

**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL  
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE  
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS –  
BIOQUÍMICA**

**RESVERATROL E GLIOMA C6: UMA ABORDAGEM CELULAR,  
MOLECULAR E PROTETORA CONTRA ESTRESSE OXIDATIVO  
INDUZIDO POR PERÓXIDO DE HIDROGÊNIO**

**ANDRÉ QUINCOZES DOS SANTOS**

**ORIENTADORA: PROFA. CARMEM GOTTFRIED**

**PORTO ALEGRE**

**2007**

**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL  
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE  
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS –  
BIOQUÍMICA**

**RESVERATROL E GLIOMA C6: UMA ABORDAGEM CELULAR, MOLECULAR  
E PROTETORA CONTRA ESTRESSE OXIDATIVO INDUZIDO POR PERÓXIDO  
DE HIDROGÊNIO**

**ANDRÉ QUINCOZES DOS SANTOS**

**Orientadora: Profa. Carmem Gottfried**

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Mestre em Bioquímica.

Porto Alegre

2007

Dedico esta Dissertação aos meus pais, pelo constante incentivo e compreensão.

“A mente que se abre a uma nova  
idéia jamais voltará ao seu tamanho  
original”.

Albert Einstein

## AGRADECIMENTOS

Agradeço:

À Carmem e ao CA, meus pais científicos, pelo exemplo de dedicação à pesquisa, preocupação com o aprendizado e bem-estar de seus alunos. Em especial à Carmem, pela orientação e oportunidade de crescimento profissional, disposição para me ensinar, pela compreensão, companheirismo e amizade.

Aos meus colegas do Laboratório 33, por transformarem o ambiente de trabalho num ambiente de agradável convívio.

À minha “equipe de trabalho”: Alessandra, Alexandra, Ana Cristina, Daniela, Lúcia e Maria Caroline, que sempre estiveram dispostas a me ajudar, independente do horário e técnica a ser realizada.

À Cláudia, pela amizade desde a iniciação científica e ajuda constante no mestrado, tornando-se “minha bolsista pós-doc”, bem como pelos ensinamentos e troca de idéias.

À Patrícia, pelo coleguismo e amizade; pelo seu duplo papel, o de minha “co-orientadora”, pela passagem de conhecimentos e, o de “bolsista”, por estar sempre presente nos meus experimentos.

À Regina, pela oportunidade de Iniciação Científica e ingresso no Departamento de Bioquímica.

Ao Departamento de Bioquímica pela infra-estrutura oferecida aos seus alunos, bem como aos meus colegas e amigos de Pós-Graduação.

Aos meus amigos extra UFRGS e, principalmente, as amigas que me acompanharam desde a Faculdade de Farmácia: Bina, Clau, Gabi e Thaís, pelo incentivo e torcida.

Aos meus familiares, especialmente à tia Virgilina pela amizade e ajuda desde quando cheguei a Porto Alegre.

Aos meus irmãos Adriano e Luciane, à minha cunhada Ariceni, pelo incentivo e cumplicidade e aos meus sobrinhos Pedro e Lavínia que tornam maravilhosos os momentos em família.

E por fim aos meus pais, Inocencio e Ayda, meus grandes incentivadores, que não mediram esforços para eu estudar e realizar o mestrado; pelo amor, pela presença constante e apoio incondicional em todos os momentos da minha vida.

## RESUMO

O antioxidante resveratrol, uma fitoalexina encontrada, principalmente em uvas e também em quantidades significativas em vários tipos de vinho tinto, é um promissor produto natural, com atividades antitumoral, cardioprotetora e neuroprotetora. O objetivo do presente estudo foi investigar em células de glioma C6 o efeito do resveratrol sobre proliferação celular e alguns parâmetros específicos relacionados a astrócitos (captação de glutamato, glutamina sintetase e secreção de S100B), comumente associados com o papel protetor dessas células. Além disso, foi investigado o efeito genoprotetor do resveratrol em condições de estresse oxidativo induzido por peróxido de hidrogênio ( $H_2O_2$ ) em células C6. A proliferação celular foi significativamente reduzida após tratamento com 100 e 250  $\mu M$  de resveratrol. Uma rápida incubação com resveratrol (1 h) induziu um aumento linear na captação de glutamato e na atividade da glutamina sintetase. O dano ao DNA foi medido pelo ensaio Cometa. Para investigar os efeitos do resveratrol sobre o dano ao DNA induzido por  $H_2O_2$  utilizou-se dois modelos de indução de estresse oxidativo. A mudança de parâmetros gliais pode contribuir para o papel protetor de astrócitos em condições de injúria cerebral, reforçando o uso deste composto no arsenal terapêutico contra doenças neurodegenerativas e desordens isquêmicas. Resveratrol também foi capaz de prevenir o dano oxidativo ao DNA celular, provavelmente, devido a suas propriedades antioxidantes, isto pode ser importante para proteger o DNA em doenças relacionadas com estresse oxidativo.

## ABSTRACT

The antioxidant resveratrol, a phytoalexin found mainly in grapes and also substantial amounts in several types of red wine, is a promising natural product with anticancer, cardio-protective and neuroprotective activities. The objective of the present study was to investigate in C6 glioma cells, the effect of resveratrol on cell proliferation, cell death and some specific parameters of astrocyte activity (glutamate uptake, glutamine synthetase and secretion of S100B) commonly associated with the protective role of these cells. Furthermore, it was investigated the genoprotective effects of resveratrol under conditions of oxidative stress induced by hydrogen peroxide ( $H_2O_2$ ) in C6 cells. Cell proliferation was significantly decreased following treatment with 100 and 250  $\mu$ M resveratrol. Short-term (1 h) of resveratrol exposure induced a linear increase in glutamate uptake and in glutamine synthetase activity. DNA damage was assessed by the comet assay. For investigate the effects of resveratrol against oxidative stress induced by  $H_2O_2$  on DNA damage, two models of oxidative stress induction were used. Changes in glial activities can contribute to the protective role of astrocytes in brain injury conditions, reinforcing the use of this compound in the therapeutic arsenal against neurodegenerative diseases and ischemic disorders. Resveratrol was able to prevent oxidative damage to cellular DNA, probably, due to its antioxidant properties, it may be important in diseases for protecting against DNA damage through oxidative stress.



<b>ÍNDICE</b>	<b>PÁGINA</b>
<b>INTRODUÇÃO.....</b>	<b>1</b>
1. Resveratrol.....	1
2. Gliomas.....	5
2.1 <i>Linhagem de glioma de rato C6.....</i>	6
3. Astrócitos.....	7
4. Marcadores Gliais em astrócitos e células de glioma C6.....	9
4.1 <i>Proteína Glial Fibrilar Ácida.....</i>	9
4.2 <i>S100B.....</i>	10
4.3 <i>Neurotransmissão Glutamatérgica.....</i>	12
4.4 <i>Captação de Glutamato e Glutamina Sintetase.....</i>	13
5. Radicais Livres e Espécies Reativas de Oxigênio.....	15
5.1 <i>Estresse Oxidativo.....</i>	16
5.2 <i>Peróxido de Hidrogênio (H<sub>2</sub>O<sub>2</sub>).....</i>	17
6. Dano ao DNA e Ensaio Cometa.....	17
<b>OBJETIVOS.....</b>	<b>19</b>
<b>CAPÍTULOS – ARTIGOS CIENTÍFICOS</b>	
7. Capítulo I.....	20
RESVERATROL INCREASES GLUTAMATE UPTAKE AND GLUTAMINE SYNTHETASE ACTIVITY IN C6 GLIOMA CELLS.....	21

8. Capítulo II.....	28
RESVERATROL ATTENUATES OXIDATIVE-INDUCED DNA DAMAGE IN C6 GLIOMA CELLS.....	30
<b>DISCUSSÃO.....</b>	<b>55</b>
<b>CONCLUSÕES.....</b>	<b>67</b>
<b>PERSPECTIVAS.....</b>	<b>69</b>
<b>REFERÊNCIAS BIBLIOGRÁFICAS.....</b>	<b>70</b>
<b>ANEXO I – LISTA DE FIGURAS.....</b>	<b>93</b>

