-well culture plates (Nunc, Roskilde, Denmark) 24 h before treatment with RSV. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 5 % foetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil) and 2 g/l HEPES buffer (pH 7.4) in a humidified atmosphere with 5 % CO_2 at 37 °C.

Resveratrol Treatment

Resveratrol (Sigma Inc., St. Louis, MO, USA) was dissolved in 20 μl of ethanol (Merck, Darmstadt, Germany) to a stock concentration of 100 mM and diluted sequentially in DMEM to a final concentration of 0.1, 1, 10 and 50 μM just before use. The cells were treated during 24 and 120 h. Media without or with RSV at the aforementioned concentrations were changed daily during the 120-h experiments.

Viability and Cytotoxicity Analysis

MTT Assay

The cell viability was measured by quantifying the cellular dehydrogenases activities that reduce MTT (3-4,5-dimethylthiazolyl-2,5-diphenyl-2*H*-tetrazolium bromide, Sigma Inc.) to a purple formazan salt [18]. After RSV treatment, the cells were incubated with 1 mg/ml of MTT for 2 h at 37 °C. The cells were then lysed in dimethylsulphoxide (DMSO, Sigma Inc.) and the absorbance was quantified in a 96-well microplate spectrophotometer (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA) at 570 and 630 nm.

Sulphorhodamine B Assay

The sulphorhodamine B assay is used for cell density determination and thus, is indicative for cell survival. After

RSV treatment, the cells were washed with phosphate-buffered saline (PBS) and fixed with a 4 % paraformaldehyde solution for 15 min, stained with 0.4 % sulphorhodamine B in acetic acid and finally dissolved in 1 % SDS [19]. The absorbance was measured in a 96-well microplate spectrophotometer (Spectra Max 190) at 515 nm.

The Trypan Blue Exclusion

The cell viability was also determined by the trypan blue exclusion method. Briefly, after treatment media was removed and the cells from each well were harvested by trypsin/EDTA (Sigma Inc). A 10 % solution of trypan blue (Sigma Inc.) was added and the cells were immediately counted in a haemocytometer [20]. The dead trypan blue-positive and alive negative cells were counted. Dead cells were calculated as a percentage of total cells in each group.

Oxidative Stress Analysis

Evaluation of the Intracellular Reactive Species Production

The intracellular reactive species (RS) production was analysed using the non-fluorescent and cell membrane-permeable 2′-7′-dichlorofluorescein diacetate (DCFH-DA, Sigma Inc.). DCFH-DA is hydrolysed by intracellular esterases and then oxidised by RS to a fluorescent, polar and non-permeable 2′-7′-diclorofluorescein (DCF) compound [21]. After treatment with RSV, the cells were incubated in the dark with 10 μM DCFH-DA in DMEM without serum for 30 min at 37 °C. The cells were washed and scraped with 0.2 % Triton X-100 (Sigma Inc.). The fluorescence was measured in a 96-well microplate reader (Spectra Max Gemini XPS, Molecular Devices) after exciting at 485 nm, and collecting the emission at 520 nM. The results were expressed as fluorescence units per μg of proteins, which were quantified by the Lowry's modified assay [22].

Antioxidant Enzyme Activity Quantification: Superoxide Dismutase and Catalase

The superoxide dismutase (SOD) activity was quantified by inhibiting the superoxide-dependent adrenaline auto-oxidation, and reading the absorbance at 480 nm [23]. The results were expressed as units of SOD per mg of protein. The catalase (CAT) activity was quantified by evaluating the decrease in H_2O_2 absorbance and reading the absorbance at 240 nm [24]. The results were expressed as units of CAT per mg of protein. Both experiments were performed in a 96-well microplate spectrophotometer (Spectra Max 190).

Quantification of Cellular Thiol (–SH) Content

The cellular free thiols (–SH) were analysed using the sulphhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), also referred as Elmann's reagent, which estimate the oxidative alterations of proteins and non-protein peptides. This analysis is based on the reaction of free thiols with DTNB resulting in mixed disulphide and 2-nitro-5-thiobenzoic acid (TNB), which is quantified by the absorbance of TNB anion at 412 nm [25]. Briefly, after treatment with RSV for 24 and 120 h, cells were collected in 0.1 % of sodium dodecyl sulphate (SDS) and 0.01 M of DTNB diluted in ethanol, and then incubated at room temperature for 20 min. The concentration of –SH was calculated using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}. The absorbance was measured at 412 nm. Results were expressed as μM of –SH per mg of protein.

Quantification of Thiobarbituric Acid Reactive Species (TBARS)

Lipoperoxidation was measured as described by the TBARS method [26]. Briefly, after each treatment, the cells were washed and scraped with Tris buffer (0.05 mM, pH 7.6) and proteins were precipitated with 10 % trichloroacetic acid. The cell homogenate was mixed with 0.67 % thiobarbituric acid (TBA) and boiled at 100 °C for 30 min. The samples were centrifuged at 3,000 rpm, the lipid supernatant was extracted with butanol, and its absorbance was measured in a spectrophotometer at 512 nm. The concentration of TBARS was calculated using a molar extinction coefficient of 153,000 M^{-1} cm^{-1}. The results were expressed in μM TBARS per mg of protein.

Cell Proliferation Analysis

The [^{3}H] Thymidine Incorporation Assay

The cells were incubated during the last 24 h of each treatment with 1 μCi/ml of [6-^{3}H] thymidine (specific activity 23.0 Ci/nmol, Amersham Biosciences, Hillerod, Denmark). Then, the cells were washed with PBS, and 10 % trichloroacetic acid was added to each well for 1 h. The pellet was dissolved with 0.1 N NaOH, and the incorporated radioactive DNA was determined by scintillation counting and expressed as cpm per mg of protein [27].

The Cell Population Doubling Calculations

After RSV treatment, the incubation media were removed, the cells were tripsinized and the viable cells were immediately counted in a haemocytometer, as described before. The population doublings were calculated for each group as follows: cell population doubling = [log (post-treatment cells number/initial cell number)]/log 2, as previously described [28].

Statistical Analysis

The data were expressed as mean ± standard error of the mean. One-way ANOVA was used to analyse the effect of RSV treatment. When necessary, two-way ANOVA was used (using the RSV doses and time of treatment as factors) and post-hoc Duncan multiple range test was performed. Results were considered statistically different when the p values were equal to or less than 0.05.

Results

The Effects of Resveratrol on the GRX Cell Viability

To initiate our studies, we wanted to know if different doses and times of RSV treatments would have any effect in cell viability (Fig. 1). While low doses of RSV did not affect the GRX viability, the higher dose (50 μM) significantly decreased the cell viability (Fig. 1a), reducing considerably cell survival and population density (Fig. 1b, c). However, our attention was drawn to the fact that the percentage of trypan blue-positive cells in the 50 μM RSV group was higher only at 24 h, indicating increase in cellular membrane damage only in this treatment time condition (Fig. 1d). Interestingly, treatment with doses greater than 50 μM of RSV (75–100 μM) resulted in death of all cells along the established times of treatment (Data not shown). Altogether, these results confirmed the dose-dependent effect of RSV regarding the survival/viability of GRX cells, and were in accordance with the previous studies that showed the cytotoxic effect of RSV related to the dose of treatment [3].

The Effects of Resveratrol on Reactive Species Production

Previous works had shown that hydrogen peroxide plays an important role by inducing cell cycle arrest and inhibition of cell proliferation [29, 30]. Likewise, RS may play an important role in elevating numbers of non-viable or damaged cells via the induction of oxidative stress [31]. Based on this premise and considering the fact that RSV induced GRX cell cycle arrest [6, 7], we sought to determine if RS was also present at these doses of RSV treatment. Treatment for 24 h with doses between 1 and 50 μM of RSV triggered a dose-dependent pro-oxidant effect on GRX. A similar result was found on GRX treated for 120 h, in which RSV also promoted pro-oxidant effects since the lowest dose (0.1 μM). A two-way ANOVA demonstrated that the time of treatment [$F(1,38) = 248.0$; $p \leq 0.05$] and the RSV dose [$F(4,38) = 17.3$; $p < 0.05$] increased RS production in a dependent manner [$\beta 24h = 0.71$, $\beta 120h = 0.61$, $p \leq 0.05$]. Also, the two-way ANOVA analysis revealed an interaction between the time of treatment and RSV dose [$F(4,38) = 5.7$; $p \leq 0.05$] because the increase of the DCF fluorescence provoked by the RSV was higher in the 120-h treatment (Fig. 2).

The Effects of Resveratrol on the Enzymatic Antioxidant Activities: Superoxide Dismutase and Catalase

Since a significant increase in the RS production was induced by the RSV in both treatment times, we assessed the activities of the main enzymatic antioxidants defences. Interestingly, the analysis of SOD activity in each treatment period revealed an opposite effect of RSV. While cells treated for 24 h with 1–50 μM of RSV presented a decrease of SOD activity, all cells treated for 120 h presented an increase in this enzyme activity, thus revealing an interaction between the time of treatment and RSV dose [$F(3,33) = 13.76$; $p \leq 0.05$]. The determination of the SOD activity in the untreated (control) group revealed that the SOD activity was decreased with the culture time. A two-way ANOVA demonstrated that the SOD activity was dependent on the treatment time [$F(1,33) = 80.19$; $p \leq 0.05$] and on the RSV dose [$F(4,33) = 7.73$; $p \leq 0.05$] (Fig. 3a). Although the highest dose of RSV has visibly decreased the CAT activity, there was no statistical difference in the enzyme activity in cells treated with all RSV concentrations for 24 h. However, compared to the control group, treatment with 50 μM of RSV for 120 h was able to significantly decrease CAT activity ($p < 0.05$) (Fig. 3b). Besides these effects on the SOD and CAT activities, we found a significant increase in the SOD/CAT ratio when compared with the control (control = 1) only in the cells treated with 50 μM RSV for 120 h (Fig. 3c).

The Effects of Resveratrol on the Oxidation of Protein and Non-protein Peptides

The oxidation or reduction of thiol (–SH) content of proteins or non-protein peptides, such as glutathione (GSH), cysteine and homocysteine, is a dynamic and reversible process by which cells can modulate its redox state

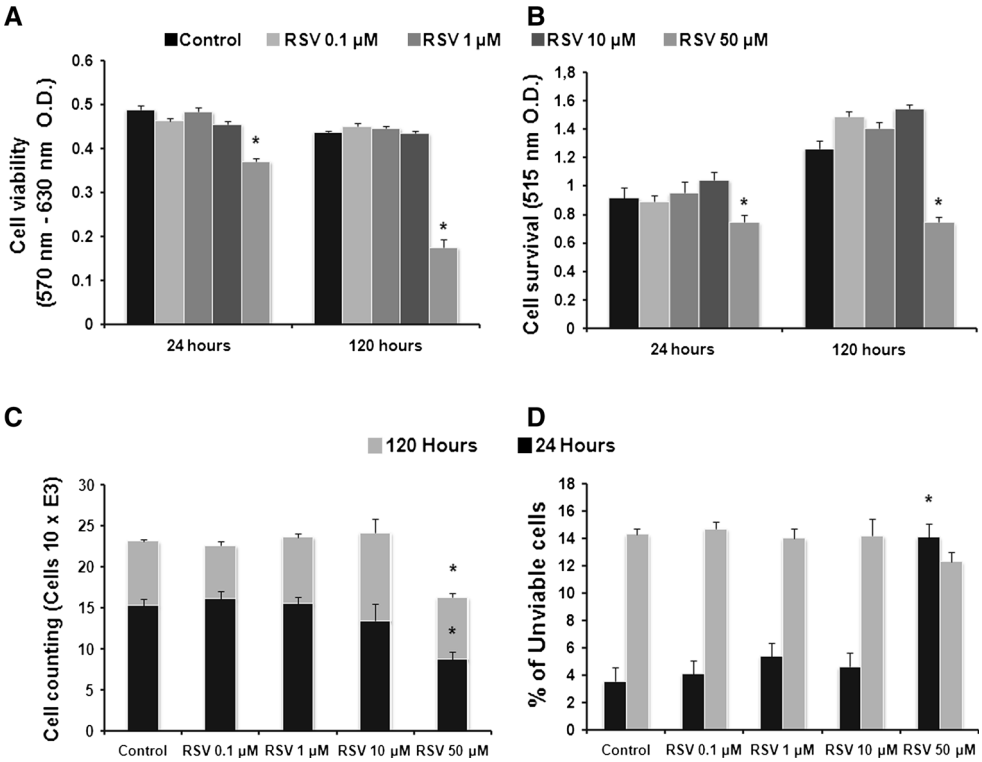

Fig. 1 The effect of resveratrol on cell viability. GRX cells were treated with 0 (control), 0.1, 1, 10 and 50 μM of RSV for 24 or 120 h. The cell viability and survival were established through MTT (**a**) and Sulphorhodamine B (**b**) assays and by Trypan Blue exclusion counting (**c**). The percent of unviable cells was calculated (**d**). *Asterisk* indicates significant differences between groups ($p \leq 0.05$). Data are expressed as mean ± SEM. ($n = 4$)

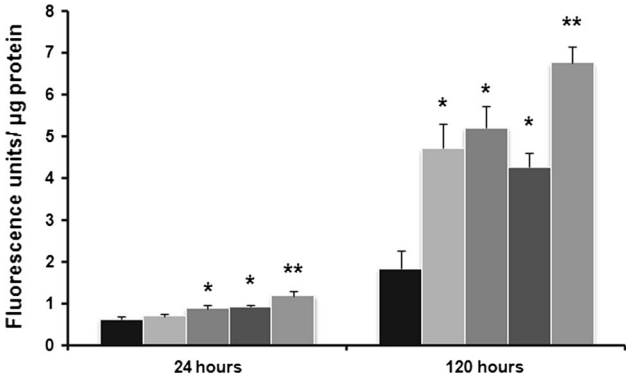

Fig. 2 The effect of resveratrol on the ROS production. GRX cells were treated with 0 (control), 0.1, 1, 10 and 50 μM of RSV for 24 or 120 h and the intracellular ROS production was detected using the non-fluorescent and cell membrane-permeable 2′-7′-dichlorofluorescein diacetate (DCFH-DA). The data were expressed as fluorescence units per μg of protein expressed as mean ± SEM. ($n = 4$). *Single asterisk* statistically significant differences from the control. *Double asterisk* represents significant differences between the 50 μM of RSV-treated group and the others (the control and RSV groups treated with lower doses) at 24 and 120 h ($p \leq 0.05$)

[32, 33]. However, despite the pro-oxidant effects that were accompanied mainly by changes in SOD activity, treatment with RSV did not induce thiols oxidation in GRX. In contrast, after 24 h of treatment, only cells that received lower doses of RSV, i.e. 0.1 and 1 μM, showed an increase of reduced thiols. At 120 h, only cells that received 50 μM of RSV showed an increase of reduced thiols (Fig. 4a).

The Effects of Resveratrol on Lipid Peroxidation

Lipid peroxidation damage occurs as a consequence of ROS over-production and cell antioxidant defences failure. As such, the malondialdehyde production was quantified by the TBARS method as an index of lipid peroxidation, which is related to the cell plasma membrane damage and cell death [34]. A two-way ANOVA demonstrated that the TBARS generation was dependent on the treatment time [$F(1,26) = 14.69$; $p \leq 0.05$] and on the RSV dose [$F(3,26) = 50.34$; $p \leq 0.05$]. There was an interaction between time of treatment and the RSV dose [$F(3,26) = 37.06$; $p \leq 0.05$] for the TBARS generation because 50 μM of RSV provoked a higher increase of lipid peroxidation at 24 h (Fig. 4b).

The Effects of Resveratrol on Cellular Proliferation

RSV is known to be an antiproliferative compound. We found a considerable decrease in the cell density in 50 μM-treated cells related to cytotoxicity, which may be also related to a diminished proliferation. Thus, we evaluated the RSV effects on cell proliferation. After 24 h of treatment, we observed that 50 μM of RSV promoted a decrease in the thymidine incorporation. Interestingly, in 120 h, although all groups have decreased the rate of cellular proliferation during the culture time, cells that received 50 μM of RSV continued to proliferate as

indicated by the significant increase in thymidine uptake (Fig. 5a). The proliferation ratio between 120 and 24 h (Fig. 5b) and the calculation of cell population doublings (Fig. 5c) confirmed these data.

Discussion

Molecular oxygen and its reactive derivatives play an important role in several metabolic processes of aerobic organisms. Nevertheless, they are paradoxically also related to the development of several diseases, including liver fibrosis [34]. RSV is well known because of its favourable biological effects that involve its chemopreventive and antioxidant capacity; however, recent studies have demonstrated that RSV may behave as a pro-oxidising agent [3].

The GRX cells correspond to the first cell line representing activated HSC [12]. Several studies suggest oxidative stress to be one of the factors that induce liver fibrosis through HSC activation [10, 35]. In this context, RSV can interfere in the molecular mechanisms of HSC that regulate phenotypical modulation, as well as liver fibrosis development [9, 35]. On the other hand, RSV has been previously shown to trigger apoptosis [8], which is well accepted as liver fibrosis resolution [10]. In GRX cells, treatment with small doses of RSV (0.1–1 μM) resulted in growth inhibition [7]. In this work, we aimed to investigate if this cell cycle arrest effect of RSV on GRX cells is accompanied by ROS production. As RSV has been described as a cytotoxic molecule at high doses and depending on the time of exposure, we also evaluate the viability and proliferation of GRX cells under treatment with crescent doses (0.1–50 μM) for 24 and 120 h.

In our experimental model, all results regarding viability were consistent with a decrease of living cells in response to the treatment with 50 μM of RSV. Interestingly, although our two RSV treatment times resulted in increased amounts of TBARS in the groups that received 50 μM, it should be noted that at 24 h, the lipid peroxidation was about three times higher than at 120 h. It has been suggested that lipid peroxidation might proceed in the cells' nuclear membranes close to chromosomes. The loss in the nuclear membranes integrity may make the circumstance suitable for oxygen radicals to attack chromatin and DNA. In this way, RS induce cell death by apoptosis, also providing lipid peroxidation and DNA damage. Furthermore, the increase in lipid peroxidation is also associated with the rupture of the external mitochondrial membrane and consequently, the activation of necrosis [36–39]. Considering the possible role of lipid peroxidation towards the induction of cell death, the diminished level of TBARS found in the 50 μM RSV group treated for 120 h could be directly related to the decrease in the percentage

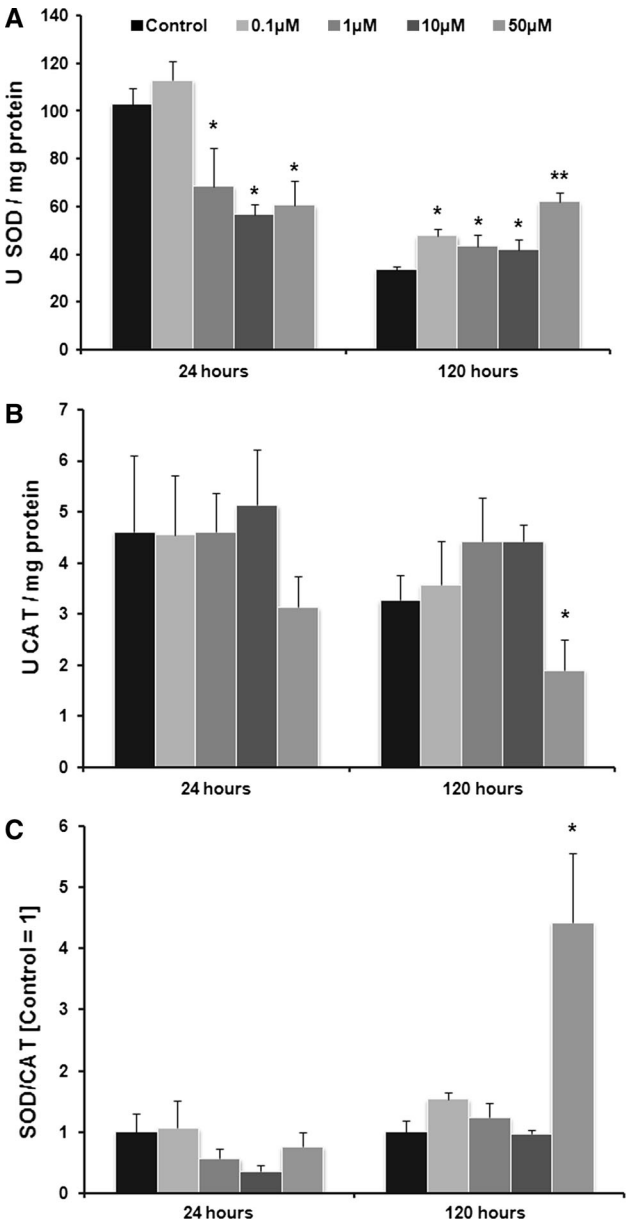

Fig. 3 The antioxidant enzyme activities. GRX cells were treated with 0 (control), 0.1, 1, 10 and 50 μM of RSV for 24 or 120 h and the SOD (**a**) and CAT (**b**) activities were determined. **c** The SOD/CAT ratio. Data are expressed as mean ± SEM. ($n = 4$). *Single asterisk* indicates statistically significant differences from control. *Double asterisk* represents significant differences between the 50 μM of RSV-treated group and the others (the control and RSV groups treated with lower doses) at 120 h ($p \leq 0.05$)

of unviable cells, thus indicating a lesser cytotoxic effect in these cells.