## LISTA DE ABREVIATURAS

AMPA - Ácido  $\alpha$ -amino-3-hidróxi-5-metil-isoxazolenopropionato ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid)

COX – Ciclooxygenase

ERO – Espécies Reativas de Oxigênio

GABA – Ácido Gama-aminobutírico

GFAP – Proteína Glial Fibrilar Ácida (Glial Fibrillary Acidic Protein)

GluRs – Receptores Glutamatérgicos

GS – Glutamina Sintetase

LDH – Lactato Desidrogenase

MAPK – Proteína Cinase Ativada por Mitógenos (Mitogen-Activated Protein Kinase)

NMDA – N-Metil-D-Aspartato

PKC – Proteína Cinase C (Protein Kinase C)

PKD – Proteína Cinase D (Protein Kinase D)

RL – Radicais Livres

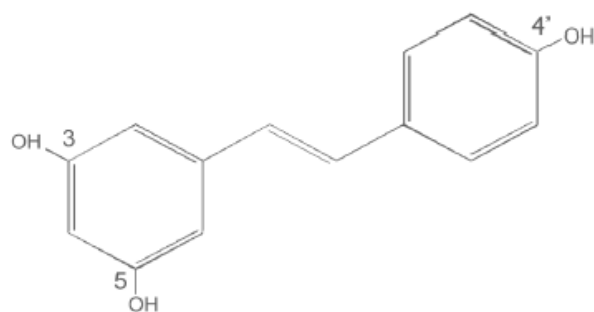
ROCK – Rho Cinase (Rho Kinase)

SNC – Sistema Nervoso Central

## INTRODUÇÃO

### 1. Resveratrol

O resveratrol (3,4',5-triidroxi-*trans*-estilbeno) (Figura 1), é um polifenol encontrado naturalmente em frutas e vegetais e possui importantes propriedades benéficas à saúde (Pervaiz, 2004).



*trans*-resveratrol

**Figura 1. Trans-resveratrol**

Os polifenóis são divididos em duas categorias principais: flavonóides e não-flavonóides. Os flavonóides são encontrados em extratos de plantas e frutas e são considerados os mais abundantes polifenóis, entretanto os estilbenos<sup>1</sup> não-flavonóides são a menor classe, mas com muitos efeitos biológicos (Doré, 2005).

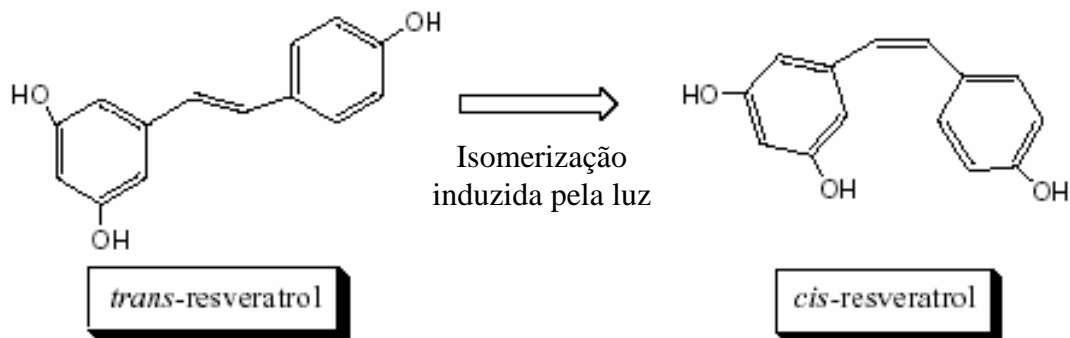
O resveratrol foi primeiramente isolado de raízes de *Veratrum grandiflorum* em 1940, e posteriormente em 1963 a partir de raízes de *Polygonum cuspidatum*, uma planta usada na medicina tradicional chinesa e japonesa (Baur & Sinclair, 2006). Além dessas plantas, o resveratrol está presente em mais de 70 espécies

---

<sup>1</sup> Os estilbenos são compostos orgânicos que contém 1,2- difeniletileno como um grupo funcional (Doré, 2005).

vegetais, sendo muito abundante em videiras de *Vitis vinifera* (Jeandet et al, 1991).

O principal significado do resveratrol na biologia da planta consiste em protegê-la de ataques externos, especialmente infecções fúngicas e radiações UV, uma propriedade que inclui o resveratrol na classe dos antibióticos das plantas, conhecido como fitoalexinas (Langeake et al, 1979; Baur & Sinclair, 2006). O resveratrol é encontrado principalmente em cascas e sementes de uvas, assim como em algumas frutas silvestres “berries<sup>2</sup>” (Soleas et al, 1997). São encontradas duas isoformas de resveratrol, os isômeros *cis* e *trans*. As uvas, aparentemente, apresentam apenas o isômero *trans*, sendo que o vinho apresenta ambos isômeros, devido provavelmente a isomerização de *trans* a *cis* pela ação da luz (Figura 2) (Doré, 2005). O isômero *trans* é o principal responsável pelos efeitos biológicos do resveratrol em mamíferos (Soleas et al, 1997).



**Figura 2. Isomerização do resveratrol.** Adaptado de Doré, 2005.

<sup>2</sup> *Berries* (do inglês antigo *berie*): Grupo de pequenas frutas vermelhas comestíveis, como morango e framboesa, contendo um pericarpo suculento – do dicionário de inglês Collins – Harper Collins Publishers (1992).

Há inúmeras lendas relatando o consumo e produção de vinhos pela humanidade. A Bíblia relata que Noé, após desembarcar de sua arca plantou um vinhedo do qual fez vinho. Já os gregos realizavam “Simpósios” que literalmente significa “bebendo junto” e dessa forma se reuniam para beber vinho em ambientes de alegre convívio (Johnson, 1999).

Existem inúmeros registros do uso medicinal do vinho pelos gregos. Hipócrates fez várias observações sobre as propriedades medicinais do vinho, que são citadas em textos da história da medicina. Galeno escreveu um tratado denominado “De antídotos” sobre o uso de preparações à base de vinho e ervas, usadas como antídotos de venenos (Johnson, 1999).

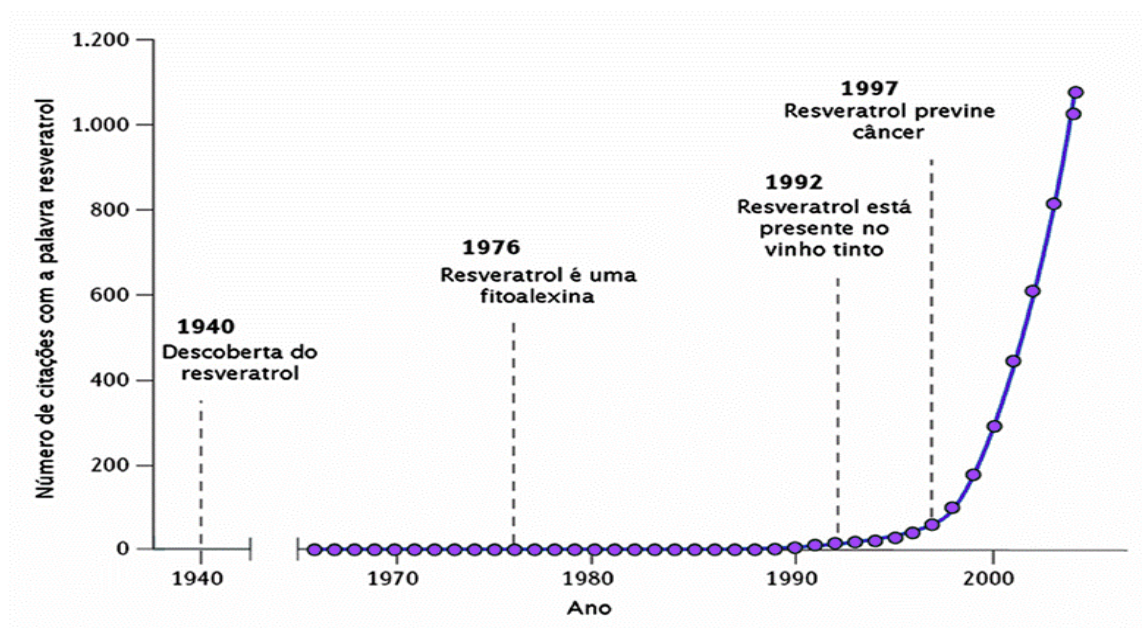
Por volta do ano de 1300, é publicado o primeiro livro sobre o vinho: “Líber de Vinis”, escrito pelo espanhol Arnaldus de Villanova. O livro cita propriedades curativas do vinho como: restabelecer o apetite e as energias, embelezar a face e manter a pessoa jovem.