The imbalance between the RS production and cellular antioxidant defences may lead to lipid peroxidation and cytotoxicity, which are related to the cell damage by oxidative stress and cell death [34, 36, 37]. Indeed, the apoptotic intrinsic pathway is often activated in response to cell stress or damage due to the exposure to cytotoxic drugs

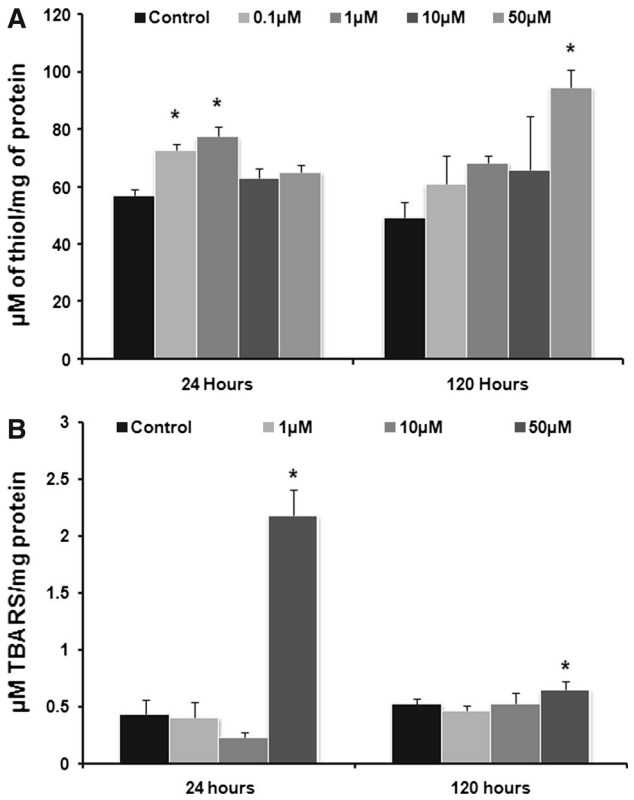

Fig. 4 The redox status of protein and non-protein compounds and the lipid peroxidation. GRX cells were treated with 0 (control), 0.1, 1, 10 and 50 μM of RSV for 24 or 120 h. The protein or non-protein redox status was measured by the determination of these compounds' reduced thiols, using Elmann's reagent (**a**). The lipid peroxidation was measured by the determination of thiobarbituric acid reactive species (TBARS) (**b**). Data are expressed as mean ± SEM. ($n = 4$). *Single asterisk* indicates statistically significant differences between groups ($p \leq 0.05$)

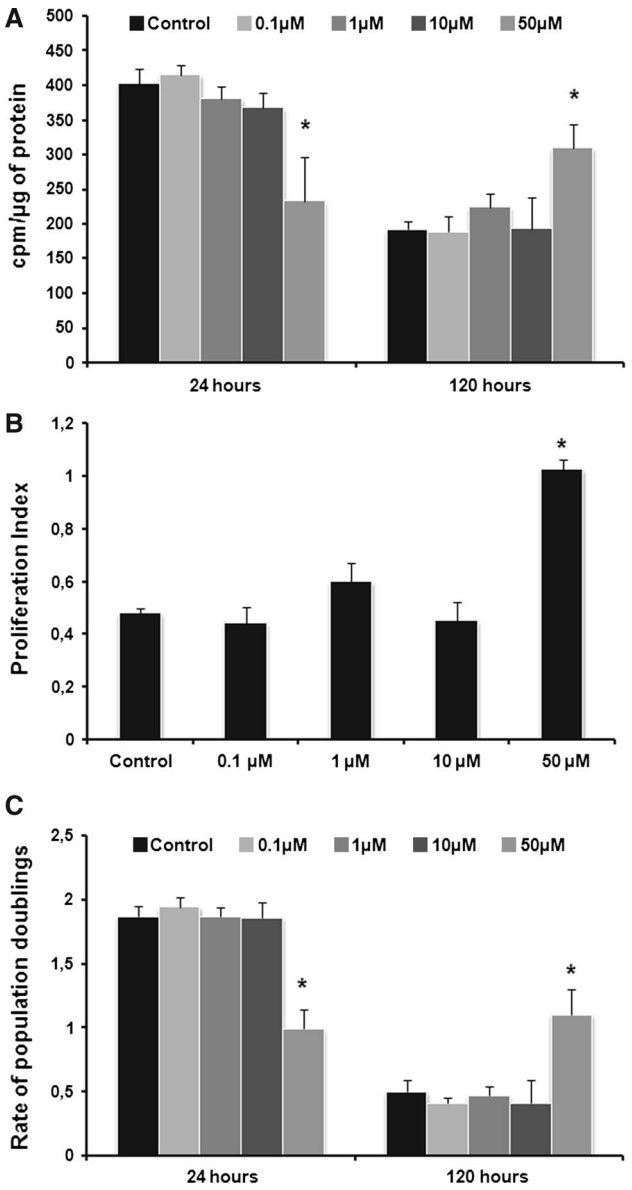

Fig. 5 The effect of resveratrol on the GRX cell proliferation. GRX cells were treated with 0 (control), 0.1, 1, 10 and 50 μM of RSV for 24 or 120 h, and the proliferation was measured by the [^{3}H]-thymidine incorporation assay (**a**). The ratio between the [^{3}H]-thimidine uptake at 120 and 24 h indicated that GRX cells treated with 50 μM of RSV remain proliferative (**b**). The calculation of the rate of population doublings corroborated these data (**c**). Data are expressed as mean ± SEM. ($n = 4$). *Single asterisk* indicates statistically significant differences between the 50 μM of RSV-treated group and the other groups ($p \leq 0.05$)

such as RSV. This situation results in mitochondria dysfunction, leading to an excessive production of RS that may first damage the organelles' membranes, which leads to the cytochrome c release into cell cytosol resulting in the caspases activation [40–43]. Interestingly, some studies also reported that the sensitivity of cells to apoptosis induction is increased upon decreasing the intracellular concentration of superoxide (O_2^{-}), which results in the cytosol acidification [44–46]. Also, the decreased levels of intracellular O_2^{-} could be related to an increase of hydrogen peroxide (H_2O_2) through SOD activity or to an increase of peroxynitrite ($ONOO^{-}$) through its reaction with nitric oxide. Consequently, H_2O_2 can be an important source for $^{\bullet}OH$ formation via the Fenton reaction or can oxidise cardiolipin, a structural mitochondrial lipid, contributing to the release of cytocrome c and the initiation of mitochondrial-mediated apoptosis. Likewise, the excessive $ONOO^{-}$ levels in mitochondria can also impair the MnSOD function, leading to this enzyme activity decrease [47–49].

Our results regarding the oxidative status of GRX showed that RSV triggers dose- and time-dependent increase of RS and mainly alters SOD activity. However, the two times of treatment with RSV triggered a paradoxical effect in this enzyme activity. Indeed, the imbalance between the increased RS production and the diminished SOD activity found at 24 h of treatment could

be harmful to cells since that excessive O_2^- can be an important source of $ONOO^-$ through reacting with nitric oxide [34]. On the other hand, another plausible explanation for this situation is that decreasing SOD activity could be owed to the consumption of this enzyme in converting O_2^- to H_2O_2. In this context, the increased production of H_2O_2 along with the possible production of $ONOO^-$ in GRX could lead to a diminished level of O_2^-, contributing to the decrease of SOD activity and to the increase of oxidised DCF. Interestingly, our attention was drawn to the fact that, although not statistically different, the visible decrease in the CAT activity induced by the highest dose of RSV can contribute to the high cytotoxicity found in this treated group at 24 h of treatment. On the other hand, in spite of the diminished SOD activity found in cells treated with 1 and 10 μM of RSV, the unchanged CAT activity in these groups may be important for protecting cells through regulating the possible increase on the levels of H_2O_2.

The increase of RS production in all cells treated with RSV for 120 h was continuous. However, in spite of the time-dependent decrease of the SOD activity in all groups including control, we found that RSV significantly prevents this decrease. In this sense, the smaller decrease in this enzyme activity, which was more significant in the 50 μM-treated cells, could be related to an increase on the O_2^- production along the 120 h of treatment and could be an important cell defence mechanism, contributing to a lower cell damage in the long-term treatment. Interestingly, treatment with 0.1–10 μM of RSV did not change CAT activity, and this fact may also be important for regulating the levels of intracellular H_2O_2. However, the highest dose of RSV decreased the CAT activity. In this sense, the SOD/CAT ratio has also been referred to as an important factor in the establishment of the redox state of cells. Furthermore, the deleterious consequences of the overproduction or excessive activity of SOD were already reported [48]. Since we discovered an imbalance favouring SOD activity in relation to CAT activity in the cells treated with 50 μM RSV, we speculate that this situation could also be related to a possible increase of H_2O_2 in this group.

Thiol-based redox couples are important compounds for regulating developmental events and are closely linked to changes in the intracellular redox potential. Among these, glutathione (GSH) is the most prevalent cellular thiol that plays an essential role in preserving a reduced intracellular environment, and thus guarding cells against oxidative injury. Indeed, glutathione mostly exists in its reduced form and can be oxidised by H_2O_2 in a reaction catalyzed by glutathione peroxidase, producing glutathione disulphide (GSSH) and water. Furthermore, GSH can scavenge reactive nitrogen species such as peroxynitrite ($ONOO^-$) with or without the help of glutathione peroxidase [50–53]. Interestingly, some studies reported that RSV can trigger an increase of GSH levels and an increase in the activity of glutathione reductase and glutathione peroxidise [54–56]. Curiously, our attention was drawn to the fact that RSV did not induce oxidation of thiols compounds at both treatment times. In contrast, cells treated with the lower doses of RSV for 24 h showed an increase of reduced thiols, and this fact suggests a cellular protective effect against the intracellular pro-oxidant environment induced by RSV. Interestingly, there was an increase of reduced thiols in the cells treated with 50 μM of RSV for 120 h. This situation could be important to these cells for counterattacking the more prominent pro-oxidant effects of this dose of RSV contributing to the balance between RS production and cellular antioxidant defences. In this way, the increase in the reduced intracellular thiol content could compensate the decrease in the CAT activity and could contribute to the reduction of lipid peroxidation and to the reduction of the percentage of unviable cells on this group at this treatment time.

As expected, treatment for 24 h with 50 μM RSV resulted in decreased [^{3}H] thymidine incorporation when compared to the other groups. Paradoxically, we observed at 120 h of treatment an increase in the [^{3}H] thymidine incorporation. The calculation of the population doubling revealed that the surviving cells of the first 24 h of treatment continued to proliferate. Interestingly, lipid peroxidation is very low or totally missing in quickly proliferating tissues such as tumours. This fact suggests that an increase of lipid peroxidation may contribute to the cell proliferation decrease [36]. Thus, these results regarding the GRX proliferation could be interrelated to that found in the TBARS assay, since the treatment with 50 μM of RSV triggered a greater lipid peroxidation at 24 h that was significantly attenuated at 120 h. In this way, the decreasing cell number showed by MTT, sulphorhodamine B, and live-trypan cell counting results found at 120 h could represent the cells that survived the significant cytotoxicity of the first 24 h of treatment, remaining viable and proliferative. Beside this, the smaller confluence after 24 h of treatment with 50 μM of RSV could also be a reason for the continuous proliferation until 120 h of treatment.

The antioxidant potential of RSV has been widely discussed because of its RS-scavenging capacity [3, 57]. However, another plausible hypothesis can be the RSV's capacity to induce a series of signals leading to an upregulation of the cell antioxidant defence systems. Recent evidence suggests that RSV could act as a signalling molecule within tissues and cells in modulating the expression of genes and proteins through the activation of redox-sensitive intracellular pathways. In this regard, the cellular tolerance to the oxidative environment could be related to some gene expression alterations as well as to an

increase in the synthesis and action of antioxidant defence systems that could result in cell survival and adaptation [58–60]. On the other hand, it was already discussed that there is an interesting correlation among the pro-oxidant activities and cytotoxicity of dietary polyphenols, such as RSV. Furthermore, it has become a consensus that every antioxidant is in fact a redox agent and thus might become a pro-oxidant, accelerating lipid peroxidation and/or inducing DNA damage under special conditions. In this way, it has been proposed that such pro-oxidant action could be an important action mechanism of RSV anticancer and apoptotic-inducing properties [3].

The current literature shows numerous targets for RSV; thus, the molecular actions of this phytoalexin have not yet been unequivocally determined. In this way, the possible capacity of RSV in modulating different pathways can result in several and opposite biological effects, depending on its concentration or treatment time [61, 62]. In this sense, it was already discussed that three distinct cellular responses appear to result from exposure to polyphenols such as RSV, with each response dependent upon the concentration and pro-oxidant nature of these polyphenols: (a) a mild exposure causes mild oxidative stress and thereby ignites cellular antioxidant defence systems; (b) an intermediate to high exposure gradually overwhelms the antioxidant defence systems and induces apoptotic cell death; and (c) a very high exposure quickly overwhelms the cellular antioxidant defences and causes oxidative damage leading to cell death by necrosis [63]. Furthermore, these controversial effects of RSV treatment reported by several studies were recently related to the *hormesis* concept [61, 64, 65], which refers to a favourable biological response to low and harmless doses of some chemical compounds that could initiate an adaptive stress response that renders cells/organism resistant against high (and normally harmful) doses of the same agent, allowing the stressed cells to avoid death or damage [61, 66].

The treatment with antioxidant molecules could be interesting as a preventative strategy for liver fibrosis development because the oxidative balance plays an important role in HSC activation [10]. Despite the wide discussion about the antioxidant properties of RSV, it was already reported that RSV can lead to DNA damage as well as a reversible or irreversible interruption of the cell cycle mediated by a pro-oxidant effect [60]. Along with the possibility that the pro-oxidant effect of RSV can trigger a reversible interruption of the cell cycle, our attention was drawn to the paradoxical effect of this phytoalexin on SOD activity. Taking into account that RSV could induce apoptosis and the role of intracellular O_2^- towards this form of cell death [44, 45], we speculate that the intracellular concentration of O_2^- could interfere in SOD activity. In this way, it is possible that even low doses of RSV (from 1 μM) can be more harmful to cells under 24 h of treatment since this condition can favour apoptosis. However, although RSV also triggers a pro-oxidant effect in cells treated for 120 h, the RS production at this treatment time could be more balanced and could be more effectively counterattacked by cellular antioxidant defences; thus, these facts could be important for the cell resistance against oxidative stress.

In summary, our findings showed that even low doses of RSV triggered a pro-oxidant effect in GRX cells and this fact may be related to the arrest cycle found in our previous work. However, although a dose-dependent pro-oxidative effect of RSV leading to oxidative stress in the cells treated with 50 μM was shown, the less expressive cytotoxicity found in this group at 120 h of treatment could suggest that the surviving cells seemed to be more resistant to the RSV-induced damage, which seemed to be attenuated with time of treatment. Nevertheless, more studies are necessary to better understand these paradoxical effects of RSV regarding the SOD activity and the time-dependent cell damage reduction. In the same way, the need for elucidating the additional targets of RSV in these cells, including other antioxidant defences, cell death pathways or cell pro-survival mechanisms, has become evident to evaluate the real benefit of this phytoalexin towards the prevention of HSC activation or liver fibrosis resolution through an effective induction of apoptosis in the activated HSC.

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II.2 THE INTERPLAY BETWEEN APOPTOSIS, MITOPHAGY, AND MITOCHONDRIAL BIOGENESIS INDUCED BY RESVERATROL CAN DETERMINE ACTIVATED HEPATIC STELLATE CELLS DEATH OR SURVIVAL

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THE INTERPLAY BETWEEN APOPTOSIS, MITOPHAGY, AND MITOCHONDRIAL BIOGENESIS INDUCED BY RESVERATROL CAN DETERMINE ACTIVATED HEPATIC STELLATE CELLS DEATH OR SURVIVAL

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ABSTRACT

Resveratrol has been the focus of numerous studies reporting opposite effects that depend on its concentration. The GRX is an activated hepatic stellate cells model used to study liver fibrosis development and resolution. We recently showed that GRX treatment with RSV (0.1 to 50 µM) for 24 hours triggered dose-dependent pro-oxidant effects, resulting in cytotoxicity and cell damage only at the highest concentration. Here, we evaluated whether the pro-oxidant effect of resveratrol treatment is accompanied by alterations on the GRX mitochondrial metabolism, and whether the concomitantly autophagy/mitophagy induction can influence on cell death or survival. We demonstrated that all concentrations of resveratrol promoted increasing on GRX cell death signals, altering the mitochondrial dynamics and function. Cells treated with all resveratrol concentrations presented higher autophagy/mitophagy features, but only treatments with 1 and 10 µM of resveratrol induced mitochondrial biogenesis. Since cell damage was higher and there was no mitochondrial biogenesis in GRX treated with 50 µM of resveratrol, we suggest that these cells failed to remove and replace all damaged mitochondria. In conclusion, the cytotoxic effect of resveratrol that effectively promotes cell death could be related to the interrelation between the concomitant induction of apoptosis, autophagy/mitophagy, and mitochondrial biogenesis in GRX.

Keywords: Autophagy, cell death; cytotoxicity; GRX; hepatic stellate cells; liver fibrosis; mitophagy; resveratrol.

1. INTRODUCTION

Resveratrol (3,5,4'-trihydroxystilbene; RSV) is a flavonoid characterized as a phytoalexin, an anti-infectious compound produced by several plant species, including peanuts and grapes, in response to pathogenic infection and environmental stresses [1]. This polyphenolic compound has been associated to beneficial effects in normal cells through its chemoprevention activity [2-3] among which: the induction of neuroprotection under adverse conditions such as oxygen/glucose deprivation [4], cardioprotection by preventing coronary heart diseases or damage to cardiac tissue due to ischemia [3, 5], and protection to pancreatic β cells contributing to an anti-diabetic effect [6]. These RSV effects are usually associated to its anti-inflammatory, anti-apoptotic, and antioxidant activities [3]. Nonetheless, RSV can also exert cytotoxicity through modulation of several pathways and induction of different mechanisms of cell death and growth inhibition, which may be accompanied by reactive species production [3, 7]. This RSV capacity can be related to its beneficial effects on the treatment of several types of carcinogenic cells and models [8]

Hepatic stellate cells (HSC) are known to play an important role in liver wound healing through fibrogenic regulation. In the resting state, these cells maintain a quiescent or lipocyte phenotype characterized by the presence and storage of retinol droplets in the cytoplasm. Changes in the surrounding extracellular matrix, as well as exposure to lipid peroxides and the products of damaged hepatocytes, as a consequence of liver injuries, lead to an inflammatory process followed by the activation of HSC. Once activated, these cells display a myofibroblastic phenotype in which they lose their lipid droplets and proliferate more rapidly. Under acute inflammatory response, HSC protect hepatocytes from damaged areas, favouring

the repair of injury tissue and promoting the restoration of hepatic homeostasis. However, under chronic inflammatory response, liver may not recover its homeostatic balance, and thus HSC become the main drivers of liver fibrosis development by promoting an excessive production of extracellular matrix that result in liver fibrosis, which in turn entails changes in the organ architecture, leading to cirrhosis and liver cancer. Therefore, strategies that decrease the stimuli leading to HSC phenotypic modulation in chronic liver injuries play an important role in preventing this pathological state. Likewise, inhibition of proliferation and clearance of activated HSC by apoptosis remains the major antifibrotic therapy in chronic liver diseases [9-11].

The GRX cell line was the first established model for the study of HSC phenotype modulation [12]. These cells were obtained from livers of C3H/HeN mice that were infected by transcutaneous penetration of *Schistosoma mansoni* cercariae [13]. In standard cultures, GRX cells express an activated feature [14]. Our early studies regarding the RSV effects in GRX showed that even at low doses (0.1 to 1 µM), this phytoalexin inhibits GRX growth by arresting cell cycle at the S phase [15], a positive effect towards the resolution of liver fibrosis [16]. We recently demonstrated that treatment for a 24-hour period at doses between 1 and 50 µM triggered a dose-dependent pro-oxidant effect in GRX cells that was accompanied by a decreased activity of superoxide dismutase. However, only treatments with 50 µM of RSV were found to be considerably cytotoxic to GRX, triggering cell oxidative damage by lipoperoxidation and decreased proliferation that results in a decreased number of living cells. Interestingly, the pro-oxidant effects of RSV seemed to be better counterattacked and the highest dose was less harmful to GRX after 120 hour of treatment, when surviving cells showed less

damage by lipoperoxidation and remained proliferative. These paradoxical results suggest a possible adaptive mechanism in which GRX cells could display resistance against the pro-oxidant and cytotoxic effects of RSV [17].

Damage to the mitochondria or more catastrophic stresses can trigger the main signalling pathways of programmed cell death such as intrinsic/extrinsic apoptosis and regulated necrosis [18]. In addition, the cell redox state plays an important role in apoptosis since that reactive species produced by mitochondria can be involved in cell damage and death [19]. On the other hand, autophagy seems to be one of the crucial cellular response against stress situations, and may contribute for cell adaptation and survival in adverse conditions [20]. Particularly, mitochondrial autophagy (also referred as *mitophagy*) appears to be a useful mechanism by removing the impaired mitochondria, thus avoiding the reactive species overproduction and this organelle-mediated cell death [18, 21]. Likewise, mitochondrial dynamics and biogenesis must be important synchronized events, contributing to maintain both the organelles integrity as its appropriated number, respectively. Hence, the cross-talk between apoptosis,autophagy/mitophagy, and mitochondrial dynamics/biogenesis seems to be critical to the overall fate of cells towards death or survival [22].

In this study, we aimed to evaluate whether the pro-oxidant effect of RSV on the GRX cells, found after 24 hour of treatment in our previous study, can be accompanied by cell death signalling and mitochondrial metabolism alterations. It was also investigated the RSV effects on inducing autophagy and mitophagy, considering these events as an important mechanism by which this phytoalexin could regulate cell death or survival. Here, we found that RSV mainly triggered caspase-mediated apoptosis, indicating an alteration on the mitochondrial function

of GRX. However, the cytotoxic effect of RSV that effectively promotes cell death was dependent on the interrelation between the concomitant induction of apoptosis, autophagy/mitophagy, and mitochondrial biogenesis.

2. MATERIALS AND METHODS

2.1 Cell culture and Resveratrol treatment

The GRX cell line was obtained from the Cell Bank of Rio de Janeiro (HUCFF, UFRJ, RJ). The cells were seeded (10^5/cm^2 or 3 x 10^4/cm^2, respectively) on 6-well culture plates or 12-well culture plates (Corning, Tewksbury, MA, USA). For transmission electron microscopy analysis, cells were cultured (10^6/ml) on 25 cm^2 culture plastic flasks (Corning); for laser-scanning confocal microscopy, cells were seeded under sterile coverslips placed on 12-well culture plates. The 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% of CO_2 at 37 °C. Resveratrol (Sigma, St. Louis, MO, USA) was dissolved in 20 µL of ethanol (Merck, Darmstadt, Germany) to a stock concentration of 100 mM and diluted sequentially in DMEM until reach a final concentration of 1, 10, and 50 µM. DMEM plus ethanol, in a quantity equal to that found in the medium containing the highest RSV concentration, was used to treat control cells. GRX was treated or not during 24 hours and then submitted to the analysis.

2.2 RNA extraction, cDNA synthesis and Real-time PCR

The RNA was isolated using TRIzol Reagent (Invitrogen). RNA was quantified using the BioPhotometer Plus (Eppendorf, Hamburg, Germany) to measure the absorbance at 260 nm relative to that at 280 nm. It was added 2 µg of total RNA to each cDNA synthesis reaction, using SuperScript®-III RT First-Strand Synthesis

SuperMix (Invitrogen). The gene sequence information was collected from databases (www.ncbi.nlm.nih.gov and www.ensembl.org). The specifics primers for each gene were designed using IDT Design Software (Integrated DNA Technologies Inc., USA), and care was taken to avoid primers that could generate secondary structures. For mitochondrial dynamics analysis [25],primers for the following genes were used: mitofusin 1 (MNF1), mitofusin 2 (MNF2), optic atrophy protein 1 (OPA1), dynamin-related protein 1 (DRP-1), and mitochondrial fission 1 protein (FIS1). For mtDNA quantification, the primer for mitochondrial NADH dehydrogenase subunit 1 (ND1) was used [26]. Along with the autophagic parameters evaluation, it was also quantified the mRNA expression of autophagy-related protein 7 (ATG7). The primer for β-2-microglobulin was used as the internal control gene for all relative expression calculations. All primer sequences are listed in Table 1. Real-time PCR reactions were carried out in a Step One Plus real-time cycler (Applied-Biosystem, New York, NY, USA) and performed in triplicates. Reaction settings were composed of an initial denaturation step of 5 min at 94°C followed by 40 cycles of 10 s at 94°C, 15 s at 60°C, 15 s at 72°C and 35 s at 60°C for data acquisition; samples were kept for 2 min at 40°C for annealing and then heated from 55 to 99°C at a rate of 0.1°C/sec to produce the denaturing curve of the amplified products.Real-time PCR was carried out in a 20 µl final volume, composed of 1 µl of each cDNA diluted 1 to 10 times, 2 µl of 10X PCR buffer, 1.2 µl of 50 mM $MgCl_2$, 0.2 µl of 5 mMdNTPs, 0.8 µl of 10 µM primer pairs, 12.75 µl of water, 2.0 µl of SYBR green (1:100,000; Invitrogen), and 0.05 µl of Platinum Taq DNA polymerase (5 U/µl) (Invitrogen).The specificity of amplification and absence of primer dimers were confirmed using melting curve

analysis at the end of each run.All results were analyzed by the $2^{-\Delta\Delta CT}$ method [27].

2.3 Western blotting analysis

For western blotting analysis, control and treated cells were lysed in Tris-HCl buffer (50 mM, pH 6.8) with 2% SDS, 10% glycerol and 2- β-mercaptoethanol (200 mM). Equal amounts of protein, previously measured according to Peterson [28], were separated on 10% SDS–PAGE, transferred to nitrocellulose membranes (Hybond ECL Nitrocellulose Membrane, Amersham, USA); which were probed overnight with polyclonal rabbit antibody against the LC3-I and LC3-II A/B isoforms I (Cell Signaling Technology Inc, MA, USA) before incubating with HRP conjugated anti-rabbit-IgG antibody (Santa Cruz Biotechnology, CA, USA) and detected by chemiluminescence (PerkinElmer Life Sciences, CA, USA) on X-ray film. For constitutive protein stain, membranes were stripped in NaOH 1M for 10 min, and then probed with β-actin, before incubating with HRP conjugated anti-mouse-IgG antibody (both from Santa Cruz Biotechnology). Western blot bands intensities were quantified by densitometry using Alpha Ease FC software (Genetic Technology Inc, FL, USA).

2.4 Analysis of mitochondrial mass and activity by fluorometry

MitoTracker™ Green FM (MTG) and MitoTracker™ Orange CM-H2TM ROS (MTO) (Invitrogen) are widely used for staining mitochondria of live cells depending respectively on the organelle lipid content (mitochondrial mass) and oxidative activity (mitochondrial membrane potential). Therefore, it was possible to establish a relationship between MTG and MTO staining for accessing the rate of mitochondrial function [29]. Briefly, after RSV treatment, GRX cells were washed on PBS saline and harvested using trypsin. Then, cells were re-suspended with

100 nM of MTG and MTO diluted into (37°C) pre-warmed DMEM for 20 minutes in the dark. The cellular fluorescence was measured by microplate fluorimeter reader (M5, Molecular Devices, USA) after exciting at 490 nm and 554 nm, and collecting the emission at 516 nM and 576 for MTG and MTO, respectively. The results were normalized by corresponding protein content, which was measured according to Peterson [28], and referred as fluorescence units per µg of protein.

2.5 Flow Cytometry Analysis

2.5.1 Analysis of cell death

Cell death analysis was performed by FITC Annexin V and Propidium Iodide (PI) Kit (Invitrogen). This assay allows measuring the mid or late-stage of apoptosis and necrosis [30]. Briefly, after RSV treatment, the samples were prepared according to the manufacturer's instructions before flow cytometry analysis. The probe CaspACE™ FITC-VAD-FMK (Promega, San Luis, CA, USA) was used to determine caspases involvement in apoptosis induction. Briefly, after RSV treatment, cells were incubated with 5µM of FITC-VAD-FMK for 20 minutes at room temperature and then, centrifuged and washed in 1ml PBS. A total of 10,000 events were acquired by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). For the first assay, the green fluorescence (FL-1 channel) for Annexin V and the red fluorescence (FL-3 channel) for PI were measured. For the second assay, the green fluorescence (FL-1 channel) was measured. Staurosporine (Sigma), a mitochondrial membrane depolarization and apoptosis inducer [31] was used as positive control. Briefly, Staurosporine was dissolved in DMSO (Sigma) to 5mg/ml, and then diluted in medium at 1 µM. Then, cells were incubated for 90 minutes before the flow cytometer analysis. All data analyses

were performed with FCS Express 4 software (De Novo, Software, Ontario, Canada).

2.5.2 Analysis of mitochondrial membrane polarization (∆ψm)

For mitochondrial membrane polarization *(∆ψm)* analysis, cells were washed on PBS and harvested using trypsin prior to the 20-minute incubation with MitoScreen JC-1 (BD Biosciences), according to the manufacturer's instructions. The cells were pelleted by centrifugation and re-suspended in 500 µL of the MitoScreen buffer. A total of 10,000 events of each sample were acquired by FACSCalibur flow cytometer (BD Biosciences), measuring both green (FL-1 channel) and red fluorescence (FL-2 channel). Relative degrees of mitochondrial polarization were quantified by measuring the red-shifted JC-1 aggregates, which are favoured under conditions of high membrane potential, and green-shifted monomers, which tend to predominate under conditions of low membrane potential [32]. Staurosporine (Sigma) was used as positive control. All data analyses were performed with FCS Express 4 software (De Novo).

2.5.3 Analysis of autophagosome development

Acridine Orange (AO; Invitrogen) is a marker that fluoresces green in the whole cell except in acidic vacuolar organelles, where it fluoresces red. Development of acidic vacuolar organelles indicates an efficient autophagic process because only mature/late autophagosomes are acidic [33]. Briefly, after RSV treatment, cells were resuspended and incubated with 2.7 µM of AO diluted in medium for 15 minutes [8]. A total of 10,000 events were acquired by the aforementioned flow cytometer, measuring both green (FL-1 channel) and red fluorescence (FL-3 channel), by which stained mature autophagosomes were

quantified. All data analyses were performed with FCS Express 4 software (De Novo).

2.6 Microscopy Analysis

2.6.1 Mitochondrial dynamics and mitophagy analysis by Laser-Scanning Confocal Microscopy

The mitochondrial dynamics was evaluated by incubating GRX cells that were seeded under coverslips with MTG and MTO, as aforementioned. For assessment of mitophagy, cells were incubated with MTG and Lysotracker Red DND-99 (LYSR, Invitrogen), a lysosome fluorescent probe. Briefly, LYSR was diluted in (37°C) prewarmed DMEM at 75 nM, and then incubated with 100 nM of MTG for 20 minutes in the dark. Images were collected using Olympus FV1000 laser-scanning confocal microscopy. Ten single confocal sections of 0.7 µm were taken parallel to the coverslip (*xy* sections) with an x 60 (numeric aperture 1.35) oil-immersion objective (Olympus, U plan-super-apochromat, UPLSAPO60XO). For each sample, images of six fields were acquired and processed with Olympus FluoView FV1000 software. The MTG fluorescence was measured after being excited by a 473 nm laser beam and emission scan collected at 520 nM. The MTO and LYSR fluorescences were measured after laser exciting at 559 nm and emission collected at 598 nm. Colocalization analysis was performed using *Intensity Correlation Analysis* plugin of imageJ software, a public domain Java Image processing programme (http://rsb.info.nih.gov/ij/). For laser-scanning confocal analysis, all experiments were performed at least four times for each sample.

2.6.2 Autophagosome stain by Acridine Orange

For AO stained cells visualization, cells were seeded under coverslips and incubated with this probe as previously described. Confocal images were collected using Olympus F1000 laser-scanning confocal microscopy after laser exciting at 559 nm and emission collection at 598 nm, as previously described. Images were acquired and processed by Olympus Fluoview FV1000 software.

2.6.3 Ultrastructural analysis by Transmission Electron Microscopy

Semi-confluent GRX cells were collected by trypsinization. The cells were harvested by centrifugation and washed twice before 0.1 M phosphate buffered (pH 7.3) addition. After, the cells were fixed in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde buffered with 0.1M phosphate (pH 7.3) at room temperature, and then postfixed in osmium tetroxide in the same buffer for 45 minutes before dehydration. Dehydration was done in a graded acetone series (30-100%) and embedding in araldite (Durcupan ACM, Fluka) for 72h at 60C°. Thin sections (70nm) were stained with 2% uranyl acetate followed by lead citrate. Ultrastructural analysis was performed in untreated and treated cells (10 and 50 µM of RSV) using the JEM 1200EX II transmission electron microscopy. The mitochondria size and the autophagosome number were quantified in fifty images correspondent to randomly selected fields of control and RSV-treated (50µM) groups, using the ImageJ software, a public domain Java Image processing programme (http://rsb.info.nih.gov/ij/). The data obtained were elaborated by Microsoft Excel™.

2.7 Statistical analysis

The quantitative data were expressed as the means ± standard error of the mean. One-way ANOVA was performed to analyse the effects of increasing doses

of RSV treatment. When indicated, a post-hoc Duncan multiple range test was performed. Student's t-test was performed to determine the significance between untreated cells and RSV 50 μM treated cells on transmission electron microscopy images analysis. The results were considered statistically significant when *p* values were equal to or less than 0.05.

3. RESULTS

3.1 All Resveratrol doses induce caspase-mediated apoptosis but only the highest dose promotes necrosis

RSV is well known to exert cytotoxic effects by inducing cell apoptosis, a death event related to intracellular reactive species increase [7]. Furthermore, our previous results showed the dose-dependent pro-oxidative effects of RSV that may be related to cell cycle arrest at lower doses of treatment [15] and to oxidative damage on cells treated with 50 μM of RSV that drastically reduced GRX proliferation and viability [17]. Therefore, we first sought to determine the RSV effects in inducing cell death by apoptosis or necrosis. All doses of RSV induced apoptosis in GRX [Figure 1A], which was mediated by caspases activation [Figure 1B]. We also observed a significant increase in cell death by necrosis only in the group treated with the highest dose of RSV [Figure 1A], which may be related to the evident decreasing of living cells and to the oxidative damage found in our recently published study [17].

3.2 Resveratrol alters the expression of mitochondrial dynamics mRNA

The mitochondrial morphology is constantly changing due to fission and fusion events, resulting in an interconnected and dynamic mitochondrial network. Interestingly, all RSV treatment affected the mitochondria movement along cell cytoplasm. Through imaging analysis obtained on laser-scanning confocal

microscopy, we observed perinuclear mitochondria in control cells. In contrast, RSV-treated cells presented uniformly distributed mitochondria in the cytoplasmic area with an elongated shape, indicating morphological changes in these organelles [Figure 2 and supplementary figure 1]. Thus, we sought to measure the mRNA expression of classical proteins related to mitochondrial fusion (MFN1, MFN2, and OPA1) and fission (DRP1 and FIS1). Except for FIS1, we found alterations in all mitochondrial dynamics tested genes. Treatment with 1 μM of RSV increased the mRNA expression of mitochondrial fusion genes MFN1 and MFN2. Despite the fact that there was no change induced by 10 μM of RSV treatment on the MFN1 mRNA expression, this concentration increased the mRNA expression of MFN2. Interestingly, 50 μM of RSV significantly reduced both MFN mRNA expressions. The pattern of mRNA expression of OPA1 was similar to the MFN2, while all RSV doses increased mRNA expression of DRP1 [Figure 3].

3.3 Resveratrol induces loss of mitochondrial membrane potential (∆ψm) and mitochondrial mass increase due to the organelle swelling and biogenesis

Cell death through apoptosis or necrosis is usually accompanied by mitochondrial swelling and damage that could respectively lead to mitochondrial mass increase and loss of mitochondrial potential [18, 34]. Interestingly, all doses of RSV induced an increase of the relative MTG fluorescence. However, the relative MTO fluorescence did not statistically change. The ratio between MTG and MTO fluorescence showed an increase in the mitochondria mass compared to their activity in the 10 and 50 μM RSV-treated cells, suggesting an increase of swollen and non-functional mitochondria [Figure 4A]. Thus, we also evaluated mitochondrial membrane polarization (∆ψm) by JC-1.Treatment with 10 and 50 μM

of RSV significantly reduced the number of cells with JC-1 red-shifted aggregates, confirming the decreased mitochondrial membrane potential [Figure 4B]. As RSV has been shown to induce mitochondrial biogenesis [24], the expression of ND1 mRNA was used as a marker of mitochondria number. Cells treated with 1 and 10 µM of RSV showed an increase in organelle biogenesis; however, 50 µM did not change the expression of ND1mRNA [Figure 4C].

3.4 Resveratrol promotes changes in the mitochondrial ultrastructure

Resveratrol induces alterations in mitochondria network of GRX that affect the activity of these organelles, triggering an increase in mitochondrial mass that could be due to swelling, especially when administrated at the highest dose. To confirm this, we evaluated organelle morphology through ultrastructural analysis. The transmission electron microscopy images revealed that RSV treatment clearly promotes changes in mitochondrial structures. Control cells presented small well-delimited mitochondria with defined cristae. The 10 µM RSV-treated cells presented mitochondria clustering and cytoplasm re-modelling. However, despite this morphological change in mitochondria, it is still possible to visualize the well-defined organelle membranes. The 50 µM RSV-treated cells presented the major changes in mitochondrial morphology, which was characterized by greater size, altered shape and undefined cristae. Indeed, cells treated with this concentration seemed to be drastically affected since significant morphological changes were found in cell cytoplasm [Figure 5A and B]. The measurement of the mitochondrial area confirmed the significant increase in the size of this organelle [Figure 5C]. Likewise, a greater number of autophagosomes was observed in these cells [Figure 5D].

3.5 Resveratrol stimulated autophagosome formation but ATG7 mRNA was decreased in cells treated with the highest dose

To confirm the increase in the autophagosomes number found on RSV-treated cells observed through transmission electron microscopy imaging analysis, autophagosome development was assessed by AO staining. All RSV treatment doses promoted an increase of these acidic vacuoles [Figure 6A]. To confirm this data, we also quantified the light chain 3 (LC3), a protein that exists in a soluble form named LC3-I that converts to the autophagic vesicle-associated form named LC3-II. The observed increase in LC3-I and LC3-II protein [Figure 6B] support the AO results. Autophagy-related protein 7 (ATG7) is an important signalling molecule involved in the LC3-I to LC3-II conversion [35]. Thus, we also sought to evaluate whether this protein mRNA was altered by RSV treatment. Interestingly, treatment with 1 and 10 µM of RSV did not change the ATG7 mRNA expression in GRX cells. However, the levels of ATG7 mRNA expression in GRX cells treated with 50 µM were decreased [Figure 6C].

3.6 Resveratrol induced mitochondrial removal by mitophagy

As we found an increase in mature autophagosomes in RSV-treated cells along with mitochondrial metabolism changes, we sought to investigate whether this phytoalexin could induce mitophagy by correlating the green fluorescent mitochondria and red fluorescent lysosomes through laser-scanning confocal microscopy. All RSV doses induced an increase in mitochondria and lysosomes colocalization in GRX, indicating mitophagy. The product of the difference from the mean (PDM) was used to show the positive correlations between the green and red probes in each image. The PDM images showed colocalization in the positive PDM channel (yellow) and exclusion, or “anti-correlation”, in the negative channel

(purple) [Figure 7]. Table 2 shows the Pearson's and Mander's coefficients used to quantify the correlation [36].

4. DISCUSSION

During the last years, RSV has been the focus of numerous *in vitro* and *in vivo* studies. Based on RSV action on several pathologic models, we believe that this phytoalexin is a useful tool in the resolution of liver fibrosis by promoting death of activated hepatic stellate cells (HSC), the main responsible for developing this disease. We already demonstrated promising results regarding the treatment of activated HSC with RSV that could favour the resolution of liver fibrosis. However, despite the pro-oxidant effects and cell cycle arrest promoted by lower doses of RSV in GRX, our activated HSC model, only doses above 50 µM were remarkably cytotoxic, drastically reducing the amount of living cells [15, 17]. Among numerous effects, RSV was found to trigger mitochondrial-mediated cell death, mitochondrial biogenesis, and autophagy [7, 19, 24, 39-41]. Thus, we hypothesized that a possible interaction between these three cellular responses could be important for triggering the effects of RSV towards effective reduction of the living cells number.

Apoptotic intrinsic pathway is often activated in response to cell stress or damage due to exposure to cytotoxic drugs. In this situation, mitochondrial dysfunction leads to the excessive production of reactive species that may damage their membranes. This event is usually followed by an increase of pores in mitochondrial outer membrane that leads to organelle swelling and cytochrome c release into cell cytosol, a defined event during apoptosis that results in caspases activation. Likewise, more catastrophic stresses can lead to a drastic opening of mitochondrial permeability pore accompanied by massive release of reactive species that culminates in necrosis [18, 21, 34, 42]. We firstly showed that

treatment with 1, 10 and 50 µM of RSV for 24 hours promoted an increase of GRX featuring apoptosis signals that were mediated by caspases activation. Moreover, treatment with the highest dose also promoted GRX necrosis, thus characterizing a more elevated cell stress. In this regard, our previous result showing the dose-dependent pro-oxidant effect of RSV that resulted in higher cytotoxicity characterized by lipid peroxidation [17] can undoubtedly be related to this more elevated cell death induction.

Mitochondria play a crucial role in cell metabolism. Many intracellular and extracellular signals, including oxidative stress and mtDNA quality, can modulate fusion and fission events, altering the mitochondrial dynamics within cells and promoting positive or negative regulation of programmed cell death [43-46]. In this regard, mitochondrial fusion facilitates exchange of biomolecules among these organelles, helping on repairing defective mitochondria that could contribute to cell death signalling. Thus, mechanisms allowing complementation of damaged mtDNA and other contents (such as lipids, proteins or metabolites) with the components of healthy mitochondria maintain normal organelle activity. Mitochondrial fission is an important mechanism on removing impaired mitochondria through mitophagy, thus favouring cellular homeostasis, or can be an early event during apoptosis [42, 44-45, 47]. All RSV treatments resulted in changes on mitochondrial morphology and network. In accordance, except for FIS 1, all RSV treatment triggered changes in mRNA related to mitochondrial dynamic.

As demonstrated, the mRNA expression of fusion proteins was mainly upregulated in GRX treated with 1 and 10 µM of RSV and downregulated in cells treated with 50 µM; while the mRNA expression of DRP1, a fission protein, was upregulated in all tested concentrations of RSV. Particularly, the fusion protein

OPA1 plays an important role in maintaining mitochondrial structures (cristae), avoiding organelle swelling and formation of pores in mitochondrial outer membranes that could promote apoptosis through cytochrome c release [42, 44-45, 47]. In contrast, the fission protein DRP1 was found to be recruited to the mitochondrial outer membranes, mediating cytochrome c release during apoptosis [44-45, 48]. Interestingly, GRX treated with all RSV concentrations presented an evident unbalance between the mRNA expression of these proteins that favoured DRP1 rather than OPA1 [Figure 8A]. In this sense, we hypothesized that this situation could be related to a higher mitochondrial injury in these cells, which could contribute to cytochrome c release and caspases-mediated apoptosis. It was worthwhile to observe that the higher unbalance between the mRNA expression of DRP1 and OPA1 proteins found in cells treated with 50 µM of RSV can be related to a drastic reduction on mitochondrial quality, contributing to the loss of organelles' function and to the significant increase of cell death in this group.

Mitochondrial swelling and the consequent loss of mitochondria function are important features of apoptotic cells [49-50]. To confirm the hypothesis that RSV treatment could lead to mitochondrial injury in GRX, we measured mitochondrial mass and activity through MTG and MTO ratio. In addition, we evaluated mitochondrial membrane potential by JC-1 assay. All RSV doses increased mitochondrial mass in GRX. However, only cells treated with 10 and 50 µM of RSV presented an increase of non-function mitochondria. Considering that RSV at 1 µM triggered caspases mediated apoptosis in GRX and assuming that this mechanism is due to mitochondrial injury that led to swelling, it would be expected that cells treated with this concentration have loss of mitochondria function. About this situation, we hypothesised that mitochondrial biogenesis, along with dynamics,

could contribute to the maintenance of organelle function in cells treated with the lowest concentration.

Indeed, several studies have reported that RSV may induce mitochondrial biogenesis, augmenting the absolute number of organelles as well as their DNA copies [24, 51-52]. This mechanism may be an important way whereby cells could replace non-function mitochondria [18], and may also contribute to the organelle mass increase. Through measuring the ND-1 mRNA expression as a parameter of cell mitochondria number [26], we found that treatment with 1 and 10 μM of RSV induced an increase in mitochondrial biogenesis. Thus, it is possible that mitochondrial biogenesis can compensate the increase of injured and non-functional organelles in 1 μM treated-cells, maintaining the mitochondrial function at control levels in these cells. Interestingly, RSV did not alter the mitochondrial biogenesis in GRX when given at the highest dose. This situation can indicate that these cells mainly had mitochondria swelling, which may result in severe damage in the organelles structure.

Ultrastructural analysis of GRX cells treated with 10 and 50 μM of RSV were performed to confirm our hypothesis regarding mitochondrial swelling. In accordance to our previous results, the general features of GRX cells were consistent with a lesser cytotoxic effect of RSV doses until 10 μM, although some changes regarding oxidative status [17], mitochondrial function or cells death are evident in these cells. Indeed, 10 μM of RSV promoted mitochondrial clustering that could be associated to the aforementioned increase in the organelle dynamics. However, this RSV dose did not significantly alter the mitochondrial morphology in GRX, which still presented defined membranes and cristae. On the other hand, treatment with 50 μM of RSV led to a more drastic change in

mitochondria structure, characterized by undefined cristae and significant increased size. In this sense, since necrotic cells usually show significant mitochondrial swelling [53-54], it is possible that this observed ultrastructural feature can also be related to the increasing of necrosis in GRX cells treated with 50 µM of RSV.

Autophagy is a highly conserved cellular process that involves the sequestration and delivery of damaged or unnecessary cytoplasmic material to lysosomes, where it is degraded and recycled. Interestingly, apoptosis and autophagy are both tightly regulated biological processes that may be triggered by common upstream signals that could result in combined responses or in two independent responses [55-58]. In this way, while autophagy has been reported as a cell death partner at some studies, this mechanism may also exert a cytoprotective function, ensuring cell survival under adverse conditions such as oxidative stress and cell toxicity [20, 22]. In our experiments, all RSV doses also induced maturation of autophagosomes that must be related to the increase on the LC3-I and II contents. Indeed, recent *in vivo* and *in vitro* studies revealed that RSV-induced autophagy can attenuate apoptosis, revealing a crosstalk among these cellular mechanisms that could regulate cellular responses to promote this phytoalexin-mediated cell survival or death [8, 39, 59]. In this sense, the elimination of dysfunctional mitochondria through mitophagy appears to be crucial for maintaining cellular homeostasis through the avoidance of organelle-mediated cell death [18, 46, 60]. As expected, the autophagy induction by RSV treatment was accompanied by an increased mitophagy in GRX cells.

A set of autophagy-related (ATG) proteins is hierarchically recruited in the construction of the autophagosome. Considering the involvement of ATG7 in the

LC3 I to LC3 II conversion [35], it seemed to be relevant that its mRNA expression level has decreased on cells treated with 50 µM of RSV. Interestingly, it was already shown that mouse embryonic fibroblasts with deficient ATG7 under prolonged metabolic stress had increased p53 binding at promoters of pro-apoptotic target genes [61]. Likewise, it was recently reported that an inhibition of autophagy as a consequence of ATG7 interference can enhance the sensitive to apoptosis induction by cisplatin in a human carcinoma cells model [62]. Lastly, the percentage of apoptotic cells significantly increased after inhibition of autophagy by ATG7 knockdown and chloroquine pre-treatment in hepatocellular carcinoma [63].