As concentrações de resveratrol encontradas nos diferentes tipos de vinho variam em função da infecção por fungos, cultivo da uva, origem geográfica, tipo de vinho e práticas enológicas (Lamuela-Raventos et al, 1999). Souto et al (2001) verificou que a média de resveratrol nos vinhos brasileiros fica em torno de 2,57 mg/L, sendo uma das mais altas do mundo. Esses valores, provavelmente devem ser resultantes da alta umidade dos solos da Serra Gaúcha, que favorecem a proliferação de fungos (LangKage & Price, 1976).

Em 1979, Leger et al relatou, após um estudo em diversos países, que o consumo de vinho estaria correlacionado negativamente com a incidência de doenças cardíacas isquêmicas. A população do sul da França possuía o menor

índice dessas doenças, apesar da ingestão de uma dieta rica em gorduras saturadas. Esta controvérsia originou o termo “Paradoxo Francês” (Renauld & Lorgeril, 1992). A esse evento atribui-se o consumo moderado de vinho tinto pelos franceses.

Ainda no mesmo ano, logo após a demonstração dos efeitos cardioprotetores do vinho, foi descoberta a presença de resveratrol no vinho (Siemann & Creasy, 1992). Desde então, inúmeros trabalhos (Figura 3) têm mostrado os efeitos benéficos desta molécula, bem como seus mecanismos de ação e vias de sinalização envolvidas.



**Figura 3. Citações de resveratrol no PubMed em função do tempo.** Adaptado de Baur & Sinclair, 2006.

Entre os inúmeros efeitos biológicos do resveratrol, destaca-se a alta capacidade antioxidante, diminuindo a lipoperoxidação, atuando como um

seqüestrador (*scavenger*) de espécies reativas de oxigênio (ERO) e diminuindo a oxidação da lipoproteína de baixa densidade (Miller & Rice-Evans, 1995; Chanvitayapongs et al, 1997; Sinha et al, 2002). Estas propriedades contribuem para o efeito cardioprotetor do resveratrol e, além disso, ele diminui a agregação plaquetária (Demrow et al, 1995; Inoue et al, 2003), aumenta a lipoproteína de alta densidade, possui atividade anti-hipertensiva (Araya et al, 2001; Soares de Moura et al, 2004) e papel neuroprotetor (Draczynska-Lusiak et al, 1998; Miloso et al, 1999; Virgili & Contestabile, 2000; Sinha et al, 2002).

A atividade antiinflamatória do resveratrol deve-se a seu efeito inibitório sobre as enzimas ciclooxigenases (COX 1 e 2) e lipooxigenase. O resveratrol reduz as inflamações agudas e crônicas, evitando todas as características do processo inflamatório (Chen et al, 2005).

O resveratrol também exerce atividade antitumoral, inibindo os três estágios da carcinogênese: iniciação, promoção e progressão (Jang et al, 1997). Ele também está envolvido em várias vias de sinalização que regulam o ciclo celular e apoptose (Signorelli & Ghidoni, 2005), e recentemente foi associado ao aumento da expectativa de vida em mamíferos, envolvendo uma família de proteínas denominadas sirtuínas (Baur & Sinclair, 2006).

## **2. Gliomas**

Os tumores cerebrais representam menos de 2% de todas as neoplasias malignas, constituindo uma pequena fração entre todos os tipos de cânceres humanos (Kleihues & Cavenee, 2000). Entretanto, eles estão relacionados a um prognóstico bastante ruim, podendo desencadear muitos efeitos adversos,



envolvendo desde o sistema locomotor até o cognitivo e raramente levam à disseminação sistêmica (Taphoorn & Klein, 2004; Mourad et al, 2005).

Os gliomas são os tumores mais comuns do Sistema Nervoso Central (SNC) em humanos, apresentam origem glial e incluem os tumores constituídos, predominantemente, de células com características de astrócitos (astrocitomas), oligodendrócitos (oligodendrogliomas), células ependimais (ependimomas), e misturas de vários tipos de células gliais (por exemplo, oligoastrocitomas) (Dai & Holland, 2001). Os astrocitomas são os gliomas mais comuns e, histologicamente, são caracterizados por células com citoplasma abundante e forte marcação para a proteína glial fibrilar ácida (GFAP, do inglês *Glial Fibrillary Acidic Protein*), que constitui os filamentos intermediários de astrócitos. A origem dos astrocitomas pode ser decorrente da dediferenciação<sup>3</sup> de astrócitos maduros ou da transformação das células precursoras astrocíticas (Konopka & Bonni, 2003).

## **2.1. Linhagem de glioma de rato C6**

O glioma C6<sup>4</sup> tem sua origem datada da década de 60 e constitui-se numa grande fonte de estudos bioquímicos. Ele foi obtido após injeções em ratos do agente alquilante N-nitrosometiluréia (Benda et al, 1968). Esta linhagem é morfológicamente similar a glioblastomas, quando injetada no cérebro de ratos neonatos (Auer et al, 1981). A comparação de modelos experimentais derivados

---

<sup>3</sup> Dediferenciação, um estágio intermediário, em que uma célula especializada torna-se uma célula mais primitiva, multipotente, para então se rediferenciar em um outro tipo celular. ATCC – American type culture collection, [www.atcc.org](http://www.atcc.org)

<sup>4</sup> O termo “C6” significa células do Clone 6, sexto clone pós agente alquilante (Benda et al, 1971).

de C6 indica que tumores gerados de células transplantadas em ratos Wistar possuem características próximas a glioblastomas naturais.

A linhagem C6 constitui-se como uma linhagem neural, apresentando características de oligodendrócitos, astrócitos e neurônios (Parker et al, 1980), dependendo do número de passagens<sup>5</sup>. A expressão das proteínas gliais GFAP e S100B atesta o caráter astrocitário (Benda et al, 1971).

Embora com algumas limitações, o modelo de estudo C6 é muito útil para estudar o crescimento, a invasibilidade e a angiogênese de glioblastomas. Estas células são também largamente utilizadas em pesquisas visando a regulação de fatores de crescimento, bem como inúmeras vias de transdução de sinal (Grobber et al, 2002; Kim et al, 2006).

Por apresentar características de uma célula astrocitária, a linhagem C6 também é utilizada para investigações de características bioquímicas e metabólicas que envolvam funções astrocíticas (Mangoura et al, 1989; Feng et al, 2004; Cechin et al, 2005; Funchal et al, 2005).

### **3. Astrócitos**

Rudolph Virchow foi quem descreveu pela primeira vez, em 1846, a neuroglia, do inglês *nerve glue* (por parecer-se com uma cola entre os neurônios), como um tecido funcional, anatomicamente distinto do neuronal e do conectivo do

---

<sup>5</sup> O número de passagens significa quantas vezes uma população de células foi tripsinizada e semeada novamente, após o primeiro cultivo. O processo envolve ação de tripsina, uma protease empregada para ressuspender uma cultura aderida, para poder cultivá-la novamente, ATCC – American type culture collection, [www.atcc.org](http://www.atcc.org).

SNC (Jacobson, 1991). Hoje, se reconhecem no SNC três grandes grupos de células gliais: (1) astrócitos e oligodendrócitos, ambos de origem ectodérmica, que formam a macroglia; (2) a microglia, de origem mesodérmica; (3) células endodérmicas, de origem ectodérmica (Young, 1991).

Os astrócitos constituem aproximadamente 50% do número total de células do SNC. Eles são divididos em dois tipos: os protoplasmáticos, na substância cinzenta (freqüentemente ramificados e com largas expansões) e os fibrosos, na substância branca (com menos ramificações, cilíndricas e longas) (Young, 1991). Sabe-se que os astrócitos e neurônios apresentam um sofisticado sistema de comunicação recíproca que pode regular a liberação de neurotransmissores, a excitabilidade neuronal e a transmissão sináptica (Carmignoto, 2000).

As funções desempenhadas pelos astrócitos no SNC são diversas, tais como: (1) manutenção da homeostase de íons extracelulares, especialmente o  $K^+$ ; (2) metabolismo de neurotransmissores, particularmente o glutamato e o GABA (ácido gama-aminobutírico) (Jessen, 2004; Gee & Keller, 2005); (3) atuam como guias na migração de neurônios nos estágios iniciais do desenvolvimento (McKay, 1989); (4) síntese e liberação de fatores tróficos e substâncias neuroativas para os neurônios e para outros astrócitos (Hansson & Rönnbäck, 1995); (5) participação na resposta imune no cérebro; (6) participação na resposta a injúrias ao SNC, tornando-se reativos (gliose reativa<sup>6</sup>); regulação do espaço extracelular através da variação do volume astrocítico (Kimelberg et al, 1992; Hansson & Rönnbäck, 1995).

---

<sup>6</sup> Gliose reativa se refere à hiperplasia e/ou hipertrofia das células gliais após injúrias ao SNC formando uma “cicatriz” no local da lesão (Shao & McCarthy, 1994).

#### **4. Marcadores Gliais em Astrócitos e Células de Glioma C6**

Como anteriormente citado, os astrócitos participam de vários processos fisiológicos e metabólicos responsáveis por manter a homeostase do SNC. Insultos físicos e metabólicos provocam rápidas alterações nas células gliais e este fenômeno é denominado gliose reativa, que é caracterizado entre outras alterações, por um aumento na expressão de marcadores gliais tais como GFAP e S100B (Baydas et al, 2003).

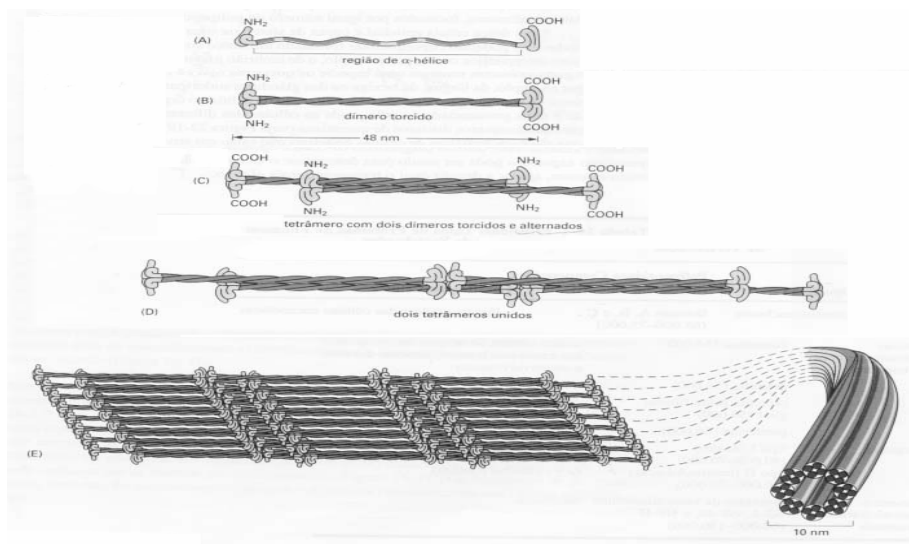
##### **4.1. Proteína Glial Fibrilar Ácida (GFAP)**

A GFAP é uma proteína estrutural e constitui a subunidade protéica de filamentos intermediários do tipo III do citoesqueleto glial (Rodnight et al, 1997; Gomes et al, 1999). A imunoreatividade da GFAP no SNC maduro se restringe aos astrócitos protoplasmáticos na substância cinzenta, astrócitos fibrosos na substância branca, glia radial no cerebelo (Glia de Bergmann) e em astrócitos subependimais adjacentes aos ventrículos cerebrais (Eng et al., 2000). Inicialmente isolada de lesões cerebrais (placas) de pacientes com esclerose múltipla (Eng et al, 2000), a GFAP é reconhecida e amplamente utilizada como marcador de astrócitos e de tumores de linhagem astrocítica (Pekny & Pekna, 2004).

A GFAP possui peso molecular aparente de 50 kDa, sendo composta por três regiões distintas. Uma região amino-terminal não-helicoidal com 35 resíduos de aminoácidos e uma estrutura em conformação  $\beta$  que contém oito resíduos de

arginina, caracterizando esta região como básica. Uma região carboxi-terminal não-helicoidal que contém cerca de 50 resíduos de aminoácidos e uma estrutura globular que pode estar envolvida em interações da GFAP com outras proteínas. A região central é formada por uma extensa  $\alpha$ -hélice cuja seqüência de aminoácidos é conservada em relação a outras proteínas de filamentos intermediários (Eng et al, 2000; Alberts et al, 2002).

A polimerização da GFAP envolve vários passos (Figura 4). Inicialmente, um dímero é formado através do pareamento entre dois monômeros idênticos. Posteriormente, dímeros dispostos antiparalelamente interagem, ou seja, de um mesmo lado encontramos um terminal carboxi e um amino formando um tetrâmero ou protofilamento, dois tetrâmeros se associam e finalmente formam o polímero (filamento intermediário) (Alberts et al, 2002).



**Figura 4. Representação esquemática da formação de um filamento intermediário: A) monômero; B) dímero; C) tetrâmero formado de dois dímeros dispostos antiparalelamente; D) dois tetrâmeros associados; E) filamento intermediário com 10 nm de diâmetro. Segundo Alberts et al, 2002.**

## 4.2. S100B

A S100B é um membro da família de proteínas S100, encontrada principalmente na forma homodimérica (com cerca de 21 kDa). É uma proteína ligante de cálcio do tipo EF-hand (hélice-loop-hélice) com dois sítios de ligação ao cálcio por monômero (Van Eldik & Wainwright, 2003). Foi isolada há mais de 40 anos (Moore, 1965) a partir de um extrato de cérebro bovino. Posteriormente verificou-se que este extrato continha duas proteínas muito similares, a S100A1 e a S100B, e a mesma foi identificada também em tecidos extracerebrais e hoje são conhecidos 21 membros da família S100 presentes nos mais diversos tipos celulares de vertebrados (Donato, 2003) os quais possuem aproximadamente 50% de homologia na sua seqüência de aminoácidos (Zimmer & Van Eldik, 1988). As proteínas da família S100 receberam esta denominação por serem solúveis mesmo em 100% de sulfato de amônio.

A S100B é produzida e secretada principalmente por astrócitos e exerce efeitos autócrinos e parácrinos sobre outras células gliais e neurônios apresentando funções intra e extracelulares como fosforilação de GFAP e manutenção da integridade do citoesqueleto (Donato, 2003; Frizzo et al, 2004).

A proteína S100B tem um efeito duplo dependente da concentração, ou seja, em concentrações na ordem nM exerce efeito neurotrófico promovendo crescimento de neuritos, aumentando a sobrevivência de neurônios durante o desenvolvimento e em situações de injúria ao SNC (Tramontina et al, 2002; Gottfried et al, 2003; Leite et al, 2006) e protegendo neurônios contra a excitotoxicidade do glutamato (Ahlemeyer et al, 2000; Tramontina et al, 2006). O

efeito trófico também é exercido em astrócitos. Já em concentrações na ordem de  $\mu\text{M}$  exerce efeito neurotóxico induzindo apoptose (Van Eldik & Wainwright, 2003).

Culturas primárias de astrócito e células de glioma C6 são comumente utilizadas para estudar a expressão e a secreção de S100B (Van Eldik & Zimmer, 1987; Pinto et al, 2000; Davey et al, 2000). No entanto, estas células apresentam diferentes mecanismos de expressão para o gene da S100B (Castets et al, 1997).