Altogether, the increased mRNA expression of fission protein DRP1 and the diminished mRNA expression of fusion proteins and ATG7 suggested that GRX treatment with 50 µM of RSV could favour the induction of apoptosis rather than autophagy. Since mitochondrial damage seemed to be dependent on the concentration of RSV, and considering the autophagy role towards cell surviving under stress condition, we hypothesized that the autophagic features found in cells treated with 50 µM could be related to an unsuccessful response against a higher mitochondria-mediated death signalling. Indeed, a simple ratio between annexin/PI and AO results confirmed that treatment with 50 µM of RSV induced an evident unbalance favouring apoptosis rather than autophagy in GRX [Figure 8B], which could lead to the drastic reducing in the number of living cells in this group [Figure 8C]. This fact suggests that the balancing between the concomitant induction of autophagy and cell death mediated by mitochondrial injury could determine the dose-dependent cytotoxicity of RSV. Furthermore, mitochondrial biogenesis induction by lower doses of RSV may have an important role by replace the

damaged mitochondria, contributing for cell resistance and homeostatic reestablishment.

The phytoalexin RSV has been widely associated to numerous beneficial effects. The present study reveals new insights on the use of RSV regarding liver fibrosis therapy. In view of the possibility that RSV could exert contradictory effects depending on its concentration, it seems to be of utmost importance determining the better dose that aims to effectively promote the activated HSC apoptosis, especially if considering that the concomitant induction of autophagy and mitophagy could attenuate cell death. Nevertheless, further investigations of RSV effects on the molecular interactions that trigger these presented results are undoubtedly necessary.

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FIGURES

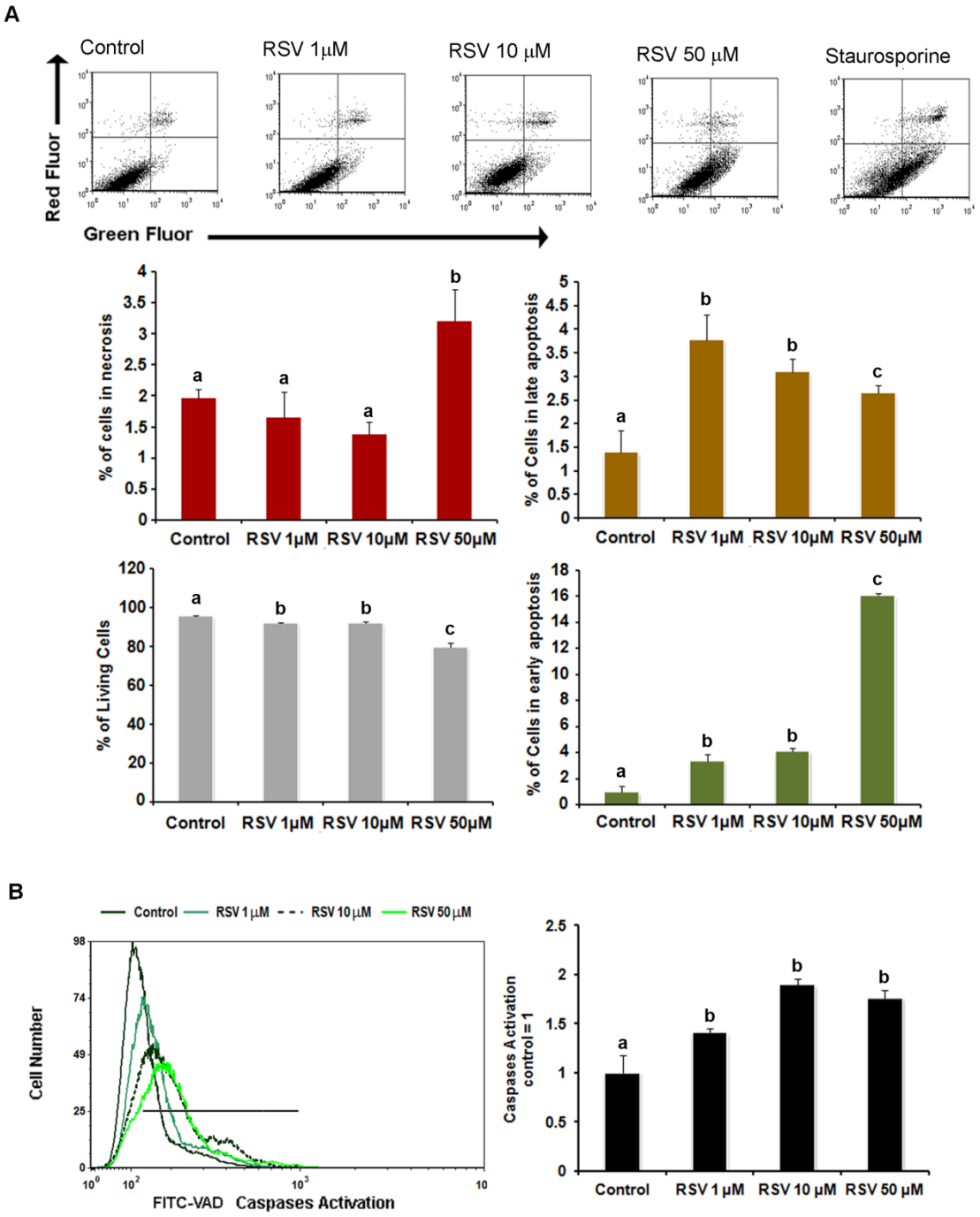


Figure 1 The profile of cell death in untreated and RSV treated GRX. The Annexin V and PI assay revealed that all RSV treatments triggered an increase of GRX featuring apoptosis signals while the highest concentration also promoted an increase of GRX featuring necrosis signals (A). The FITC-VAD-FMK assay revealed that all RSV concentrations triggered caspase activation (B). Data is expressed as mean ± S.E.M. (n = 4). Means with different letters (a,b,c) are statistically different at $p \leq 0.05$.

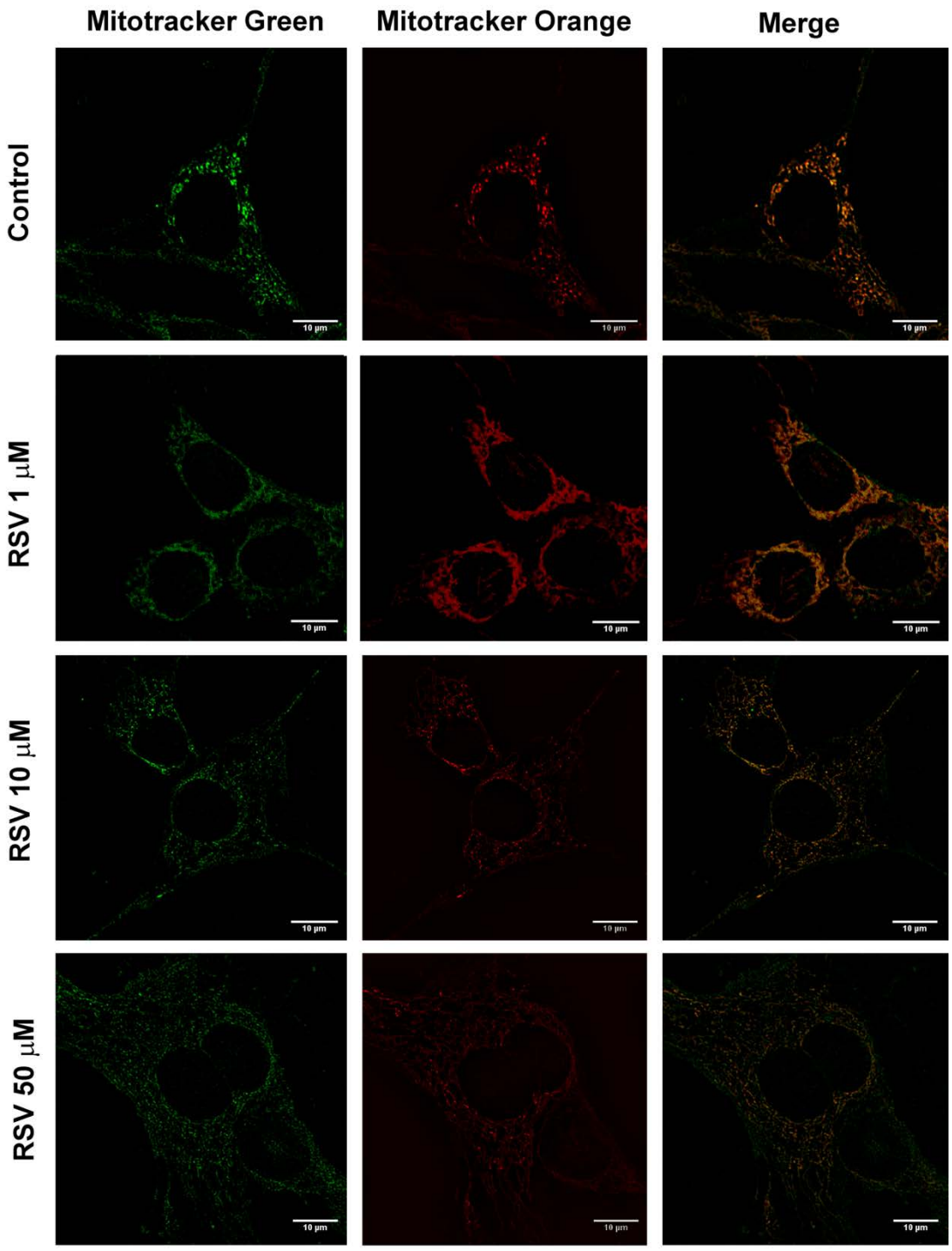


Figure 2 The mitochondrial morphology and distribution along GRX cytoplasm through Laser-Scanning Confocal Microscopy. GRX cells probed with MTG and MTO revealed that all RSV treatments changed the organelle aspect from a round perinuclear feature to a network well-distributed feature. Scale bar = 10μm.

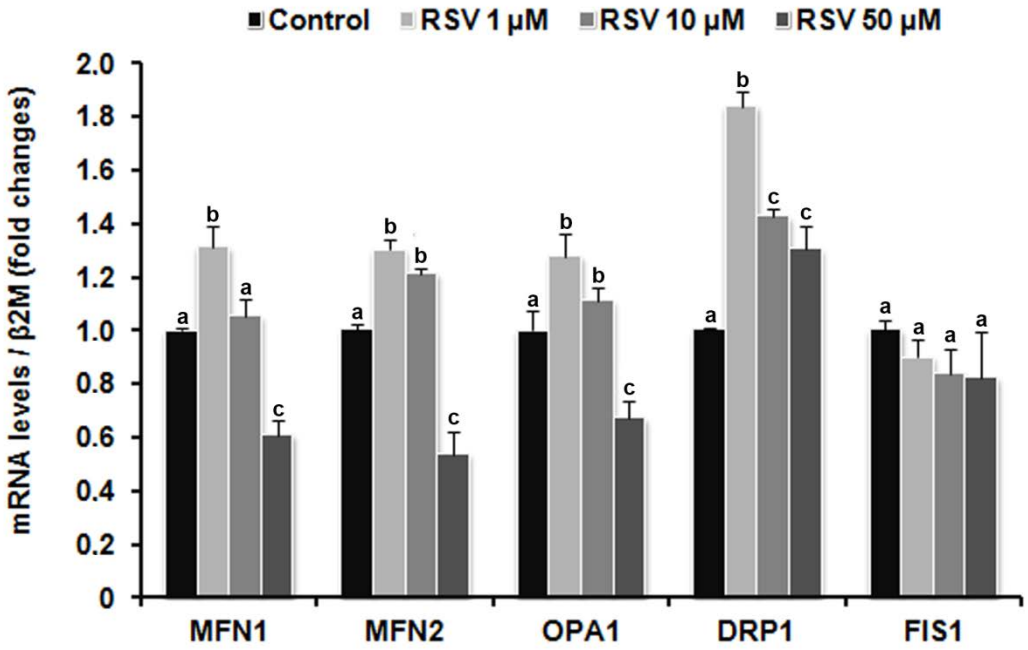


Figure 3 The mRNA expression profile of mitochondrial dynamics proteins. Except for FIS1, which was not changed, and DRP1, which had all mRNA expression upregulated, treatment with 1 and 10 µM of RSV mainly increased mRNA expression of fusion proteins (MFN1, MFN2 and OPA1), while treatment with 50 µM decreased. Data is expressed as mean ± S.E.M. (n = 3). Means with different letters (a,b,c) are statistically different at $p \leq 0.05$.

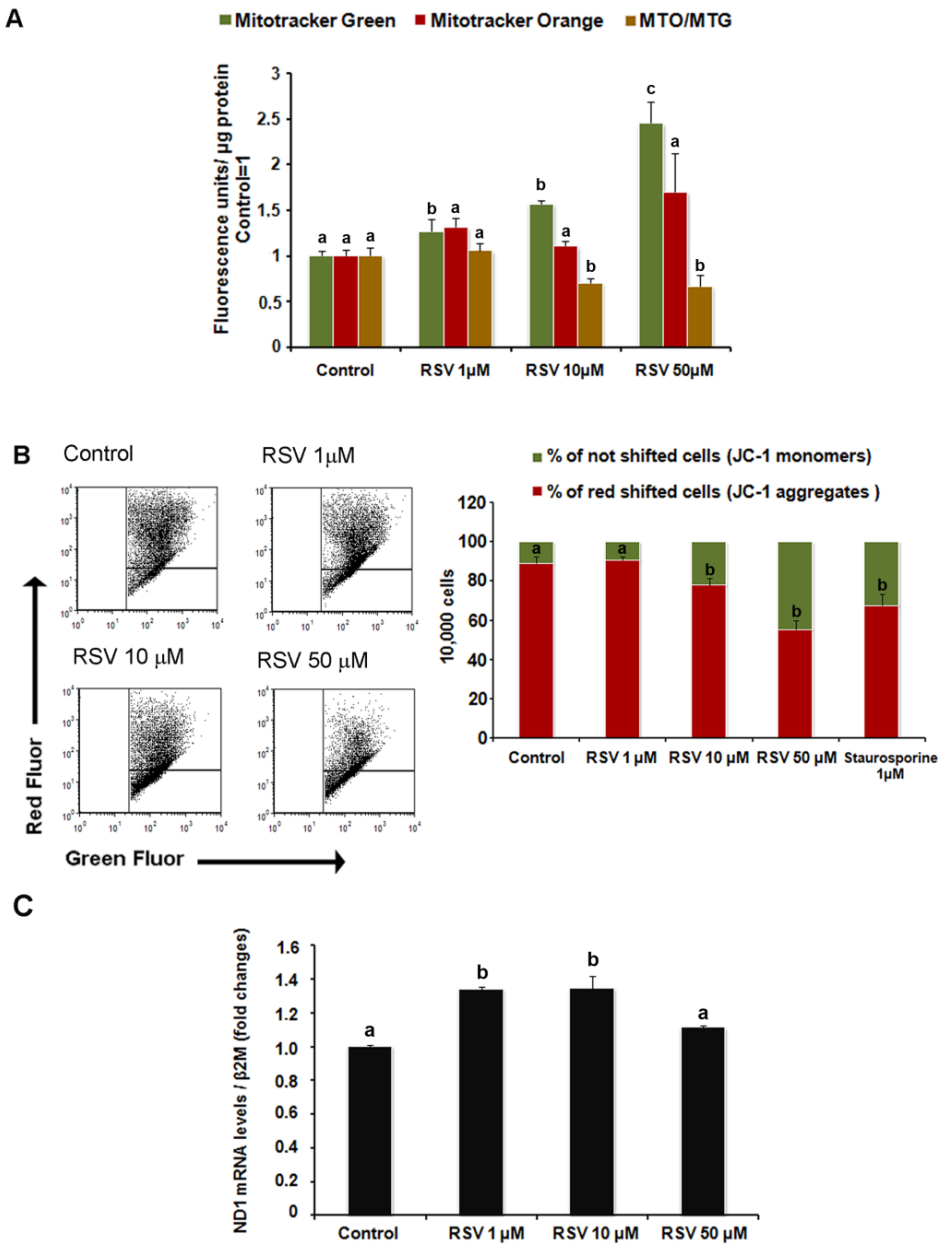


Figure 4 The evaluation of mitochondrial mass, function, and biogenesis. The MitoTracker Green FM (MTG) probe revealed an increase in mitochondrial mass in all RSV treated cells while MitoTracker Orange CM-H2TM ROS (MTO) probe showed that the organelle function did not alter. The ratio between MTG and MTO indicated the loss of mitochondrial function in cells treated with 10 and 50 µM of RSV (A). The JC-1 probe confirmed the MTG and MTO ratio results (B). The measure of mtDNA (ND1) revealed that treatment with 1 and 10 µM of RSV triggered mitochondrial biogenesis. However, the highest dose did not alter the mtDNA content in GRX (C). Data is expressed as mean ± S.E.M. (n = 4), $p \leq 0.05$. Means with different letters (a,b,c) are statistically different at $p \leq 0.05$.

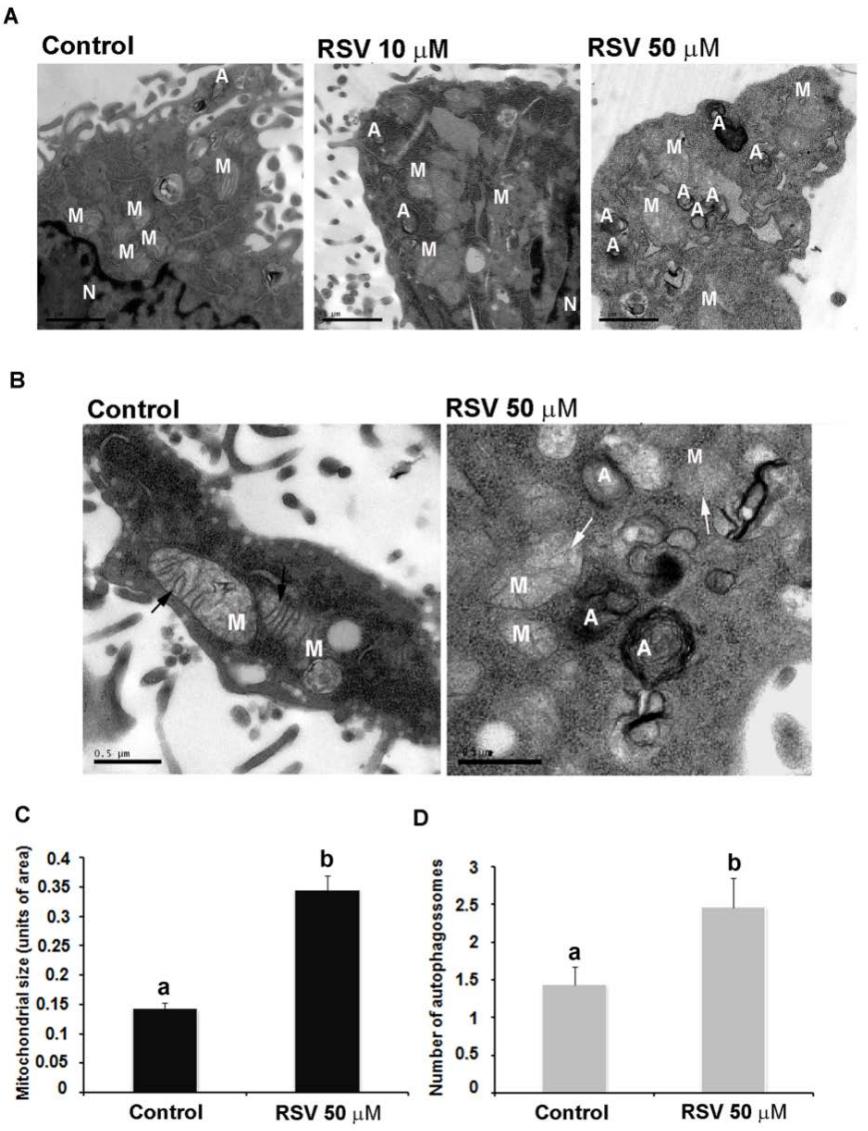
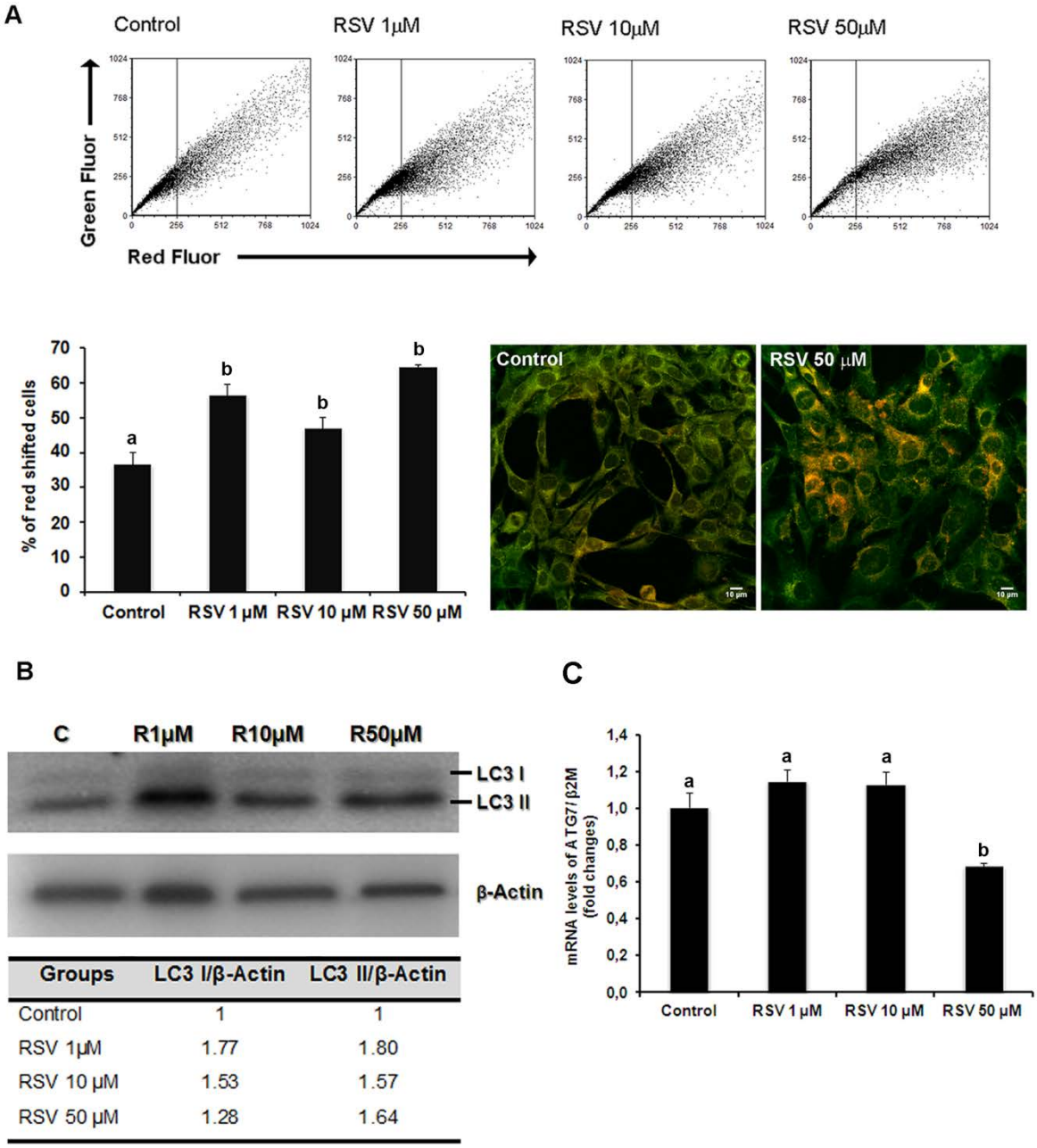


Figure 5 Ultrastrucural analysis of GRX. Transmission electron microscopy images revealed that treatment with 10 µM of RSV promoted mitochondrial clustering while the highest dose significantly altered the organelle morphology by promoting cristae disruption and swelling. Furthermore, the high dose of RSV also increased the number of autophagosomes in GRX cells. Scale bar = 1µm (A). The GRX cytoplasm detailed. Scale bar = 0.5µm (B). Treatment with 50 µM of RSV triggered a significant increase in the mean of mitochondrial size (C) and autophagosome number (D). Data is expressed as mean ± S.E.M, $p \leq 0.05$. Means with different letters (a,b) are statistically different at $p \leq 0.05$. **M** = mitochondria; **N** = Nucleus; **A** = autophagossomes; **Black arrows** indicates the well-delimited mitochondrial membranes with organized cristae. **White arrows** indicates the more undefined mitochondrial membranes and shapeless cristae.