### **4.3. Neurotransmissão Glutamatérgica**

O glutamato é o principal neurotransmissor excitatório do SNC de mamíferos e exerce um importante papel na plasticidade neural e neurotoxicidade (Nakanishi, 1992). O glutamato medeia vários processos vitais, tais como: desenvolvimento das células nervosas, incluindo proliferação e migração (McDonald & Johnson, 1990), modulação de mecanismos de aprendizado e memória (Izquierdo & Medina, 1997) e envelhecimento (Segovia et al, 2001). Os receptores glutamatérgicos (GluRs) têm papel fundamental na plasticidade e no desenvolvimento neural, bem como nos processos de neurodegeneração e transmissão sináptica. A ativação excessiva dos GluRs durante episódios de estresse cerebral, tais como: isquemia, traumatismo craniano, surtos epilépticos e doenças neurodegenerativas levam à morte de neurônios (neurotoxicidade). Os GluRs estão envolvidos intimamente na fisiopatologia das funções cerebrais (Nakanishi et al, 1998; Ozawa et al, 1998; Anderson & Swanson, 2000; Loureiro et al, 2005).

Os GluRs são divididos em duas classes distintas: receptores ionotrópicos e metabotrópicos (Stanimirovic et al, 1999). Os receptores ionotrópicos são assim

denominados por serem canais iônicos permeáveis a cátions e são subdivididos em N-metil-D-aspartato (NMDA) e não NMDA, que compreende os receptores ácido  $\alpha$ -amino-3-hidróxi-5-metil-isoxazolenopropionato (AMPA) e cainato. Os receptores metabotrópicos (mGluRs) pertencem a uma família de receptores que estão acoplados a proteínas G. A concentração de glutamato no espaço extracelular determina o grau de ativação de seus receptores, por isso, é essencial manter os níveis extracelulares desse neurotransmissor normais.

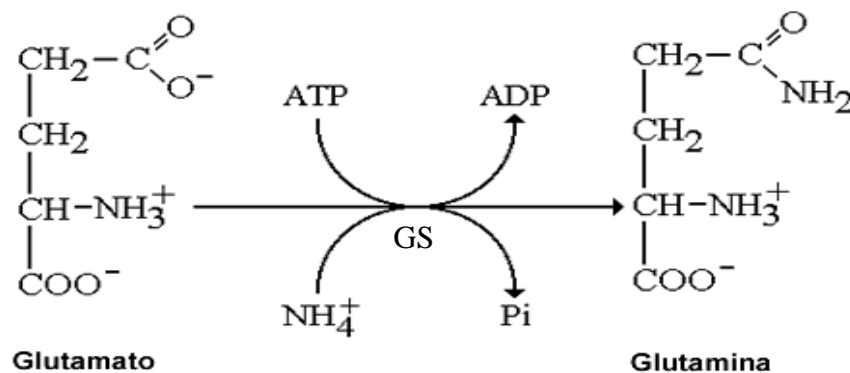
#### **4.4 Captação de Glutamato e Atividade da Glutamina Sintetase**

O glutamato, após ser sintetizado, é estocado pelo sistema de transporte presente nas vesículas que se encontram no terminal pré-sináptico. Quando ocorre a despolarização dos terminais sinápticos glutamatérgicos, o glutamato que se encontra nas vesículas é liberado para o meio extracelular (fenda sináptica) para interagir com seus receptores ionotrópicos e/ou metabotrópicos que estão localizados nas membranas pré e pós-sinápticas e também nas membranas gliais (Gallo & Ghiani, 2000; Scannevin & Huganir, 2000). Após, o glutamato é removido da fenda sináptica principalmente por sistemas de transporte que são dependentes de sódio, localizados nos neurônios e principalmente nas células gliais (Robinson & Down, 1997; Anderson & Swanson, 2000; Danboldt, 2001; Amara & Fontana, 2002; Gottfried, 2002; Martini et al, 2006). A captação astrocítica é a mais eficiente para manter normal a concentração extracelular de glutamato. Os astrócitos captam glutamato e intracelularmente ele é convertido em glutamina pela enzima glutamina sintetase (GS). A glutamina é liberada pelos



astrócitos e captada por neurônios para ser novamente convertida em glutamato, ciclo este conhecido como glutamina-glutamato (Matthews et al, 2005).

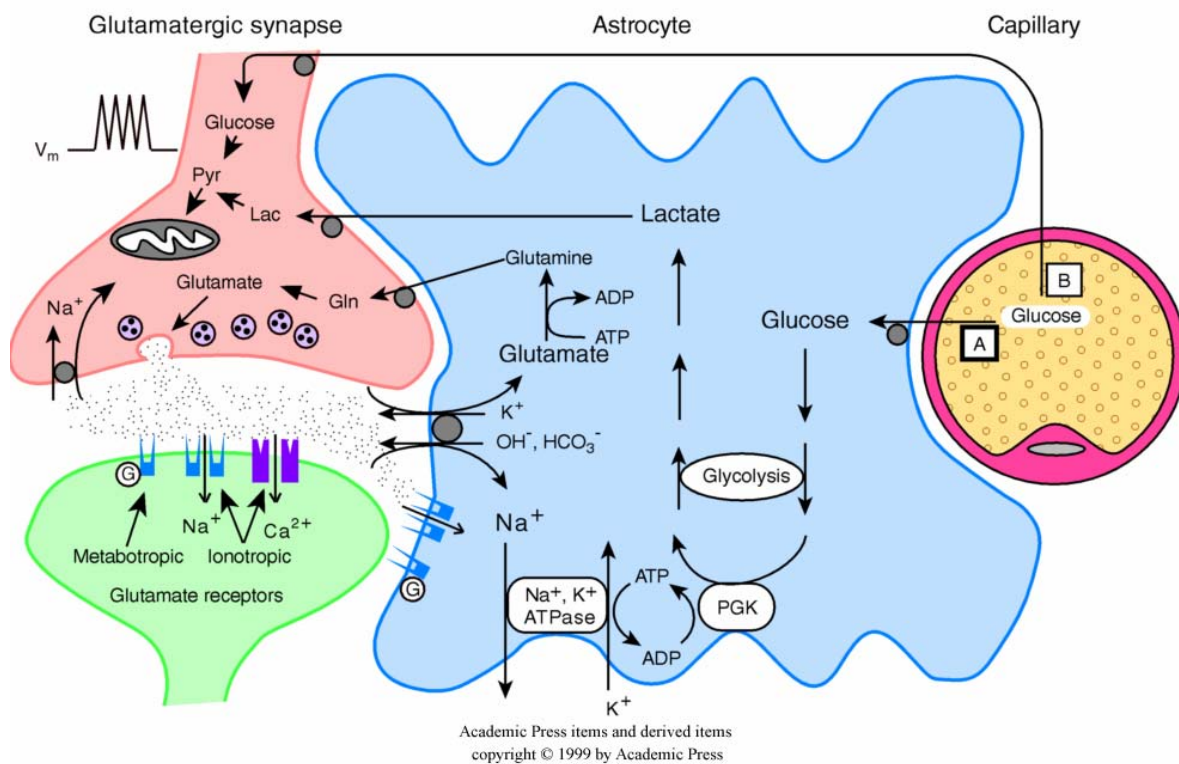
A glutamina-sintetase é uma enzima chave em duas importantes vias bioquímicas: no fígado a GS catalisa a detoxificação da amônia e no cérebro, além deste papel, garante a reciclagem do neurotransmissor glutamato, estando localizada nos astrócitos (Figura 5) (Matthews et al, 2005).



**Figura 5. Reação catalisada pela enzima Glutamina Sintetase**

Atualmente, cinco tipos de transportadores de glutamato dependentes de sódio estão bem identificados e caracterizados: GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4 e EAAT5 (Amara & Fontana, 2002). Os transportadores de glutamato localizados nas membranas das células gliais (Figura 6) são de fato os responsáveis pela manutenção dos baixos níveis extracelulares de glutamato, garantindo dessa forma a homeostase celular (Andreson & Swanson, 2000; Tanaka 2000; Amara & Fontana, 2002; Emanuelli et al, 2003; Porciúncula et al, 2004).

A linhagem celular C6 pode apresentar os seguintes transportadores de glutamato dependentes de sódio, GLAST, GLT-1 e EAAC1 (Takano et al, 2001). Também é expresso em C6 o trocador cistina-glutamato independente de sódio (sistema XC) (Ye & Sontheimer, 1999; Takano et al, 2001). A principal função do sistema XC é a captação de cistina e manutenção dos níveis de glutatona intracelulares (Takano et al, 2001).