Groups	LC3 I/β-Actin	LC3 II/β-Actin
Control	1	1
RSV 1μM	1.77	1.80
RSV 10 μM	1.53	1.57
RSV 50 μM	1.28	1.64

Figure 6 The evaluation of autophagy induction. The Acridine Orange (AO) probe revealed an increase of mature autophagosomes in all RSV treated cells (A). Imunnoblotting revealed an increase in both forms of LC3, an important protein that regulates the maturation of autophagosomes, corroborating the AO data (B). Treatment with 50 μM of RSV significantly decreased mRNA expression of ATG7 (C). Data is expressed as mean ± S.E.M. (n = 4), $p \leq 0.05$. Means with different letters (a,b) are statistically different at $p \leq 0.05$.

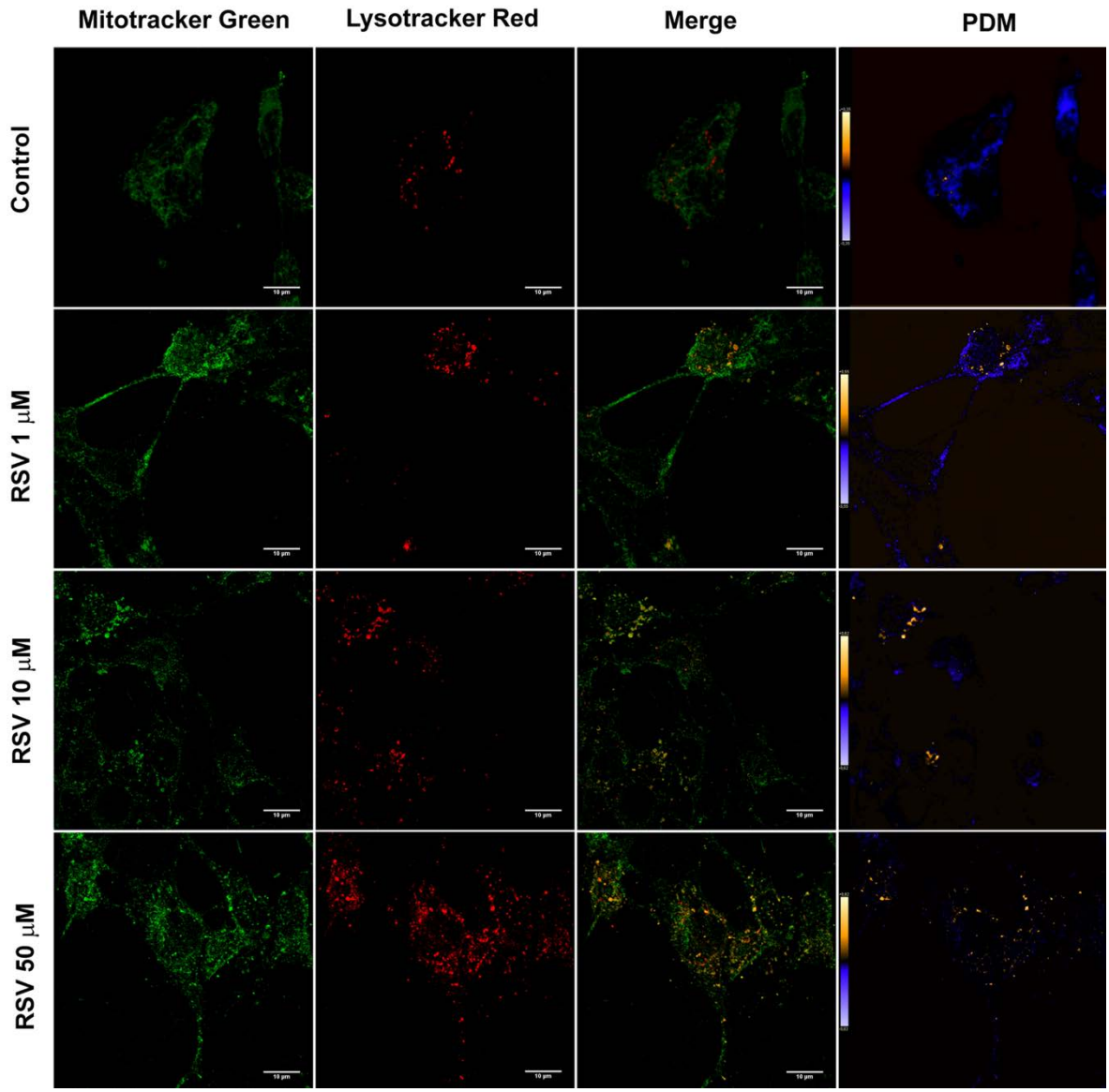


Figure 7 Mitophagy induction. Confocal images were obtained after probing GRX cells with MTG for mitochondria and LysoTracker Red DND-99 (LYSR) for lysosomes. All RSV treatments increased the positive correlation between the two organelles. PDM images show colocalization in the positive PDM channel (yellow) and “anti-correlation” in the negative channel (purple). Scale bar = 10μm.

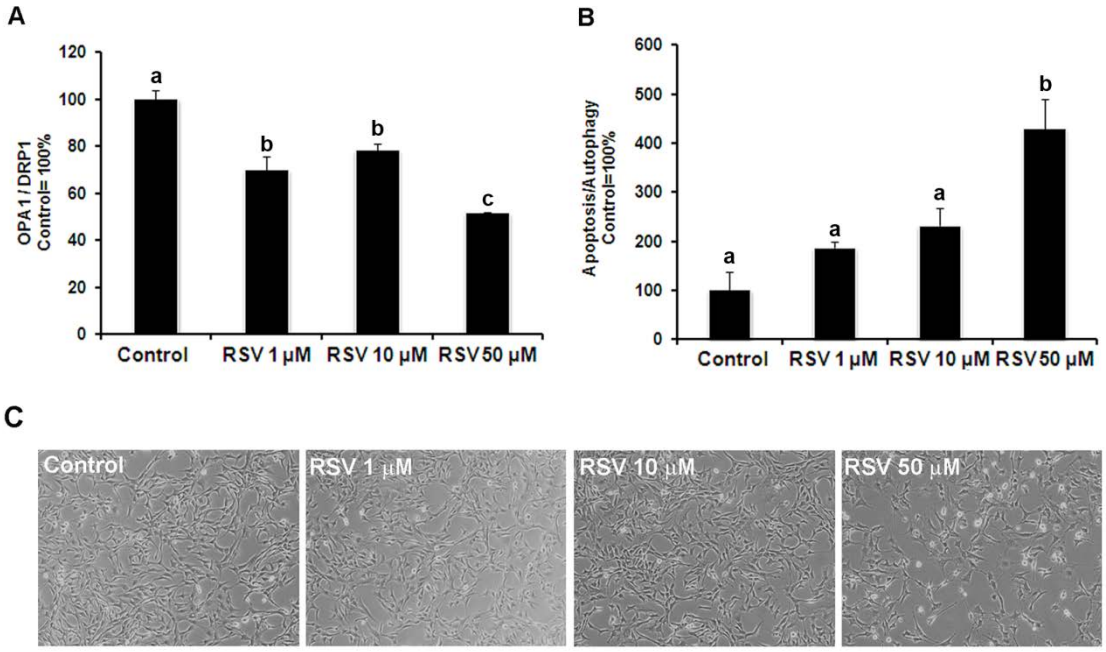
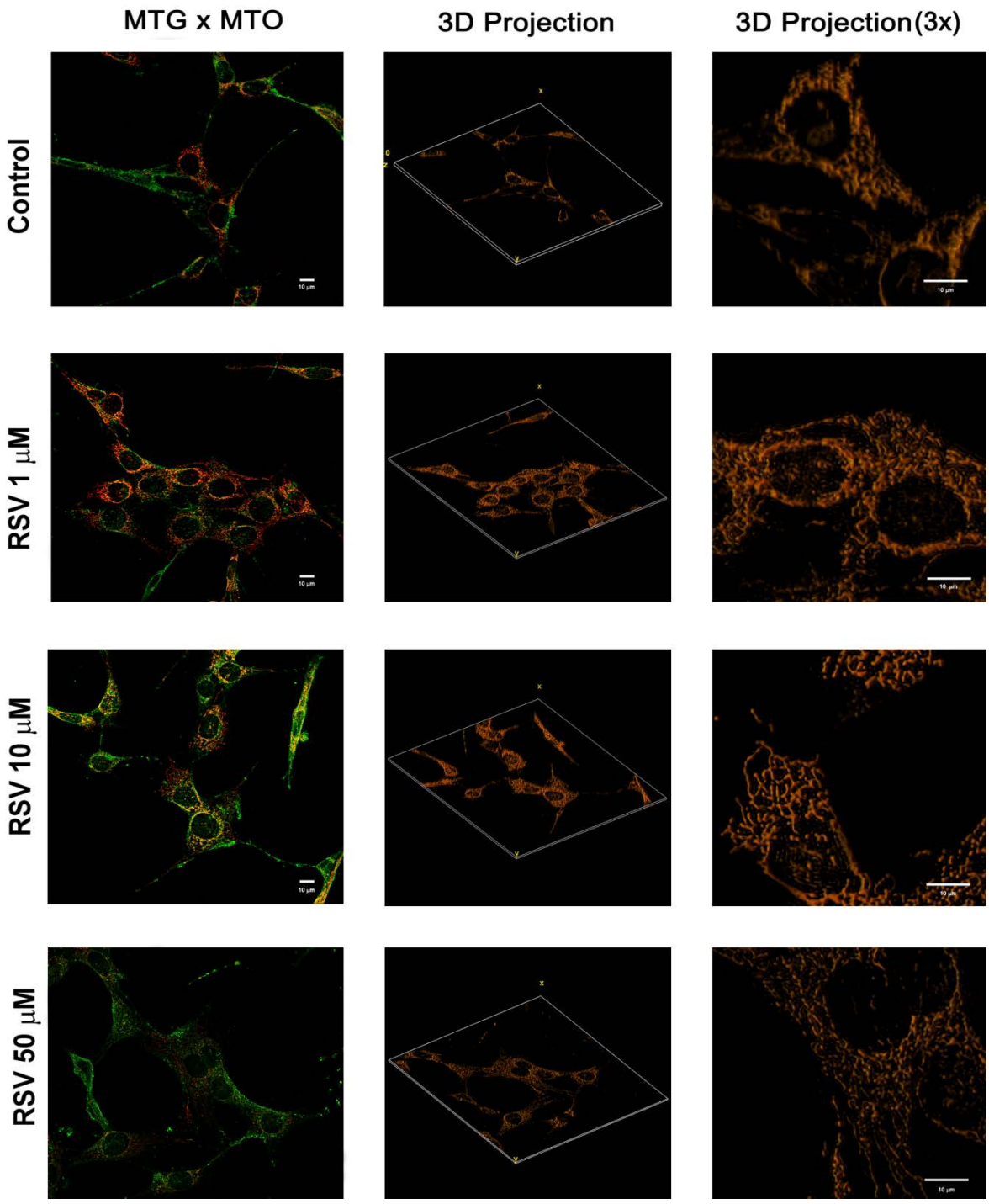


Figure 8 Apoptosis versus autophagy. GRX treated with all doses of RSV presented an evident unbalance between the mRNA expression of DRP1 and OPA1 proteins that contributes to the reduction on the mitochondrial quality, favouring apoptosis (A). The ratio between apoptosis (annexin/PI results) and autophagy (Acridine Orange results) showed that cell death induction was significantly favoured in GRX treated with 50 µM of RSV (B). Optical microscopy (100x) showed that only treatment with 50 µM of RSV effectively changed cell morphology and number (C). Data is expressed as mean ± S.E.M., $p \leq 0.05$. Means with different letters (a,b,c) are statistically different at $p \leq 0.05$.



Supplementary Figure 1 The mitochondrial morphology and distribution along GRX cytoplasm through Laser-Scanning Confocal Microscopy. GRX cells were probed with MTG and MTO. 3D projection of cells was obtained using the *volume viewer* plugin of imageJ software. All RSV treatments changed the organelle aspect from a round perinuclear feature to a network well-distributed feature. Scale bar = 10μm.

II.3 RESVERATROL REGULATES THE RELEASE OF TUMOR NECROSIS FACTOR-α, INTERLEUKIN-6, AND INTERLEUKIN-10 BY ACTIVATED HEPATIC STELLATE CELLS

(Manuscrito a ser submetido como *short communication*)

RESVERATROL REGULATES THE RELEASE OF TUMOR NECROSIS FACTOR-α, INTERLEUKIN-6, AND INTERLEUKIN-10 BY ACTIVATED HEPATIC STELLATE CELLS

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ABSTRACT

Hepatic stellate cells (HSC) play an important role in liver wound-healing by favouring the repair of injured tissue. However, chronic liver injuries can increase cytokines activity, contributing to perpetuate HSC activation and leading to liver fibrosis. Interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) participate on HSC activation. Oppositely, interleukin-10 (IL-10) has anti-fibrogenic activity, inducing activated HSC to apoptosis. HSC not only respond to these cytokines but also secrete them, revealing a tightly regulated interaction. Resveratrol is known for its anti-inflammatory properties but can also exert cytotoxicity through inducing the cell death and growth inhibition, two positive effects on treating several pathological cell models. We previously showed promising results regarding the cytotoxic effects of resveratrol on treating GRX cells, an activated HSC model. Here, we evaluated the effects of resveratrol (0.1 to 50 μM) in the GRX ability on releasing TNF-α, IL-6, and IL-10 in the culture media. We found that while resveratrol mainly induced a reduction in IL-6 concentration, the TNF-α and IL-10 concentration was increased in culture media of GRX cells treated for 24 and 120 hours. These results reveal a new investigation perspective on studying the anti-inflammatory effects of resveratrol in HSC and its possible role in autocrine signalling.

Keywords: Hepatic stellate cells, Interleukin-6, Interleukin-10, Resveratrol, Tumor necrosis factor-α.

1. INTRODUCTION

Hepatic stellate cells (HSC) are known to store retinol droplets in the cytoplasm, a condition that characterizes their quiescent phenotype. As one of their physiological features, HSC undergo to an activated phenotype in response to paracrine stimulation. At this condition, these cells lose their retinol droplets and become myofibroblast-like cells, playing an important role in liver wound-healing through protecting healthy areas from damaged areas. HSC are also thought to influence the function of hepatocytes and sinusoidal cells through releasing biologically active mediators, favouring the repair of injured tissue and promoting the restoration of hepatic homeostasis. Nonetheless, continuous damage to the liver results in a chronic inflammatory response in which hepatic environment may not recover its homeostatic balance. In this sense, increased production/activity of cytokines may be critical for both autocrine and paracrine perpetuation of HSC activation, which contributes to an excessive extracellular matrix accumulation that leads to liver fibrosis [1-3].

Strategies that promote activated HSC death or decrease the stimuli leading to cells activation play an important role on treating chronic liver injuries. At this context, the reduction on liver proinflammatory condition appears to be an interesting way for decreasing HSC activation, thus preventing fibrosis development [1-3]. Among others, Tumour Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) are important proinflammatory mediators, participating on HSC activation [4-7]. On the other hand, Interleukin-10 (IL-10) was found to have anti-inflammatory and anti-fibrogenic activity, also inducing activated HSC to apoptosis [1, 6, 8]. Interestingly, HSC not only respond to these cytokines but also secrete them, revealing the importance of tightly regulated local control of cytokine action

[1]. 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, present at relevant concentrations in red wine, has been related to numerous beneficial effects on health through its chemoprevention activity, which is largely associated to its anti-inflammatory and antioxidant effects. Interestingly, RSV can also exert cytotoxicity through inducing the cell death and growth inhibition, two positive effects on treating several pathological cell models [9-10].

Our previous studies already demonstrated promising results regarding the cytotoxic effects of RSV on treating the murine cell line GRX, an activated HSC model [11]. Firstly, we found that RSV at doses between 0.1 and 1 μM was able to inhibit GRX growth through promoting cell cycle arrest in the S phase [12]. Further, we found that RSV treatment triggered a dose-dependent pro-oxidant effect on GRX [13]. Recently, we showed that the dose-dependent cytotoxicity of RSV on GRX, which culminates in the evident cell damage and death at the highest concentration, may be related to an imbalanced interplay among the concomitant induction of mitochondria damage leading to caspases-mediated apoptosis, mitophagy, and mitochondrial biogenesis [unpublished data].

Besides these positive effects suggesting that RSV can reduce the number of activated HSC, its ability on modulating cellular inflammatory response can also be interesting for treating this disease [1, 10]. Thus, we evaluated the effects of RSV in the GRX ability on releasing IL-6, IL-10, and TNF-α in the culture media. We found that while RSV mainly induced a reduction in IL-6 concentration, the IL-10 and TNF-α concentration was increased in culture media of GRX cells treated for 24 and 120 hours. Based on previous literature and considering these results, we

sought to find a new perspective to investigate/explore the RSV effects towards liver fibrosis resolution from the point of view of HSC fibrogenic regulation or cells activation through an autocrine signalling.

2. MATERIALS AND METHODS

2.1 Cell culture and Resveratrol treatment

The GRX cell line was obtained from the Cell Bank of Rio de Janeiro (HUCFF, UFRJ, RJ). The cells were seeded (1.5 x 10^4/cm^2) on 24-well culture plates (Nunc, Roskilde, Denmark). The 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% CO_2 at 37 °C. Resveratrol (Sigma Inc., St. Louis, MO, USA) was dissolved in 20 µL of ethanol (Merck, Darmstadt, Germany) to a stock concentration of 100 mM and diluted sequentially in DMEM to a final concentration of 0.1, 1, 10, and 50 µM just before use. The cells were treated during 24 hours or 120 hours. Media without (control) or with RSV was changed daily during the 120-hour experiments. Media from the last 24 hours of treatment was used to perform the 120-hour experiments.

2.2 Tumour necrosis factor-α, interleukin-6, and interleukin 10 measurement

IL-6 and IL-10 concentration were quantified in 500 µL of cell culture media using Quantikine ELISA Kit and protocol (R&D Systems, Minneapolis, MN, USA). TNF-α concentration in the culture media was determined using Sigma ELISA Kit (Sigma Inc.), accordingly to the manufacturer's protocol. RSV at the highest concentration exerts an evident cytotoxic effect, drastically reducing the amount of live cells from 24 hours of treatment [13]. For allowing a more precise estimate on the RSV effects on releasing of these cytokines, cell quantity had to be

considered. Results were then normalized by protein content, measured according to Peterson [14] at the end of RSV treatments, and were expressed as pg/µg of cell protein.

2.3 Statistical analysis

The data was expressed as mean ± standard error of the mean (S.E.M). One-way ANOVA was used to analyse the effect of RSV treatment and *post-hoc* Duncan multiple range test was performed. Results were considered statistically different when the *p* values were equal to or less than 0.05.

3. RESULTS AND DISCUSSION

Understanding liver fibrosis focuses primarily on events that lead to activation and proliferation of hepatic stellate cells (HSC). The activation of HSC consists of two major phases: initiation and perpetuation. Under liver inflammatory condition, the paracrine stimuli from neighboring cells – namely injured hepatocytes, endothelial cells, Kupffer cells, and platelets – initiates HSC activation, rendering cells responsive to other cytokines. The pathways for perpetuating the activated HSC phenotype include the acquisition and maintenance of new functions such as proliferation, release of proinflammatory cytokines and chemokines, matrix degradation, and fibrogenesis[1-4].

Most of the new cellular functions of activated HSC are also sustained by an autocrine loop characterized by the production of several mediators and by the enhancement of cell response to these factors through both the up-regulation of their membrane receptor and the enhancement of intracellular signalling. Numerous cytokines, which may be pro- or anti-fibrogenic, have been shown to play a major role in wound-healing response during liver diseases [1, 4]. Therefore, the largely discussed anti-inflammatory property of RSV [9-10] may

interfere on signalling between cultured HSC. Thus, we evaluated the RSV effects in the GRX ability on releasing TNF-α, IL-6, and IL-10; three important cytokines that are involved in liver fibrogenesis [1, 4, 15-16].

Here, we firstly showed that all doses of RSV were able to decrease IL-6 released by GRX in culture media at both times of treatment [Figure 1A].Oppositely to the IL-6 result, we found that all RSV doses significantly increased the concentration of IL-10 released by GRX in culture media during 24 hours of treatment. During 120 hours, only cells treated with 50 μM of RSV presented a highly significant increase of IL-10 released by GRX in culture media [Figure 1B]. Finally, we found that treatment with 1, 10, and 50 μM of RSV for 24 hours significantly increased the concentration of TNF-α released by GRX in culture media. However, during 120 hours of treatment, the TNF-α concentration in culture media has increased only in GRX treated with 50 μM of RSV [Figure 2].

It is largely agreed that IL-6 and TNF-α play an important role in liver inflammatory regulation, mediating the HSC activation [1, 4, 6]. On the other hand, it is noticeably that IL-6 and TNF-α also exert beneficial effects during liver chronic diseases. Indeed, while IL-6 is thought to play an important role for inducing the hepatocytes regeneration [1, 4-5], other studies suggested that TNF-α reduces liver fibrogenesis by inducing HSC to synthesize less collagen I during their perpetuation phase [4, 6, 17]. IL-10 has been regarded as one of the most important cytokines that contributes on preventing an excessive or inappropriate inflammatory response [18]. Also, IL-10 contributes to the intracellular superoxide decrease, which may result in the cytosolic acidification, an event that favours apoptosis [18-20]. In this way, IL-10 also plays an important role on decreasing

liver fibrogenesis through regulating the HSC activation and promoting the activated HSC apoptosis [6, 21-25].