**Figura 6. Representação esquemática de uma Sinapse Glutamatérgica.**

Adaptado de Magistretti et al., 1999. *PGK, fosfoglicerato cinase.*

## 5. Radicais Livres e Espécies Reativas de Oxigênio

O termo radical livre (RL) refere-se a uma estrutura química que possui um elétron desemparelhado, ou seja, ocupando um orbital atômico ou molecular

sozinho. Isso o torna muito instável, extremamente reativo e com uma enorme capacidade para combinar-se inespecificamente com as diversas moléculas integrantes da estrutura celular ou derivados de cada uma delas (Halliwell & Gutteridge, 2000). Os RL, em geral são formados por absorção de radiação (ultravioleta ou visível), por reações redox ou por processos de catálise enzimática (Slater, 1984).

Nos mamíferos, são produzidos RL de carbono, enxofre, nitrogênio e oxigênio, mas os que ganham mais destaque devido à reatividade e aos danos que podem causar são os radicais derivados do oxigênio. O termo espécies reativas de oxigênio (ERO) é freqüentemente usado para incluir não apenas RL de oxigênio, mas também espécies que não são propriamente um RL, mas derivadas do  $O_2$ , capazes de gerar RL, como por exemplo o peróxido de hidrogênio ( $H_2O_2$ ) e o ácido hipocloroso (HClO), entre outros (Halliwell & Gutteridge, 2000).

As ERO e outros RL podem ser produzidos por fontes endógenas e exógenas. Entre as principais fontes endógenas de RL estão a cadeia de transporte de elétrons mitocondrial, a degradação de ácidos graxos nos peroxissomos, os mecanismos de detoxificação mediados pelo complexo enzimático citocromo P-450, o processo de fagocitose; entre as exógenas destacam-se as radiações, o cigarro e solventes orgânicos (Halliwell & Gutteridge, 2000).

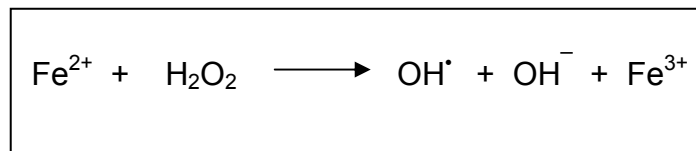
### **5.1. Estresse Oxidativo**

Os RL formam-se em condições fisiológicas em proporções controladas pelos mecanismos defensivos celulares. Entretanto, em situações patológicas,

essa produção pode aumentar substancialmente. O estresse oxidativo pode resultar de uma situação em que há uma diminuição nos níveis das enzimas antioxidantes, uma elevada velocidade de produção de ERO ou uma combinação de ambas condições (Salvador & Henriques, 2004).

## 5.2. Peróxido de Hidrogênio (H<sub>2</sub>O<sub>2</sub>)

Embora o H<sub>2</sub>O<sub>2</sub> não seja estritamente um RL por definição, ele é uma ERO importante por sua capacidade de gerar radical hidroxila (OH<sup>•</sup>) em presença de metais como ferro (Figura 7). Ele é formado principalmente na matriz mitocondrial, durante o processo de redução do oxigênio, ou pela, dismutação do radical superóxido pela enzima superóxido dismutase (Fridovich, 1998).



**Figura 7. Reação de Fenton**

## 6. Dano ao DNA e Ensaio Cometa

O dano ao DNA produzido por oxidação é considerado o mais significativo dano oriundo do metabolismo celular. Estima-se que aproximadamente 2x10<sup>4</sup> lesões oxidativas ao DNA ocorram no genoma humano por dia (Ames & Shigenaga, 1992). Acredita-se que, desta maneira, o reparo destas lesões possua um papel central na prevenção do aumento de mutações nos organismos vivos (Maluf, 2004).

O excesso de ERO (por ex.  $H_2O_2$ ) pode levar à oxidação de lipídios, proteínas e DNA, causando dano às membranas celular e nuclear. O dano irreparável ao DNA está envolvido na carcinogênese, envelhecimento e outras doenças degenerativas (Cozzi et al, 1997). Porém, devemos levar em consideração que o dano ao DNA pode ser induzido por hábitos alimentares e estilo de vida (McCord & Edeas, 2005).

O Ensaio Cometa é uma técnica simples e rápida para verificarmos de modo quantitativo, quebras simples e duplas ao DNA (Maluf & Erdtmann, 2000; Faust et al, 2004). Östling & Johanson (1984), detectaram via eletroforese o dano ao DNA induzido por radiação ionizante, sob condições de pH neutro, verificando apenas quebras duplas ao DNA. Singh et al (1988) propuseram realizar a técnica sob condições alcalinas e assim, detectaram quebras simples e duplas ao DNA. Atualmente, esta técnica sofreu pequenas modificações (Tice et al, 2000), e vem sendo amplamente utilizada para detecção de dano ao DNA.

## **OBJETIVOS**

Esta dissertação será apresentada na forma de capítulos, constituídos de artigos científicos, visando atingir os seguintes objetivos gerais:

### **Capítulo I:**

Avaliar a ação do resveratrol sobre a linhagem celular C6, através de parâmetros celulares e gliais.

### **Capítulo II:**

Avaliar o possível efeito protetor do resveratrol sobre a linhagem C6, após insulto com peróxido de hidrogênio, através de parâmetros moleculares.

## **CAPÍTULO I**

### **RESVERATROL INCREASES GLUTAMATE UPTAKE AND GLUTAMINE SYNTHETASE ACTIVITY IN C6 GLIOMA CELLS**

**ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS**

## Resveratrol increases glutamate uptake and glutamine synthetase activity in C6 glioma cells

André Quincozes dos Santos, Patrícia Nardin, Cláudia Funchal,  
Lucia Maria Vieira de Almeida, Maria Caroline Jacques-Silva, Susana T. Wofchuk,  
Carlos-Alberto Gonçalves, Carmem Gottfried\*

*Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Departamento de Bioquímica,  
Rua Ramiro Barcelos 2600 anexo, 90035-003 Porto Alegre, RS, Brazil*

Received 10 April 2006, and in revised form 9 June 2006  
Available online 28 July 2006

### Abstract

Resveratrol, a phytoalexin found mainly in grapes, is a promising natural product with anti-cancer and cardio-protective activities. Here, we investigated, in C6 glioma cells, the effect of resveratrol on some specific parameters of astrocyte activity (glutamate uptake, glutamine synthetase and secretion of S100B, a neurotrophic cytokine) commonly associated with the protective role of these cells. Cell proliferation was significantly decreased by 8% and 26%, following 24 h of treatment with 100 and 250  $\mu\text{M}$  resveratrol. Extracellular S100B increased after 48 h of resveratrol exposure. Short-term resveratrol exposure (from 1 to 100  $\mu\text{M}$ ) induced a linear increase in glutamate uptake (up to 50% at 100  $\mu\text{M}$  resveratrol) and in glutamine synthetase activity. Changes in these glial activities can contribute to the protective role of astrocytes in brain injury conditions, reinforcing the putative use of this compound in the therapeutic arsenal against neurodegenerative diseases and ischemic disorders.

© 2006 Elsevier Inc. All rights reserved.

**Keywords:** Resveratrol; Glutamate uptake; Glutamine synthetase; C6 glioma; GFAP; S100B

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene), a phytoalexin found mainly in grapes and mulberries, is a promising natural antioxidative, anti-inflammatory and estrogenic product [1–3]. Many investigations have sought to understand the molecular mechanisms of resveratrol and its diverse biological effects, particularly its cancer chemopreventive [3], cardio-protective [4–6] and neuroprotective activities [7].

Brain tissue is particularly vulnerable to oxidative damage, possibly due to its high consumption of oxygen and the consequent generation of high quantities of reactive oxygen species (ROS)<sup>1</sup> during oxidative phosphorylation [8]. More-

over, several enzymes expressed in brain, including monoamine oxidase and tyrosine hydroxylase, lead to hydrogen peroxide formation as a normal by-product of their activity. Several regions of the brain are particularly rich in iron, which promotes the production of damaging oxygen-free radical species. Furthermore, the brain is relatively poorly endowed with protective antioxidant enzymes or antioxidant compounds. ROS formation has been implicated in damage to cerebral tissue in several nervous pathologies such as ischemia-reperfusion injury and neurodegenerative disorders [9,10].