A recently *in vivo* study reported that RSV decreased the mRNA expression of IL-6 in Kupffer cells, an event that can be beneficial in treating acute liver injury by reducing hepatocytes apoptosis. However, the same study pointed that RSV can also limit the hepatocyte regeneration, possibly by reducing the hepato-mitogenic signalling [26]. For analysing our results, it is undoubtedly important to state that GRX cells are a model of isolated culture of activated HSC. At this context, it would be a plausible hypothesis that an increase on the cellular releasing of TNF-α along with a decrease on the cellular releasing of IL-6 mediated by RSV treatment may contribute to prevent a possible perpetuation on HSC activation and fibrogenesis through an autocrine way. Similarly, a decrease of IL-6 along with an increase of IL-10 in the media of RSV-treated GRX would be in accordance to the anti-inflammatory role of this phytoalexin in our model.

Another relevant state is that HSC activation depends on the activation of NFκB, a transcription factor that regulates the expression of several cytokines including TNF-α and IL-6 [6, 16]. Known inducers of NFκB activity are highly variable and include reactive oxygen species (ROS), interleukin 1-β, lipopolysaccharides and the own TNF-α [6, 27-28]. Particularly, it is known that TNF-α can mediate a pro-survival pathway depending on NFκB activation. However, TNF-α is also thought to mediate a pro-apoptotic pathway depending on the inhibition of NFκB activation. At this situation, mitochondrial potential decreases while ROS and a caspase cascade act as downstream mediators [6, 29-31]. Interestingly, RSV was already found to inhibit NFκB activity [32]. At the same, we previously showed that RSV treatment induced an increase of reactive

species [13], a decrease of mitochondrial activity, and GRX cell apoptosis [unpublished data]. Thus, it seems to be plausible to hypothesise that a possible increase on TNF-α protein along with an inactivation of NFκB could be related to our previous results. In this way, there are evidences that an increased IL-10 prevents NFκB activation by inhibiting its translocation to cell nucleus, or directly inhibits NFκB binding to DNA. Also, IL-10 could decrease the NFκB expression [6, 23].

It is a consensus that several cytokines have pleiotropic activity in cellular signalling. Though we did not precisely reveal a direct interaction between the RSV-mediated releasing of TNF-α, IL-6, and IL-10 by GRX in culture media, these results suggested that it is possible to establish an interesting hypothesis to be investigated, in which RSV could induce an anti-fibrogenic effect through its anti-inflammatory properties that could also prevent or revert HSC activation [Figure 3]. Undoubtedly, the mechanism in which TNF-α could be involved in the NFκB activation and its pro-apoptotic function depending on this transcript factor inhibition must be better investigated. In the same way, studying the RSV-mediated effects on the releasing of other cytokines or on the interaction between cytokines and HSC receptors must be important for better understanding the possible autocrine regulation between activated HSC that would contribute to fibrogenic regulation.

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FIGURES

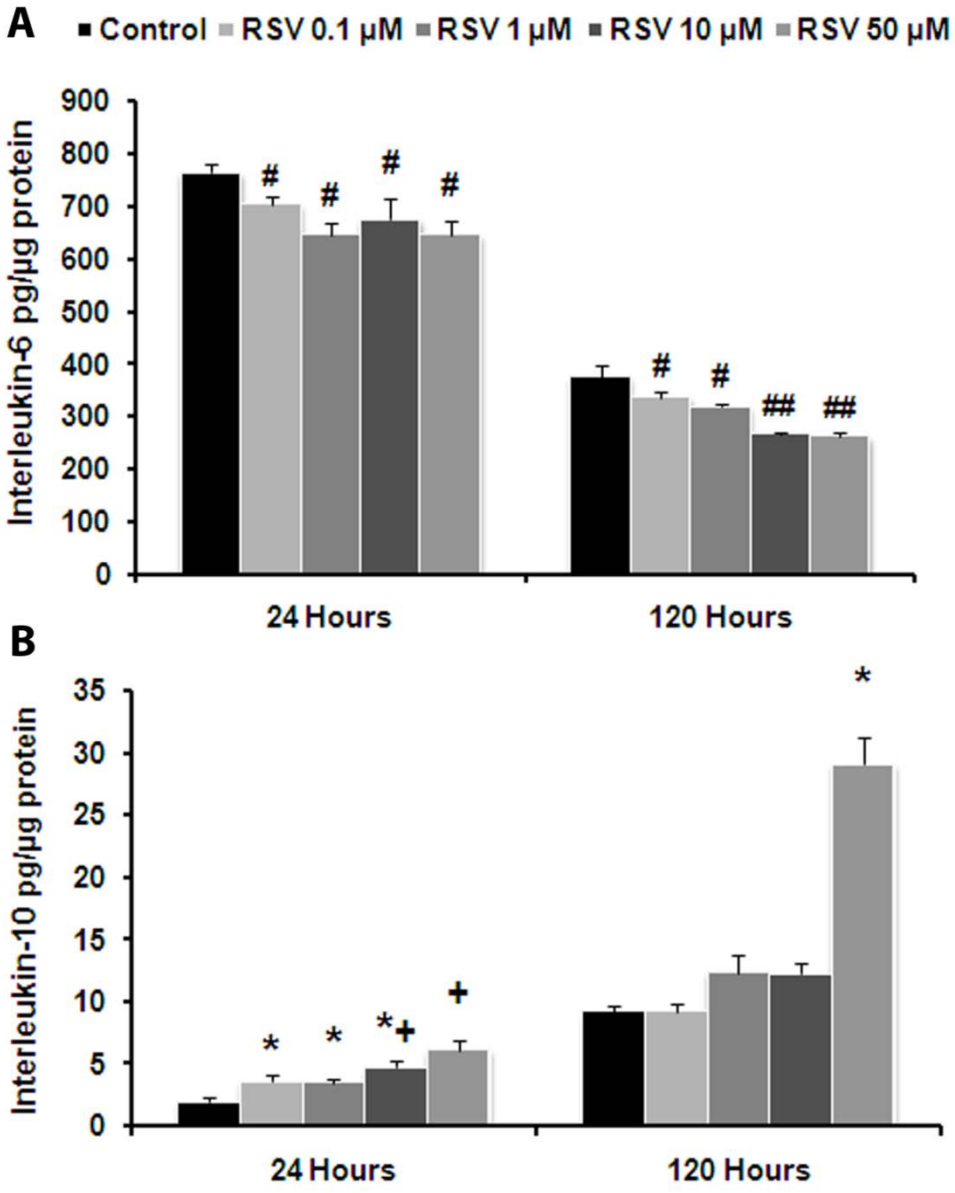

Figure 1 The effect of RSV on GRX releasing of Interleukin-6 (A) and 10 (B). GRX cells were treated with 0 (control), 0.1, 1, 10 and 50 µM of RSV for 24 or 120 hours. Similar symbols indicate which groups are statistically similar to each other ($p \leq 0.05$). Data is expressed as mean ± S.E.M. ($n = 4$).

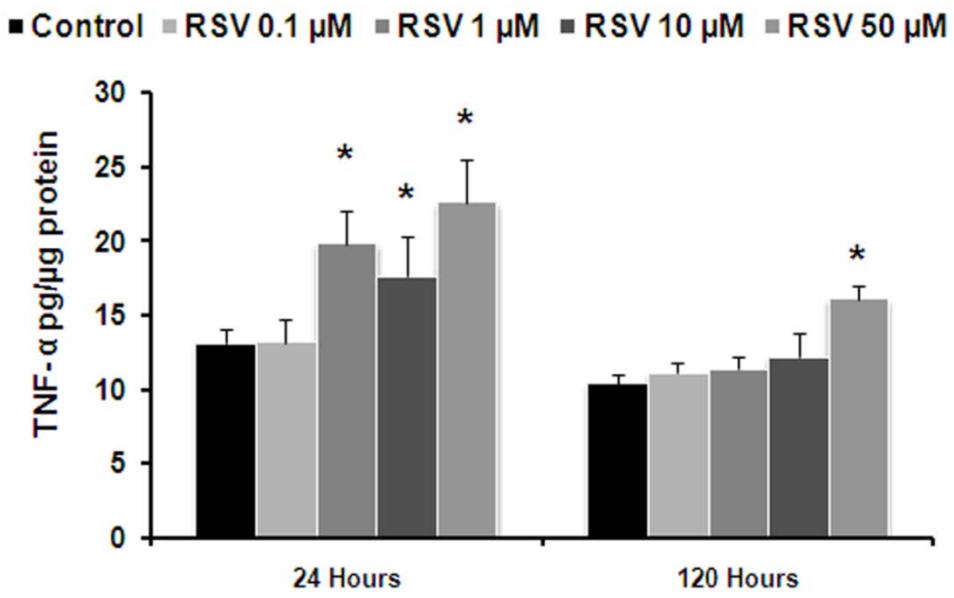

Figure 2 The effect of RSV on GRX releasing of TNF-α. GRX cells were treated with 0 (control), 0.1, 1, 10 and 50 µM of RSV for 24 or 120 hours. Single asterisk indicates significant differences between groups ($p \leq 0.05$). Data is expressed as mean ± S.E.M. ($n = 4$).

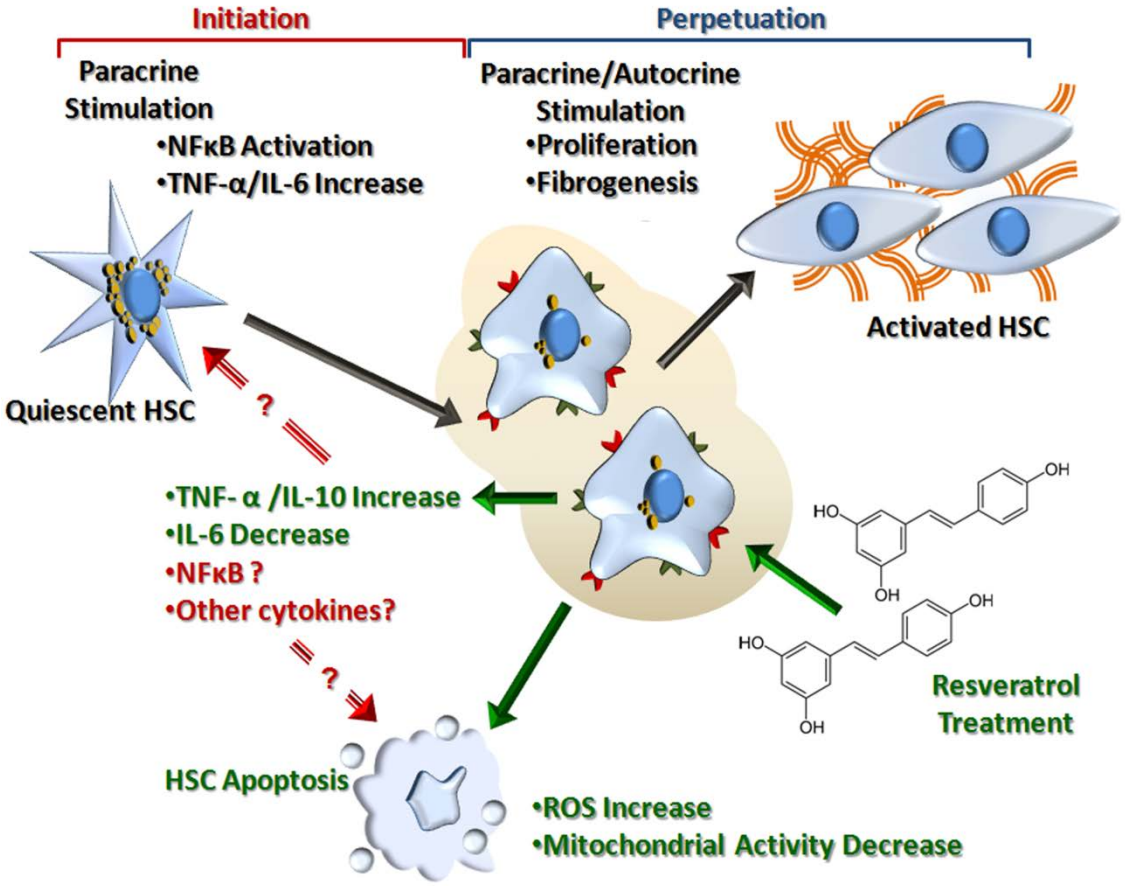

Figure 3 Liver fibrosis treatment focuses on the activated HSC resolution towards cell apoptosis or phenotypical reversion. Whether resveratrol could regulate NFκB or could favour other cytokines releasing appears to be an attractive way for understanding the mechanisms by which this molecule could exert its effects in our model. In the same way, how these events could be interrelated and their importance towards activated HSC apoptosis or phenotypical reversion must be investigated.

II.4. RESULTADOS COMPLEMENTARES

II.4.1 Os efeitos do Resveratrol sobre a produção de superóxido mitocondrial

O oxigênio molecular (O_2) se caracteriza por possuir quatro elétrons desemparelhados na sua última camada, o que lhe confere a capacidade de participar de diversos processos metabólicos. A cadeia respiratória mitocondrial é uma etapa importante para a produção de ATP e calor, sendo essencial para as células eucariotas que necessitam de uma alta demanda energética. Neste processo metabólico, o O_2 é um aceptor de elétrons de uma série de reações de oxirredução que pode, à medida que recebe um elétron, formar uma espécie reativa diferente até a sua redução total à água. As mitocôndrias são, portanto, uma grande fonte de espécies reativas (ER), que podem causar estresse oxidativo se não dismutadas adequadamente pelas enzimas antioxidantes intracelulares. De fato, cerca de 3-5% do O_2 utilizado na cadeia respiratória não é reduzido totalmente, podendo gerar ER [40, 65-66].

A espécie reativa (ER) superóxido (O_2^-) é o produto da reação de redução do oxigênio molecular por um elétron. Trata-se de uma molécula tóxica com um papel fisiológico relevante para o sistema imune celular, relacionado ao combate a microrganismos patogênicos. O superóxido também é importante para a formação de duas ER altamente tóxicas: o peroxinitrito ($ONOO^-$), produto da sua reação com a ER óxido nítrico (NO); e o peróxido de hidrogênio (H_2O_2), produto da sua dismutação pela enzima superóxido dismutase (SOD) [65-66]. O tratamento das células GRX por 24 e 120 horas com concentrações que variaram entre 0,1 e 50 µM de RSV resultou em um aumento de espécies reativas, fato que foi atestado pelo aumento da fluorescência relativa de2’-7’-diclorofluoresceína (DCF), uma molécula que tem afinidade grande pelo H_2O_2 e pelo $ONOO^-$ [67].

Além disso, o RSV induziu uma alteração significativa e paradoxal na atividade da SOD durante os dois tempos de tratamento: enquanto as células GRX tratadas com concentrações entre 1 e 50 µM por 24 horas apresentaram uma diminuição da atividade da SOD; as células em cultura por 120 horas apresentaram uma diminuição natural da atividade desta enzima, que foi prevenida pelo tratamento com RSV nas concentrações entre 0,1 e 50 µM.

Considerando que o O_2^- pode desempenhar um papel relevante e que o tratamento com RSV também alterou a função das mitocôndrias das células GRX, a quantidade desta ER foi investigada nestas organelas usando a sonda MitoSOX^{TM} (Invitrogen, Carlsbad, CA, EUA). Este reagente é especificadamente oxidado pelo O_2^- mitocondrial e passa a emitir uma fluorescência vermelha [68]. Por citometria de fluxo (FACS Calibur, BD Biosciences, CA, EUA), foi possível medir no canal FL3, a intensidade de fluorescência relativa em 10 mil eventos (células). Os resultados obtidos foram analisados pelo *software* FCS Express 4 (De Novo, Ontario, Canada). Após 24 horas, as células tratadas com 1 µM de RSV apresentaram um aumento da quantidade de O_2^- mitocondrial. Por outro lado, as células tratadas com 10 e 50 µM de RSV apresentaram uma diminuição da quantidade desta ER nas mitocôndrias. Nos tratamentos por 120 horas, apenas as células tratadas com a dose mais elevada de RSV apresentaram uma alteração significativa representada pelo aumento da quantidade de O_2^- mitocondrial [Figura 7].

Apesar do efeito pro-oxidante causado por todas as concentrações testadas de RSV mostrado no capítulo II.1, a produção de O_2^- mitocondrial não aumentou da mesma forma. De fato, a diminuição inesperada desta ER nas mitocôndrias de células tratadas com as concentrações mais altas de RSV por 24 horas chamou a

atenção. No entanto, alguns estudos já relataram que os efeitos pró-apoptóticos do RSV estão relacionados com a diminuição do pH citosólico. Neste sentido, a diminuição da concentração de O_2^- contribui para a acidificação intracelular e, desta forma, para a indução de apoptose [19, 69]. Considerando essa premissa, existe a possibilidade da diminuição na concentração de O_2^- ter relação com a indução de apoptose mostrada como um dos efeitos do RSV na GRX no capítulo II.2 desta tese.

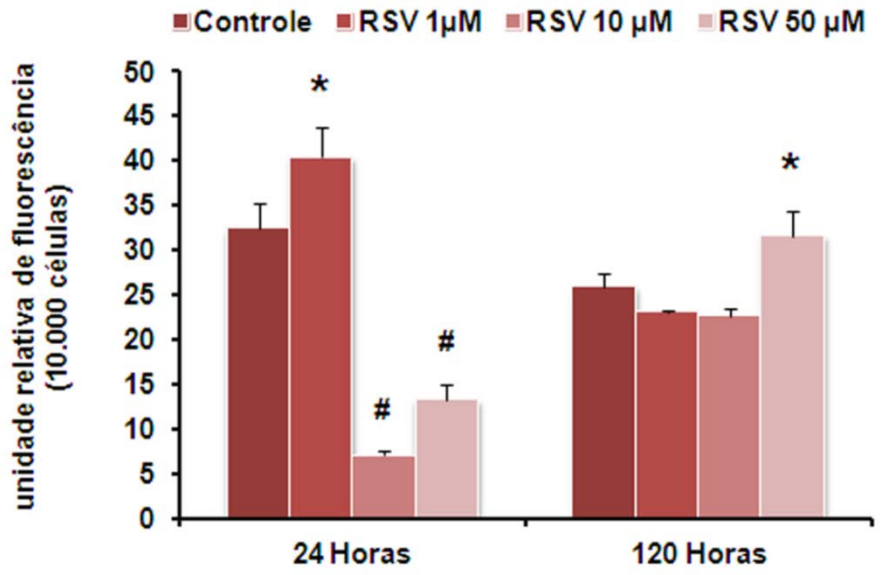

Figura 7 Efeitos do RSV na produção de O_2^- mitocondrial por citometria de fluxo, utilizando a sonda MitoSOX^{TM}. Durante 24 horas de tratamento, diferentes concentrações de RSV provocaram efeitos paradoxais quanto à produção desta espécie reativa. Durante 120 horas de tratamento, apenas a dose de 50 µM de RSV induziu uma alteração significativa ($p \leq 0,5$).

A redução considerável de O_2^- também pode estar relacionada com o aumento significativo e dependente da dose de H_2O_2 e de $ONOO^-$ intracelulares mensurados pelo DCF no capítulo II.1. Neste sentido, o H_2O_2 pode ser fonte importante do radical hidroxil através da reação de Fenton. Esta ER danifica as

cardiolipinas, lipídios estruturais das membranas mitocondriais, causando a perda de função mitocondrial, também contribuindo para a sinalização de morte celular. De maneira similar, um aumento de $ONOO^-$ pode danificar a estrutura da MnSOD, a SOD mitocondrial, contribuindo para diminuição da atividade desta enzima, conforme mostrado no trabalho anterior [70-71].

Ainda que os efeitos do RSV sobre a atividade da SOD, mostrados no capítulo II.1, não representem apenas a da SOD mitocondrial (MnSOD) e que a sonda MitoSOX^{TM} marque especificamente a quantidade de O_2^- mitocondrial, é possível pensar em uma hipótese na qual a própria concentração desta ER pode ser responsável por modular a atividade da SOD. Com base na literatura, a diminuição da concentração de O_2^- mostra um indício de que o tratamento com RSV, especialmente em doses mais altas, gera um efeito mais tóxico na GRX nas primeiras 24 horas de tratamento. De fato, ainda que o RSV tenha promovido efeitos pro-oxidantes nas células GRX nos dois tempos estabelecidos neste estudo, o maior equilíbrio observado na relação entre produção de ER e atividade das enzimas antioxidantes nas células tratadas por 120 horas deve ser fundamental para evitar ou atenuar o estresse oxidativo. Pelo exposto, é pertinente propor que a quantificação de O_2^- intracelular e de outras ER produzidas nas mitocôndrias das GRX tratadas com RSV seja uma abordagem interessante para o entendimento dos efeitos desta fitoalexina nas HSC.

II.4.2 Efeitos do Resveratrol após 120 horas de tratamento

O efeito de citotoxicidade associada a um aumento de espécies reativas é evidente na GRX tratada com 50 µM de RSV. A indução da morte celular

(associada a danos mitocondriais), da autofagia/mitofagia e da biogênese de mitocôndrias são respostas concomitantes ao tratamento da GRX com RSV por 24 horas. A relação entre estes eventos mostrou ser importante em determinar a sobrevivência ou morte destas células e deve estar relacionada com o efeito dose-dependente do RSV que acarreta em alta citotoxicidade e dano oxidativo em doses a partir de 50 µM. Os resultados seguintes são referentes aos efeitos do RSV na função mitocondrial, na morfologia da GRX e na indução da maturação de autofagossomos após 120 horas de tratamento[1]. Para estes experimentos, as células foram tratadas com 1, 10 e 50 µM de RSV. O meio de cultura com ou sem os tratamentos foi trocado diariamente até o dia dos experimentos.

II.4.2.1 Efeitos do Resveratrol na função mitocondrial da GRX

As sondas fluorescentes MitoTracker™ Green FM (MTG) e MitoTracker™ Orange CM-H2TM ROS (MTO) (ambas da Invitrogen) são amplamente utilizadas para marcar as mitocôndrias de células vivas para a visualização em microscopia de fluorescência ou confocal. O MTG emite fluorescência verde e tem afinidade pelos lipídios de membranas das mitocôndrias. O MTO emite fluorescência vermelha e tem afinidade por mitocôndrias que possuam potencial de membrana ativo. Portanto, estabelecer uma relação entre as intensidades de fluorescência destas duas sondas é uma maneira possível de se acessar a massa mitocondrial – que pode estar relacionada com o número ou com o inchaço (*swelling*) das

[1] Os resultados dos ensaios com MitoTracker™*Green/Orange*, JC1 e *Acridine Orange* referentes ao tratamento da GRX por 24 horas são os mesmos apresentados no capítulo II.2 da presente tese; portanto, não serão descritos neste capítulo. Exceto pelo ensaio com *Acridine Orange, o*s experimentos em ambos os tempos foram realizados no mesmo momento e com as mesmas condições; estão dispostos juntos com finalidade de comparação do efeito das diferentes concentrações de RSV na GRX nestes dois tempos distintos.

organelas – e a atividade mitocondrial. Quando a intensidade de fluorescência do MTO é maior do que a do MTG, o resultado pressuposto é de um aumento da atividade mitocondrial da célula. O contrário, quando a intensidade de fluorescência do MTG é maior do que a do MTO, o resultado pressuposto é a perda de função e o *swelling* mitocondrial, situação que caracteriza dano da organela e geralmente acarreta em morte celular [72].