Growing evidence indicates the prominent role of astrocytes in the defense against oxidative-stress mediated neuronal death [11]. In fact, co-culture studies show that astrocytes protect neurons from cell death induced by hydrogen peroxide and nitric oxide [12–14]. Moreover, astrocytes provide glutathione precursors to neighboring neurons [15].

\* Corresponding author. Fax: +5551 3316 5535.

E-mail address: [cgottfried@ufrgs.br](mailto:cgottfried@ufrgs.br) (C. Gottfried).

<sup>1</sup> Abbreviations used: GS, glutamine synthetase; ROS, reactive oxygen species; EDTA, ethylene-diaminetetraacetic acid; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DAPI, 4',6'-diamidino-2-phenylindole; R, resveratrol; PI, propidium iodide; GFAP, glial fibrillary acidic protein.



Although resveratrol has been proposed as a pharmacological tool for neuroprotection in several models of neuronal injury, there are only a few studies regarding the effect of this compound on astrocytes, particularly in ischemic models of injury. Resveratrol is able to prevent interleukin-6 production in astrocyte cultures exposed to oxygen and glucose deprivation [16] and, *in vivo*, resveratrol attenuated neuronal cell death and astrocyte reactivity in gerbils submitted to global ischemia [17].

The C6 glioma cell line was originally derived from rat brain tumors induced with *N*-nitrosomethylurea [18]. This cell line has oligodendrocytic, astrocytic and neuronal properties [19] and are a widely used astrocyte-like cell line [20–23]. Here, we investigated in C6 glioma cells the effect of resveratrol on some specific parameters of astrocyte activity; glutamate uptake, glutamine synthetase (GS) and S100B secretion, commonly associated with the protective role of these cells. The effect of resveratrol on cell morphology, proliferation and death was also investigated.

## Material and methods

### Materials

L-<sup>3</sup>H]Glutamate was purchased from Amersham International (UK). Resveratrol, monoclonal anti-S100B (SH-B1),  $\gamma$ -glutamylhydroxamate, propidium iodide and material for cell culture were purchased from Sigma (St. Louis MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Carlsbad, CA, USA), fetal bovine serum (FBS) from Cultilab (Campinas, SP, Brazil) and rhodamine-labeled phalloidin from Molecular Probes (OR, USA), polyclonal anti-GFAP from DAKO, polyclonal anti-S100 from DAKO, 4',6'-diamidino-2-phenylindole (DAPI) was from Calbiochem (La Jolla, CA). All other chemicals were purchased from common commercial suppliers.

### C6 glioma culture and resveratrol treatment

C6 glioma cells (clone 2B) were purchased from the American type culture collection (Rockville, Maryland, USA). Late passage cells (i.e. after at least 100 passages) were seeded in flasks and cultured in DMEM (pH 7.4) supplemented with 5% FBS, 2.5 mg/ml Fungizone® and 100 U/L gentamicin. Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95% and an atmosphere of 5% CO<sub>2</sub> in air. Exponentially growing cells were detached from the culture flasks using 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) and seeded in 24-well plates (5 × 10<sup>3</sup> cells/well). After cells reached confluence, the culture medium was removed by suction and the cells were incubated for 24 or 48 h at 37 °C in an atmosphere of 5% CO<sub>2</sub>/95% air in DMEM (pH 7.4) without serum in the absence or presence of resveratrol at indicated concentrations, assuming cells exposed to vehicle (0.25% ethanol) as controls. It is important to mention that in all parameters

analyzed the results obtained with vehicle were not different from those obtained in basal conditions without ethanol. After incubations cells were fixed for labeling studies and morphological studies were performed using phase contrast optics.

### Cell counting

C6 cells were seeded at 5 × 10<sup>3</sup> cells/well in 24-well plates and grown for 24 h in DMEM + 5% FBS. The culture medium was removed by suction and the cells were incubated for 24 or 48 h at 37 °C in an atmosphere of 5% CO<sub>2</sub>/95% air in DMEM (pH 7.4) without serum in the absence (controls) or presence of 1, 10, 25, 50, 100 or 250 μM resveratrol. At the end of this period, the medium was removed, cells were rinsed with PBS and 200 μl of 0.05% trypsin/EDTA solution was added to detach the cells, which were counted in a Neubauer chamber.

### Cell integrity

Total intracellular lactate dehydrogenase (LDH) activity after resveratrol exposure was determined in lysed cells with 0.2% Triton X-100 by a colorimetric assay kit (from Doles, Goiânia, Brazil). Cells were treated with resveratrol at indicated concentrations and incubated for 24 and 48 h. Additionally, cellular damage was assessed by fluorescent image analysis (Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory) of propidium iodide (PI, at 7.5 μM) uptake during 6 h. Optical density was determined with the Optiquant version 02.00 software (Packard Instrument Company). Density values obtained were expressed as density light unit.

### Nuclear morphology assay

C6 cells were cultured on circular glass coverslips and treated with or without 100 or 250 μM of resveratrol for 24 or 48 h. The nuclear morphology assay was carried out and cell cultures were fixed for 20 min with 4% paraformaldehyde in phosphate buffer (PBS), stained with 0.2 μg/ml of 4',6'-diamidino-2-phenylindole (DAPI) for 1 h and visualized under a fluorescent microscope (Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory). Apoptotic cells can be morphologically visualized by nuclear shrinkage and chromatin condensation or fragmentation.

### Immunocytochemistry for GFAP

Cells were cultured on circular glass and treated with or without 100 μM resveratrol for 24 or 48 h, fixed for 20 min with 4% paraformaldehyde in phosphate-buffered saline (PBS; 2.9 mM KH<sub>2</sub>PO<sub>4</sub>, 38 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 130 mM NaCl, 1.2 mM KCl), rinsed with PBS and permeabilized for 10 min in PBS containing 0.2% Triton X-100. To reduce non-specific background staining the fixed cells were then

blocked for 1 h with PBS containing 0.5% bovine serum albumin. Immunocytochemistry for GFAP was carried out as described previously [24]. Briefly, fixed cells were incubated overnight with polyclonal anti-GFAP (1:200) followed by peroxidase-conjugated IgG (Amersham, 1:200) for 2 h. Finally, the cells were treated with 0.05% diaminobenzidine (Sigma) containing 0.01% hydrogen peroxide for 20 min. Cells were viewed with a Nikon inverted microscope and images transferred to computer with a digital camera (Sound Vision Inc. Wayland, MA). All images are representative fields from at least three experiments carried out in triplicate.

#### Glutamate uptake assay

C6 cells were preincubated in the absence (controls) or in the presence of 0.01, 0.1, 1, 10, 25, 50, 100 or 250  $\mu\text{M}$  resveratrol for 1 h at 37°C in an atmosphere of 5%  $\text{CO}_2$ /95% air in DMEM (pH 7.4) without serum. Glutamate uptake was performed as previously described [25] with some modifications. Briefly, C6 glioma cells were incubated at 37°C in a Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl; 5.36 KCl, 1.26  $\text{CaCl}_2$ , 0.41  $\text{MgSO}_4$ , 0.49  $\text{MgCl}_2$ , 0.63  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 0.44  $\text{KH}_2\text{PO}_4$ , 4.17  $\text{NaHCO}_3$  and 5.6 glucose, adjusted to pH 7.4. The assay was started by addition of 0.1 mM L-glutamate and 0.33  $\mu\text{Ci/ml}$  L-[2,3- $^3\text{H}$ ] glutamate. Incubation was stopped after 10 min by removal of the medium and rinsing the cells twice with ice-cold HBSS. The cells were then lysed in a solution containing 0.5 M NaOH. Radioactivity was measured in a scintillation counter. Final glutamate uptake was obtained by discounting non-specific uptake in assays carried out in sodium-free medium, prepared by replacing NaCl with choline chloride in the HBSS.