Ao término do período de tratamento, as células foram tripsinizadas e incubadas em meio contendo 100 nM de MTG e MTO. Após 20 minutos, o meio de incubação foi descartado e as células lavadas com PBS. A medida de fluorescência foi realizada em um fluorímetro de placas (M5, Molecular Devices, EUA). A intensidade de fluorescência foi normalizada pela quantidade de proteína correspondente, que foi mensurada de acordo coma técnica de Lowry modificada por Peterson [73]. Apenas a concentração de 50 µM de RSV foi capaz de alterar a atividade mitocondrial na GRX tratada por 120 horas, enquanto que a massa da organela aumentou a partir do tratamento com 10 µM. A relação entre as intensidades de fluorescência de MTO e MTG revelou que apenas o tratamento com 50 µM de RSV induziu um aumento significativo da atividade sobre a massamitocondrial [Figura 8].

O marcador MitoScreenJC-1 (BD Bioscience, San Jose, CA, EUA) é usado para monitorar a polarização da membrana mitocondrial *(*$\Delta\psi m$). Este ensaio mede a atividade das mitocôndrias de maneira direta e a morte celular de maneira indireta, uma vez que este evento fisiológico está associado à perda de função desta organela. Trata-se de uma molécula monomérica permeável a membrana celular que emite fluorescência verde. Por ter afinidade pelas mitocôndrias ativas, se acumula formando agregados nestas organelas, mudando a emissão de

fluorescência para vermelho. Desta forma, os graus de polarização da membrana mitocondrial são quantificados pela mudança de emissão de fluorescência do JC-1 [74].

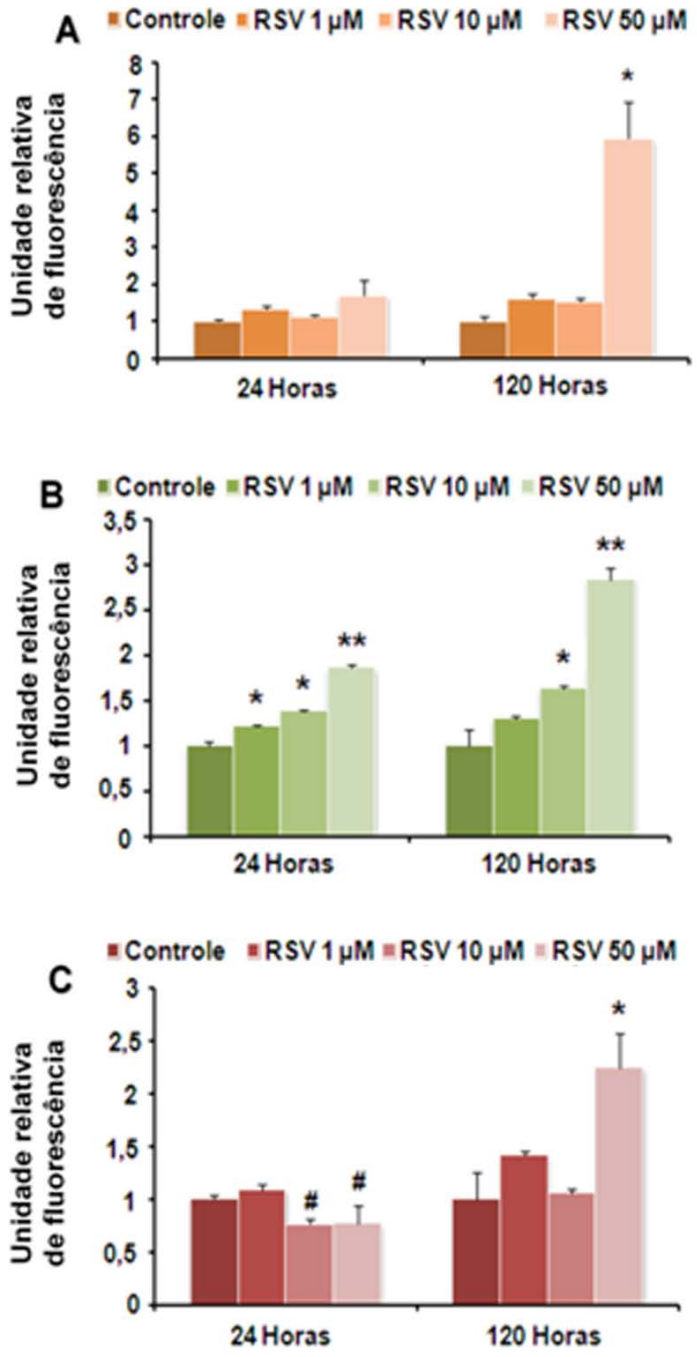

Figura 8 Efeitos do RSV na atividade (A) e na massa (B) mitocondrial da GRX. A relação entre a intensidade de fluorescência do MTO e do MTG (C) revelou um aumento significativo da atividade mitocondrial nas células tratadas por 120 horas com 50 µM de RSV (Controle=1; $p \leq 0,5$).

De maneira similar ao que foi descrito anteriormente, após o término do tratamento com RSV, as células foram tripsinizadas e suspendidas em solução contendo JC-1, preparada de acordo com as instruções do fabricante. A medida da fluorescência foi feita utilizando os canais verde (FL1) e vermelho (FL2) do citômetro de fluxo FACSCalibur (BD Biosciences), em 10.000 células. Apenas as células tratadas com 50 µM de RSV apresentaram um aumento significativo no potencial de membrana mitocondrial, indicando um aumento da atividade das mitocôndrias das células deste grupo [Figura 9]. Os resultados obtidos neste ensaio estão de acordo com os dados obtidos através da relação estabelecida entre as intensidades de fluorescência do MTO e do MTG [Figura 8].

Considerando os resultados apresentados nas figuras 8 e 9, a morfologia das mitocôndrias foi analisada por microscopia eletrônica de transmissão. A figura 10 mostra a ultraestrutura de células tratadas e não tratadas com 50 µM de RSV por 24 e 120 horas. O RSV promoveu um efeito citotóxico após 24 horas de tratamento, caracterizado pelo estresse citoplasmático e, principalmente, pela alteração da anatomia das mitocôndrias, que apresentaram uma desestruturação nas cristas e um aumento de área, caracterizando o *swelling* que acompanha a perda de função atestada pelos ensaios com MTO/MTG e JC-1. De fato, o aumento da massa associado com a diminuição da atividade mitocondrial é um indicativo de dano à organela. Nesta situação, o aumento na produção de espécies reativas e a falha no sistema antioxidante podem ser responsáveis por danificar as membranas das mitocôndrias, alterando sua morfologia. Este evento é geralmente acompanhado por um aumento de poros na membrana mitocondrial externa que leva ao inchaço da organela e à liberação do citocromo c, resultando

na ativação das caspases e, consequentemente, na apoptose [46, 54], como apresentado no capítulo II.2 deste estudo.

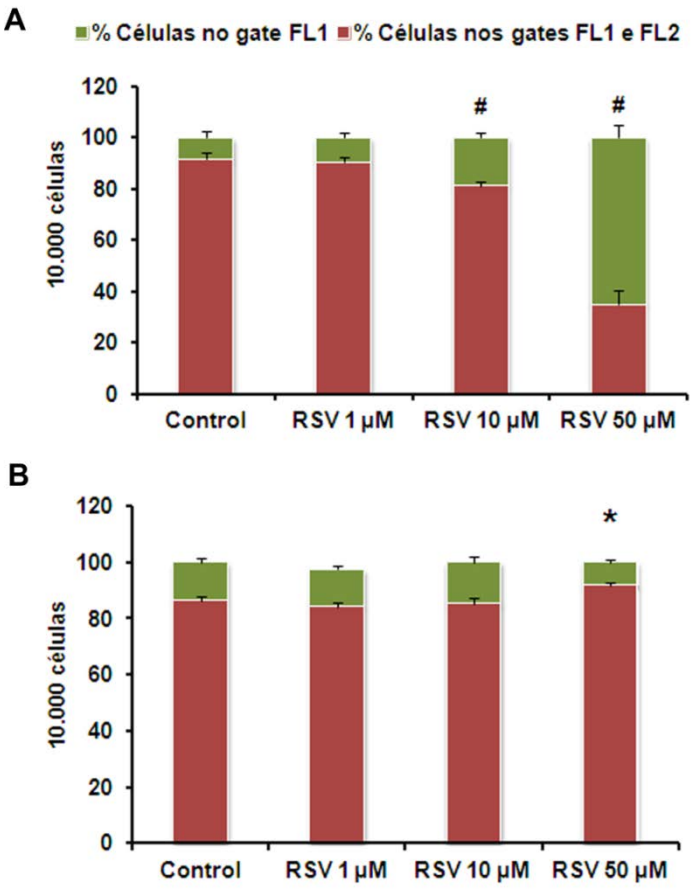

Figura 9 Efeitos do RSV na atividade mitocondrial avaliada pela marcação com a sonda JC-1. Foi medida a mudança de fluorescência – do verde para o vermelho, indicativo de potencial de membrana mitocondrial alto – em 10.000 células através de citometria de fluxo. (A) representa o gráfico com os resultados obtidos após 24 horas de tratamento. (B) apresenta os dados obtidos após 120 horas de tratamento (Controle=1; $p \leq 0,5$). Estes resultados confirmam os dados obtidos na relação entre as intensidades de fluorescência do MTO e do MTG.

Curiosamente, os efeitos do tratamento com 50 µM de RSV são visivelmente menos citotóxicos após 120 horas de tratamento. Em comparação com as imagens das células tratadas com esta concentração por 24 horas, é possível

observar uma alteração menos drástica na estrutura citoplasmática celular, além de numerosas mitocôndrias com área menor e bem delimitadas pelas suas membranas [Figura 10].

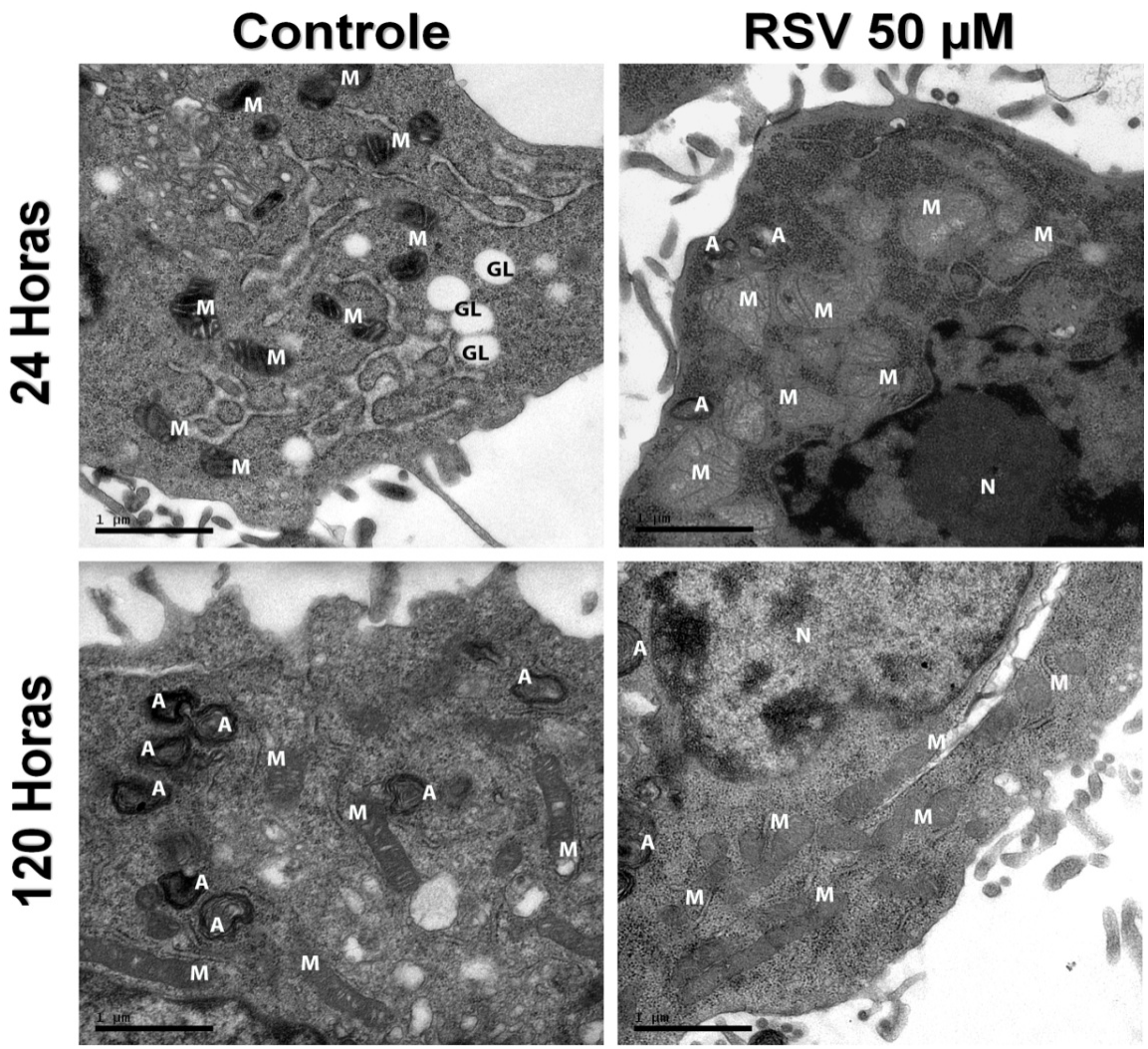

Figura 10 Efeitos do RSV na ultraestrutura da GRX. O tratamento com 50 µM de RSV por 24 horas provoca alterações mais evidentes na anatomia das mitocôndrias, caracterizando um efeito citotóxico maior. **M-**Mitocôndria; **N-**Núcleo; **A-**Autofagossomo; **GL**-Gotas lipídicas.

De fato, as imagens sugerem que há uma diminuição do dano mitocondrial e indicam uma recuperação da função destas organelas nas células tratadas com 50 µM de RSV por 120 horas. Apesar de ter sido mostrada pela relação

MTO/MTG que a atividade mitocondrial aumenta nas células tratadas com 50 µM de RSV, é importante ressaltar que a massa mitocondrial, medida pela marcação com MTG, está também aumentada. Este fato, somado a ausência de *swelling* mitocondrial nas células tratadas por 120 horas, pode sugerir que o número de mitocôndrias está aumentado neste grupo de tratamento, possivelmente devido ao aumento da biogênese de mitocôndrias. Este fenômeno deve estar relacionado com um aumento da demanda energética e pode estar de acordo com os resultados anteriores, que mostraram que estas células apresentam uma taxa de proliferação maior do que as células tratadas com a mesma concentração durante 24 horas e do que as células tratadas com as concentrações menores durante 120 horas.

Naturalmente, o aumento da função mitocondrial pode ser também responsável por um aumento da produção de ER nas células tratadas com 50 µM de RSV por 120 horas. No entanto, o dano oxidativo causado por esta concentração mais alta de RSV é menor em 120 horas do que em 24 horas de tratamento. Este fato deve estar relacionado com uma atividade mais eficiente das defesas antioxidantes celulares, como observado no capítulo II.1 desta tese, que também deve contribuir para o aumento da resistência destas células a este tratamento.

II.4.2.2 Os efeitos do Resveratrol sobre a maturação de autofagossomos na GRX e sobre a morfologia celular

As alterações nas células GRX em resposta a um desequilíbrio homeostático causado pelo tratamento com RSV podem incluir a modificação na estrutura das

organelas ou o aumento do número de autofagossomos, como atestado nos experimentos realizados em 24 horas. Curiosamente, as análises ultraestruturais da GRX em cultura por 120 horas revelaram que as células não tratadas tiveram um aumento do número de autofagossomos em relação às células não tratadas em cultura por 24 horas [Figura 10]. Por este motivo, foi realizado o ensaio com *Acridine Orange* (AO; Invitrogen), quantificado pelo citômetro de fluxo FACSCalibur (BD Biosciences), para determinar a maturação de autofagossomos na GRX em cultura por 120 horas. O AO é um marcador que emite fluorescência verde em toda célula. Em compartimentos ácidos, como dos autofagossomos maduros, o AO passa a emitir uma fluorescência vermelha. De maneira similar aos ensaios com MTO/MTG e JC-1, após as 120 horas de tratamento com as concentrações de RSV estabelecidas, as células foram tripsinizadas e incubadas em meio contendo 2,7 µM de AO por 15 minutos. O tratamento da GRX com RSV por 120 horas revelou que a quantidade de autofagossomos é similar em todos os grupos [Figura 11].

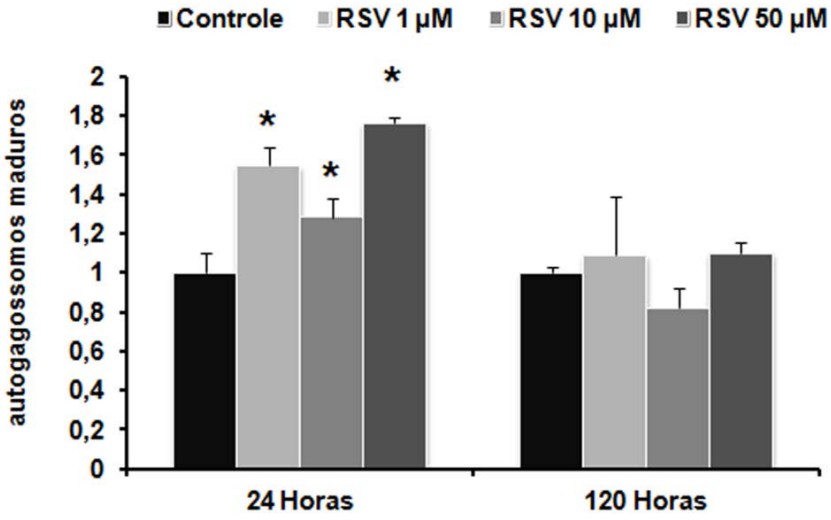

Figura 11 Efeitos do RSV na indução de autofagia avaliado pelo ensaio com *Acridine Orange*. As células tratadas por 120 horas com todas as concentrações de RSV apresentaram um número de autofagossomos similar às células não tratadas (Controle=1; $p \leq 0,5$).

As análises realizadas por citometria de fluxo também permitiram avaliar a morfologia celular através dos parâmetros que medem o tamanho (FSC; *forward scatter*) e a complexidade (SSC; *side scatter*) das células. Esta avaliação revelou que o tratamento e o tempo de cultura não alteraram o tamanho da GRX [Dado não mostrado]. No entanto, o tratamento com RSV por 24 horas induziu um aumento de complexidade citoplasmática, sendo cinco vezes maior nas células tratadas com 50 µM de RSV. Uma avaliação estatística usando ANOVA de duas vias revelou que o tempo de cultura também influenciou na alteração deste parâmetro. No entanto, apenas a dose de 50 µM induziu um aumento significativo da complexidade celular na GRX em cultura por 120 horas [Figura 12].

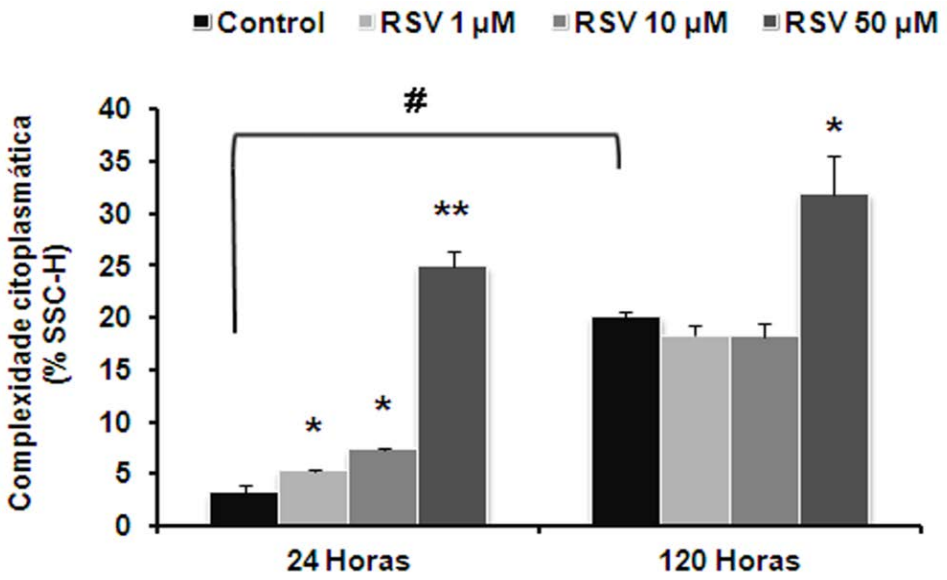

Figura 12 Efeitos do RSV na complexidade celular. Todas as concentrações de RSV alteraram a complexidade citoplasmática da GRX após 24 horas de tratamento. A avaliação por ANOVA de duas vias revelou que o tempo de cultura influenciou na alteração deste parâmetro, no entanto apenas a concentração mais alta provocou aumento na complexidade celular da GRX em 120 horas de cultura ($p \leq 0,5$).

As mudanças morfológicas na GRX, que contribuem para o aumento da complexidade celular, podem ter relação com a citotoxicidade provocada pelo RSV. Desta forma, as alterações nas estruturas das organelas, incluindo as mitocôndrias, ou o aumento do número de autofagossomos devem contribuir para aumentar a complexidade citoplasmática destas células. A possibilidade de que o tratamento com RSV por 120 horas induza biogênese de mitocôndrias, aumentando o número de organelas nestas células, é outro fator que pode contribuir para um aumento da complexidade citoplasmática e merece ser investigado. Da mesma forma, ainda que os resultados apresentados pelo ensaio com AO não tenham permitido avaliar com precisão estatística o efeito do tempo sobre a maturação dos autofagossomos, a possibilidade que o aumento da quantidade destas organelas na GRX, respaldadas pelas análises ultraestruturais da GRX em cultura por 120 horas, tenha relação com o aumento da complexidade morfológica destas células, também surge como uma perspectiva a ser melhor investigada.

PARTE III

III.1 DISCUSSÃO

O Resveratrol (RSV) é uma molécula amplamente estudada em diversos modelos biológicos e a capacidade do RSV em modular diversas vias metabólicas resulta em muitas respostas fisiológicas importantes. Apesar deste fato, os efeitos e os mecanismos de ação do RSV ainda não foram completamente desvendados. Entre estes efeitos, é notável o potencial do RSV em agir como uma molécula antioxidante e antiinflamatória que induz citoproteção para células normais, ou como um agente pro-oxidante que provoca citotoxicidade e morte de células em modelos que mimetizam patologias como, por exemplo, o câncer [17, 63].

As células estreladas hepáticas (HSC) são conhecidas por suas múltiplas funções. Uma das principais características destas células é a sua capacidade de transitar entre os fenótipos quiescente e ativado. No fenótipo ativado, as HSC desempenham um papel importante na cicatrização hepática, secretando citocinas que favorecem a regeneração de hepatócitos e a restauração da homeostase do fígado. No entanto, o estado inflamatório crônico resulta em uma ativação contínua e não controlada das HSC que tem, como consequência, o acúmulo de matriz extracelular fibrótica em razão de um desequilíbrio entre sua produção e degradação. Esta situação caracteriza a fibrose hepática, uma doença que acarreta em mudança da arquitetura do fígado e que acompanha outras patologias crônicas, como a cirrose e o carcinoma. Portanto, a compreensão do papel das HSC neste processo patológico é importante na procura por terapias antifibrogênicas adjuvantes para o tratamento das doenças crônicas hepáticas [7-9, 13]. A ação do RSV na prevenção de inúmeras doenças é relevante porque esta molécula é encontrada em diversos alimentos naturais. Diante de inúmeros

benefícios atribuídos ao uso do RSV, partiu-se do pressuposto de que esta fitoalexina deve atuar favoravelmente na prevenção ou no tratamento da fibrose hepática porque pode interferir nos mecanismos moleculares envolvidos na ativação das HSC ou induzir a apoptose das células ativadas [2, 8, 17].

No primeiro capítulo desta tese, foi descrito que o tratamento da GRX, um modelo de HSC ativadas, por 24 horas com concentrações entre 1 e 50 µM de RSV provocou um efeito pro-oxidante, diminuindo a atividade da superóxido dismutase. Apesar deste efeito, refletir um desequilíbrio do estado redox destas células, apenas a concentração mais alta efetivamente causou danos relacionados ao estresse oxidativo, diminuição de proliferação e redução drástica da população celular. O tratamento com as mesmas concentrações de RSV ao longo de 120 horas continuou a promover um efeito pro-oxidativo na GRX. No entanto, ainda que a atividade da superóxido dismutase tenha diminuído ao longo do tempo em que as células estiveram em cultura em todos os grupos experimentais, o tratamento com RSV preveniu o efeito do tempo sobre a diminuição da atividade da enzima. Além deste fato, apesar da população celular ainda ser menor do que a dos demais grupos, as células tratadas com a concentração mais alta de RSV apresentaram um dano oxidativo menor e uma maior taxa de proliferação, indicando uma recuperação destas células frente ao choque tóxico das primeiras 24 horas de exposição a esta fitoalexina.

A abordagem escolhida para entender os efeitos apresentados no capítulo II.1 foi a avaliação da função mitocondrial na GRX. Estas organelas são extremamente dinâmicas e importantes para a manutenção do metabolismo energético que garante a sobrevivência celular. Por outro lado, as mitocôndrias são fontes importantes de espécies reativas e também desempenham uma

função crucial mediando a indução de morte celular programada [44, 48-49, 75]. A função e a integridade das mitocôndrias dependem de um equilíbrio entre o tráfego destas organelas no citoplasma por meio dos processos de fissão e fusão (dinâmica mitocondrial), a autofagia (mitofagia) de organelas danificadas e a biogênese de novas organelas. Todos estes eventos normalmente são modulados pela alteração da demanda energética das células, que necessitam de um número ideal de mitocôndrias conforme a situação fisiológica em que se encontram. Neste sentido, uma consideração importante é que a diminuição ou o aumento exagerado na quantidade de mitocôndrias caracterizam diversas situações patológicas decorrentes de alterações na fisiologia/bioquímica celular, o que revela a importância da interação coordenada entre estes eventos. O aumento da necessidade energética celular leva a uma atividade mitocondrial maior. As organelas com metabolismo aumentado naturalmente produzem mais espécies reativas através da cadeia respiratória que, por sua vez, podem danificar as membranas mitocondriais e celulares. As mitocôndrias danificadas que não são eliminadas por mitofagia perdem sua função no metabolismo energético, diminuem sua atividade e acabam por mediar o início da sinalização de vias clássicas de morte celular, como as de apoptose e necrose [46, 49, 54, 76-77].

Os processos de dinâmica mitocondrial são importantes para a manutenção de organelas saudáveis: a fusão mitocondrial permite a troca de componentes danificados por componentes íntegros de outras mitocôndrias, enquanto que a fissão mitocondrial “separa” os componentes danificados em organelas que serão eliminadas por mitofagia. Neste contexto, a biogênese de mitocôndrias é um processo muito importante não apenas para atender a um aumento da demanda energética celular, o que implica no aumento do número de organelas e,

consequentemente, do DNA mitocondrial, mas para a manutenção do número ideal de organelas saudáveis, substituindo as organelas danificadas e eliminadas por mitofagia [54, 71, 78-80]. Além dos efeitos pró-apoptóticos já descritos, o RSV é conhecido por regular a função e a biogênese mitocondrial e por promover a autofagia [25-26, 60-64]. Curiosamente, estes dois últimos eventos tem relação com a sobrevivência celular frente a alterações homeostáticas drásticas, com a redução da produção de espécies reativas e com o aumento da longevidade mediado pelo RSV [46, 54, 80].

Nas células tratadas por 24 horas, todas as concentrações de RSV provocaram alterações significativas na função mitocondrial, que resultou na mudança da distribuição das organelas no citoplasma e na ativação da via de morte celular por apoptose mediada por caspases. Além deste fato, todas as concentrações de RSV induziram um aumento de massa mitocondrial na GRX, o que poderia significar um aumento no número de mitocôndrias nestas células. Entretanto, foi observada a biogênese de mitocôndrias apenas nas células tratadas com 1 e 10 µM de RSV. Nesta situação, seria esperado que pelo menos estes grupos de tratamento também apresentassem a atividade mitocondrial aumentada. No entanto, os testes que avaliaram potencial da membrana mitocondrial, indicativo de atividade da organela, não mostraram um aumento do número de organelas ativas. Pelo contrário, o tratamento com 10 e 50 µM de RSV induziu uma diminuição da atividade das mitocôndrias nas células. A partir destes resultados, foi possível supor que o RSV nas concentrações de 1 e 10 µM não apenas induziu o aumento no número de mitocôndrias, mas provocou um inchaço (*swelling*) mitocondrial. Este fenômeno pode estar relacionado não apenas com dano às mitocôndrias, diminuindo a função/atividade destas organelas, mas à

ativação das caspases por meio da liberação do citocromo c, e à indução de apoptose da GRX em resposta ao tratamento com todas as concentrações de RSV.

As análises ultraestruturais da GRX mostraram alterações nas estruturas das cristas mitocondriais nas células tratadas com 10 μM de RSV e o inchaço visivelmente maior das organelas nas células tratadas com 50 μM. Neste contexto, ainda que tenha sido observada uma diminuição na quantidade de superóxido mitocondrial nas células tratadas com as concentrações mais altas, é importante ressaltar que esta espécie reativa é uma importante fonte de outras espécies reativas consideravelmente tóxicas para membranas celulares. Portanto, é perfeitamente plausível supor que o efeito pro-oxidante do RSV tenha relação com a alteração da função e com o dano mitocondrial. Desta forma, o tratamento com RSV resultaria em uma alteração do funcionamento da cadeia respiratória mitocondrial que levaria a um aumento da produção de espécies reativas que, por sua vez, contribuem para danificar drasticamente as mitocôndrias, alterando sua morfologia. O fato de não ocorrer a biogênese de mitocôndrias nas células tratadas com 50 μM de RSV, junto ao evidente dano encontrado nestas organelas e nas demais estruturas celulares, indica que a renovação das mitocôndrias nestas células não acontece com eficiência. Esta situação resulta em uma maior perda de função mitocondrial que acarreta no aumento da debilidade celular devido à deficiência no metabolismo energético, situação que deve favorecer a morte [78, 80].

Junto com biogênese de mitocôndrias íntegras, a autofagia das mitocôndrias danificadas (mitofagia) reduz a sinalização de morte mediada por estas organelas [54]. Os experimentos realizados (*Acridine Orange* e *immunobloting* das proteínas

LC3 I e II) indicaram que todas as concentrações de RSV induziram a formação de autofagossomos maduros na GRX. As análises ultraestruturais de células tratadas com 50 µM de RSV confirmaram um aumento de autofagossomos no citoplasma das células. Paralelamente, as análises de colocalização de mitocôndrias e lisossomas na GRX tratada com RSV confirmaram um aumento de mitofagia nestas células. No entanto, apesar de todas as evidências que apontaram para a indução de autofagia/mitofagia na GRX por todas as concentrações de RSV, a expressão da proteína relacionada com autofagia 7 (ATG 7) diminuiu nas células tratadas com 50 µM de RSV.

A ATG 7 é uma das diversas proteínas que estão envolvidas no processo de autofagia, desempenhando um papel importante na maturação dos autofagossomos por induzir a fusão de membranas que formam estas estruturas[81-82]. Desta forma, a diminuição de expressão da ATG 7 nas células tratadas com 50 µM pode parecer um resultado contraditório aos demais que apontaram o aumento da formação de autofagossomos. Mas o fato é que a diminuição da expressão desta proteína pode indicar que a sinalização para induzir autofagia (em específico, a maturação dos autofagossomos) nestas células está prejudicada após as 24 horas de tratamento. Neste sentido, o aumento de autofagossomos ou de mitofagia nas células tratadas com 50 µM de RSV por 24 horas pode refletir uma tentativa destas células em reestabelecer o equilíbrio homeostático que não foi eficaz; e, neste caso, a indução de autofagia não evitaria a morte celular. Portanto, a maior produção de espécies reativas nas células tratadas com 50 µM de RSV pode estar relacionada às alterações da função mitocondrial, às falhas no sistema antioxidante celular e à falha da mitofagia. Em conjunto, estes eventos podem ter relação com o dano oxidativo e

morte mais evidentes nestas células, reforçando a ideia de um efeito “dose-dependente” no tratamento com esta fitoalexina.

Recentemente, as respostas controversas ao tratamento com RSV em diversos modelos de estudo tem sido atribuídas a um efeito denominado *hormesis* [21, 23, 83]. Este fenômeno caracteriza as respostas biológicas favoráveis a doses inofensivas de compostos químicos e outros fatores de estresse. Desta forma, os compostos que estimulam a *hormesis* induzem o início de uma resposta adaptativa ao estresse que tornam as células (ou organismos) resistentes a doses elevadas (e geralmente prejudiciais) do mesmo agente. Na teoria, a *hormesis* pode ser um mecanismo importante pelo qual as células em condições de estresse evitam o dano e, em última instância, a morte [21, 84]. De fato, várias moléculas de sinalização e muitos processos fisiológicos celulares estão envolvidos neste fenômeno [41]. Ainda que as espécies reativas estejam relacionadas com danos às estruturas celulares, é amplamente aceito que estas moléculas também podem participar de vias de sinalização redox-sensíveis que resultam em efeitos pró-sobrevivência celular [83]. De fato, o aumento da formação de espécies reativas pode resultar na biogênese de mitocôndrias [85], na autofagia/mitofagia [54, 56] e na regulação positiva da expressão de genes de proteínas antioxidantes [86]. Estes efeitos contribuem para uma reposta adaptativa *hormética* através da qual a resistência ao primeiro impacto tóxico culmina na redução do estresse oxidativo e da morte celular em longo prazo [87]. A resposta da GRX ao tratamento com RSV tem semelhanças como fenômeno descrito como efeito *hormesis*. De fato, o tratamento com 1 e 10 µM de RSV por 24 horas também promove citotoxicidade e aumento da produção de ER. No entanto,é possível que estas células exibam uma resposta de resistência ao

estresse mais eficiente após as primeiras horas de exposição ao RSV, gerando um restabelecimento homeostático mais rápido do que o observado nas células tratadas com a concentração de 50 µM.

Apesar do efeito citotóxico alto do tratamento com 50 µM de RSV por 24 horas, caracterizado pelo grande dano oxidativo e pela morte celular considerável, as células sobreviventes permaneceram proliferativas ao longo de 120 horas de tratamento com esta concentração. Curiosamente, estas células não apresentaram danos oxidativos tão significativos quanto os observados em 24 horas de tratamento, indicando que os efeitos desta concentração de RSV são relativos ao tempo de tratamento. Os testes realizados nas células tratadas por 120 horas revelaram que a função mitocondrial em resposta ao tratamento com 10 µM de RSV foi restabelecida. Nas células tratadas com 50 µM de RSV por 120 horas, a atividade mitocondrial é maior. Este fato pode estar relacionado com os resultados apresentados no capítulo II.1, em que se observou que as células tratadas com a maior concentração de RSV por 120 horas apresentavam uma taxa de proliferação alta. Esta situação também caracteriza o fenômeno *hormesis*, em que as células que sobreviveram se adaptaram ao alto impacto tóxico das primeiras 24 horas de tratamento.

As análises ultraestruturais das células tratadas com 50 µM por 120 horas confirmaram que suas organelas, em especial as mitocôndrias, apresentam uma morfologia semelhante às células não tratadas, o que sugere a recuperação de funcionalidade. Naturalmente, a atividade mitocondrial mais elevada nestas células deve contribuir para a maior produção de espécies reativas, como atestado pela mensuração das espécies reativas intracelulares (capítulo II.1) e do superóxido mitocondrial (capítulo II.4). Neste sentido, a resistência destas células

à ação das espécies reativas deve estar relacionada com a resposta antioxidante mais eficiente e, possivelmente, com o equilíbrio entre a biogênese mitocondrial e a autofagia/mitofagia. Esta situação que corresponde a uma resposta adaptativa do metabolismo mitocondrial em reposta ao aumento de espécies reativas tem sido denominada como *mitohormesis* [87]. Seguramente, outros testes devem ser realizados para a melhor compreensão dos eventos envolvidos nessa possível adaptação ou resistência mitocondrial aos danos evidentes observados em 24 horas.

A perpetuação da ativação das HSC inclui a aquisição e a manutenção das funções que envolvem, por exemplo, a secreção de citocinas inflamatórias. Esta capacidade das HSC ativadas é importante porque algumas destas citocinas são responsáveis pela sinalização autócrina que mantém seu estado de ativação[8, 12]. Entre as citocinas envolvidas na modulação fenotípica e na atividade das HSC, o fator de necrose tumoral-α (TNF-α) e a interleucina-6 (IL-6) são mediadores pró-inflamatórios que desempenham um papel importante na ativação das HSC. No entanto, ambas citocinas também desempenham funções favoráveis ao fígado na resposta a um dano: enquanto que o TNF-α também estimula a diminuição da produção de colágeno tipo I pelas HSC, a IL-6 é a principal citocina envolvida no estímulo de regeneração de hepatócitos. A interleucina-10 exerce uma função antiinflamatória que promove a regulação da ativação das HSC e a apoptose de células ativadas [88-92]. Considerando o papel antiinflamatório do RSV, o presente estudo também avaliou a capacidade da GRX em liberar TNF-α, IL-6 e IL-10.

O tratamento da GRX por 24 horas com 1, 10 e 50 μM de RSV resultou no aumento da liberação de TNF-α no meio de cultura das células. No entanto,

apenas a concentração mais elevada provocou este efeito na GRX tratada por 120 horas.É importante ressaltar que o modelo experimental utilizado é a cultura de uma linhagem celular e que a sinalização inflamatória que intermedeia o desenvolvimento das doenças crônicas do fígado envolve outros tipos celulares hepáticos. Essas considerações servem para salientar que os resultados deste estudo permitem acessar a função das HSC na sinalização autócrina e não sua função na fisiologia hepática. Portanto, diante dos resultados apresentados no capítulo II.3, poder-se-ia hipotetizar que o aumento da liberação do TNF-α pela GRX, induzido pelo tratamento com RSV, esteja relacionado com uma sinalização que favorece a diminuição de fibrogênese, inibindo a produção do colágeno tipo I[88], algo que deve ser avaliado.Curiosamente, o TNF-α pode mediar a via pró-apotótica dependendo da inativação do NFκB, fato que resulta em diminuição da função mitocondrial, aumento da produção de espécies reativas e ativação da via das caspases [93-94], resultados que estão de acordo com os apresentados no capítulo II.2, ainda que a ativação do NFκB ainda reste por ser também avaliada na GRX. O tratamento da GRX por 24 e 120 horas com 0,1, 1, 10 e 50 µM de RSV provocou a diminuição da liberação da IL-6 no meio de cultura. Por outro lado, a secreção de IL-10 aumentou em células tratadas nas mesmas condições por 24 horas. Nas células tratadas por 120 horas, apenas 50 µM de RSV induziu o aumento da liberação de IL-10.Considerando o papel destas citocinas, o RSV repetiu o efeito antiinflamatório descrito em outros trabalhos.Este efeito deve contribuir para a prevenção ou redução do estímulo para a ativação da GRX, fato que também deve ser considerado uma perspectiva interessante a ser investigada para o tratamento da fibrose hepática.

O tratamento de HSC ativadas com RSV modulou eventos importantes que podem determinar não apenas o estimulo de ativação, que inclui o aumento de espécies reativas, mas o destino destas células quanto à morte ou à sobrevivência. A indução de apoptose da GRX, um modelo bem estabelecido de HSC ativadas, é uma das perspectivas almejadas para a resolução da fibrose hepática; no entanto, a ativação concomitante da autofagia e da biogênese de mitocôndria parece favorecer a sobrevivência das células. Uma vez que as HSC constituem uma população heterogênea que, em estado de ativação controlado, desempenha funções importantes na regeneração de hepatócitos e na detoxificação hepática, a manutenção destas células em baixa proliferação (ou em uma população reduzida) e o possível controle do estímulo de ativação ou da perpetuação da ativação (efeito de quimioprevenção) é interessante, especialmente considerando que a fibrose acompanha outras doenças crônicas do fígado, como a cirrose. Portanto, os estudos que visam estabelecer parâmetros para a utilização do RSV como adjuvante no tratamento da fibrose hepática devem considerar a redução do estímulo para a ativação, controlando o estado pró-inflamatório ou mesmo a sinalização autócrina e pró-fibrogênica destas células, e a escolha de uma dose de RSV que favoreça a apoptose ao invés da autofagia ou da biogênese de mitocôndrias, para que haja uma redução efetiva da população de células ativadas.

Apesar de induzir efeitos promissores quanto à resolução da fibrose hepática, a necessidade de se entender outros alvos de ação do RSV na GRX ou em outros modelos de HSC ativadas – ou não – é evidente. Assim, outros estudos são necessários para obtermos respostas mais amplas que possibilitem a utilização do RSV no tratamento desta patologia. Até o presente momento, os trabalhos

publicados nas diversas áreas apresentam efeitos variados (muitos são paradoxais) que dependem da concentração e da forma em que o RSV é administrado. Indubitavelmente, estes efeitos do RSV devem ser testados em modelos mais abrangentes de estudo das doenças hepáticas crônicas.

III.2 CONCLUSÕES

Ao contrário do efeito antioxidante mostrado em inúmeros trabalhos que estudam os efeitos do resveratrol, esta fitoalexina induziu um aumento de espécies reativas no nosso modelo de estudo, a linhagem celular GRX, representativas de HSC ativadas. De fato, o aumento de espécies reativas é um fator determinante para o desenvolvimento de diversas patologias, incluindo a fibrose hepática.

No presente trabalho, no entanto, este efeito pro-oxidativo do resveratrol foi relacionado com a diminuição de viabilidade da GRX que acarretou na diminuição da população destas células, fato que consiste em um dos objetivos de tratamento da fibrose hepática. Este resultado deve estar associado à indução da parada do ciclo celular [35] e da apoptose, observados nas células tratadas com todas as concentrações de resveratrol, e ao estresse oxidativo, observado nas células tratadas com a concentração de 50 μM. As alterações provocadas por esta fitoalexina no metabolismo mitocondrial da GRX resultam no aumento de espécies reativas intracelulares; e a interação entre a indução de apoptose mediada por mitocôndrias danificadas, mitofagia e biogênese de mitocôndrias e são determinantes para estabelecer o destino das células quanto à morte ou sobrevivência.

Os efeitos do resveratrol na GRX são explicados pelo fenômeno definido como *hormesis*, pelo qual as concentrações tóxicas e não letais de uma determinada molécula bioativa tornam células ou organismos mais resistentes a uma exposição crônica ou a uma exposição a concentrações mais altas a mesma molécula. As concentrações mais baixas de resveratrol parecem promover um

efeito hormético pelo qual as células se recuperam mais rapidamente à alteração homeostática promovida pelo tratamento. A concentração de 50 µM de resveratrol também parece promover o efeito hormético, porém as células necessitam mais tempo para se recuperar. De fato, alguns parâmetros avaliados após 120 horas de tratamento, como a ultraestrutura da GRX e a atividade das mitocôndrias, sugerem que as células tratadas com 50 µM de resveratrol se recuperam.

O tratamento com resveratrol alterou a secreção das Interleucina-10 e Interleucina-6 na GRX, sugerindo uma ação antiinflamatória do resveratrol. De maneira similar, o aumento da secreção de TNF-α pela GRX pode ser um efeito potencialmente antifibrogênico, considerando a possibilidade de que esta molécula esteja relacionada com a inibição da secreção de colágeno tipo I.Estes efeitos são condizentes com a ação quimiopreventiva do RSV descrita em inúmeros trabalhos. Em conjunto, considerando que a GRX é um modelo patológico de estudo, estes resultados sugerem que o resveratrol é um candidato promissor para futuras terapias anti-fibróticas que visem induzir a diminuição de HSC ativadas e controlar a ativação das HSC diminuindo a sinalização pro-inflamatória também mediada por estas células. No entanto, determinar uma concentração que efetivamente promova a apoptose de HSC ativadas é relevante.

III.3 PERSPECTIVAS

Em relação ao apresentado nesta tese, aprofundar o estudo sobre os efeitos do RSV quanto ao estímulo de resistência à citotoxicidade nas HSC tratadas por mais de 24 horas ainda resta como uma grande perspectiva. Neste sentido, o estudo do ciclo de vida mitocondrial, da autofagia/mitofagia e da sinalização de morte ao longo de um tratamento mais longo deve ser avaliado. Da mesma forma, os mecanismos moleculares comuns, que determinam a interação entre estes fenômenos, surgem como outra perspectiva importante de estudo. Além destas metas, o estudo dos efeitos antiinflamatórios do RSV precisa ser ampliado, assim como seus efeitos sobre a função fibrogênica das HSC. Quanto ao estudo da fibrose hepática, avaliar os efeitos dose-dependentes do RSV em células primárias, outras linhagens celulares (como por exemplo, as linhagens de HSC humanas LX-1 E LX-2) e em um modelo animal faz-se necessário. Sob este aspecto, tanto os efeitos citotóxicos do RSV podem ser explorados/estudados para o tratamento de indivíduos com fibrose hepática, quanto os efeitos quimiopreventivos desta fitoalexina podem ser avaliados em indivíduos saudáveis que possam ser induzidos a desenvolver esta patologia.

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ANEXOS

LISTA DE FIGURAS

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II.1 ARTIGO 1

II.2 ARTIGO 2

II.3 ARTIGO 3

II.4 RESULTADOS COMPLEMENTARES