#### GS activity

C6 cells were preincubated in the absence (controls) or in the presence of 0.01, 0.1, 1, 10, 100 or 250  $\mu\text{M}$  of resveratrol for 1 h at 37°C in an atmosphere of 5%  $\text{CO}_2$ /95% air in DMEM (pH 7.4) without serum. The enzymatic assay was performed as previously described [26] with some modifications. Briefly, homogenate (0.1 ml) was added to 0.1 ml of reaction mixture containing (in mM): 10  $\text{MgCl}_2$ ; 50 L-glutamate; 100 imidazole-HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine-HCl; 10 ATP and incubated for 15 min at 37°C. The reaction was stopped by the addition of 0.4 ml of a solution containing (in mM): 370 ferric chloride; 670 HCl; 200 trichloroacetic acid. After centrifugation, the supernatant's absorbance was measured at 530 nm and compared to the absorbance generated by standard quantities of  $\gamma$ -glutamylhydroxamate, treated with ferric chloride reagent.

#### S100B measurement

The culture medium was replaced by DMEM without serum. S100B was measured after 24 or 48 h in the absence

or in the presence of resveratrol (10, 50, 100 or 250  $\mu\text{M}$ ). Cells were washed and scraped at 24 h for measurement of intracellular S100B content. ELISA for S100B was carried out as described previously [27] with modifications. Briefly, 50  $\mu\text{l}$  of sample plus 50  $\mu\text{l}$  of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1, from Sigma). Polyclonal anti-S100 (from DAKO) was incubated for 30 min and then peroxidase-conjugated anti-rabbit was added for another 30 min. The color reaction with *o*-phenylenediamine was measured at 492 nm.

#### Protein determination

Protein concentrations were determined as previously described [28], using bovine serum albumin as standard.

#### Statistical analysis

Data from the experiments are presented as mean  $\pm$  SE and were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey test. Values of  $P < 0.05$  were considered to be significant. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

## Results

#### Effect of resveratrol and C6 glioma proliferation

The anti-proliferative effect of resveratrol was assessed by incubating pre-confluent cells with resveratrol at indicated concentrations (Fig. 1). Proliferation was significantly decreased by 8% and 26%, following 24 h of treatment with 100 or 250  $\mu\text{M}$  resveratrol, respectively ( $P < 0.01$ ). This effect was more pronounced after 48 h of

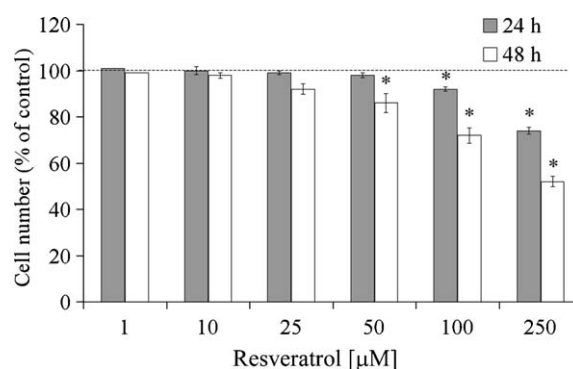


Fig. 1. Influence of resveratrol on C6 cell proliferation. Cells were treated with resveratrol at indicated concentrations for 24 and 48 h in DMEM without serum. At the end of the incubation, medium was removed followed by addition of 0.05% trypsin/EDTA solution to detach the cells, which were counted in a Neubauer chamber. The data represent the means  $\pm$  SE values from three independent experiments performed in triplicate. Significant differences from control (0.25% ethanol) values is indicated by \* $P < 0.01$ .

treatment. Proliferation decreased by approximately 14%, 28% and 48%, following 48 h of treatment with 50, 100 and 250  $\mu\text{M}$  resveratrol, respectively ( $P < 0.01$ ). It is important to emphasize that controls were treated with 0.25% ethanol vehicle.

*Cell integrity and death*

The LDH assay indicated that resveratrol impairs the membrane integrity only at 250  $\mu\text{M}$  at 24 and 48 h of treatment (Fig. 2). Ethanol *per se* (at 0.25%) did not cause LDH loss in C6 cells when incubated for 48 h. PI incorporation (Fig. 3) was increased by resveratrol exposure only at 250  $\mu\text{M}$  for 24 and 48 h (about 14 times and 20 times, respectively). Microscopic observations following DAPI incubation (Fig. 4) showed that resveratrol at 250  $\mu\text{M}$  (in 24 and 48 h) was able to induce cell death possibly by necrosis, indicated by the absence of nuclear fragmentation. In order to confirm absence of cell damage with resveratrol at 100  $\mu\text{M}$  we carried out immunocytochemistry in intermediate filaments using anti-GFAP antibody (Fig. 5). No alterations were observed in phase contrast and in intermediate filaments induced by 100  $\mu\text{M}$  resveratrol during exposure for 24 and 48 h.

*Glutamate uptake and GS activity in C6 cells*

Basal glutamate uptake under our conditions was 0.2 nmol/mg prot/min (Fig. 6) and was not affected by 0.25% ethanol. Resveratrol from 0.1 to 100  $\mu\text{M}$  induced a linear increase in glutamate uptake ( $R^2 = 0.92$ ). Interestingly, at 250  $\mu\text{M}$ , resveratrol also induced an increase of glutamate uptake; however, we observed a decrease when compared to 100  $\mu\text{M}$  resveratrol. It is important to mention that the glutamate uptake measurement was carried out in the first hour of resveratrol exposure; therefore, the decrease with 250  $\mu\text{M}$  resveratrol would not be due to cell death. Basal GS activity

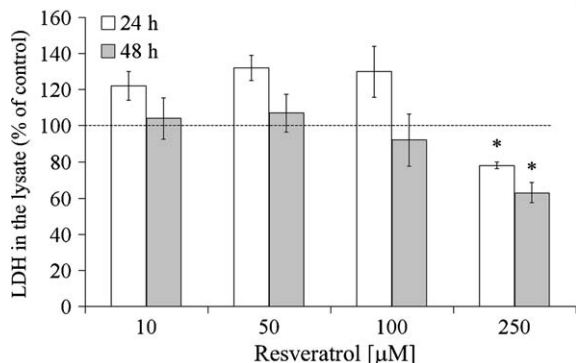


Fig. 2. Effect of resveratrol on C6 cell damage measured by LDH. This assay was evaluated by measuring the amount of the remaining intracellular enzyme, LDH, after resveratrol exposure at indicated concentrations for 24 and 48 h. The data represent the means  $\pm$  SE values from three independent experiments performed in triplicate. Significant difference from control (0.25% ethanol) values is indicated by \* $P < 0.01$ .

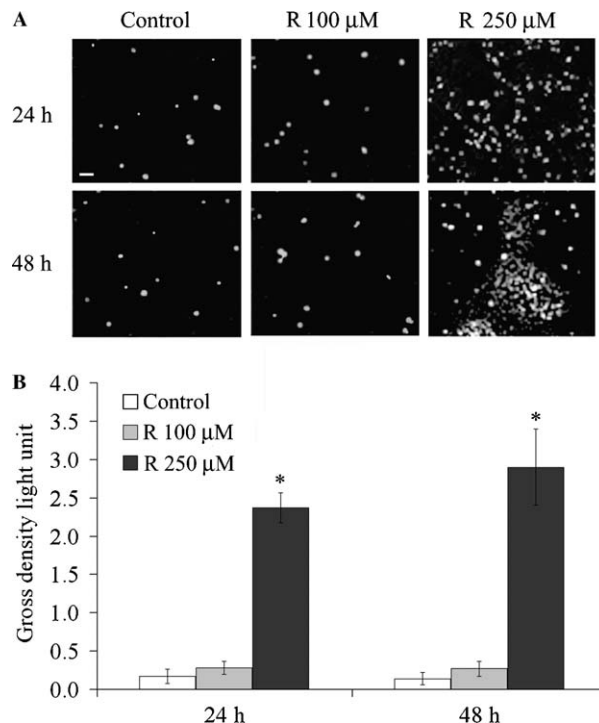


Fig. 3. Influence of resveratrol on membrane integrity measured by propidium iodide (PI) uptake. PI uptake assay was carried out to quantify loss of membrane integrity after exposure to resveratrol (R) at indicated concentrations for 24 and 48 h. (A) Representative fluorescent images with PI. (B) Bars are values (mean  $\pm$  SE) from three independent experiments performed in triplicate. Significant difference from control (0.25% ethanol) values is indicated by \* $P < 0.01$ . Scale bar: 50  $\mu\text{m}$ .

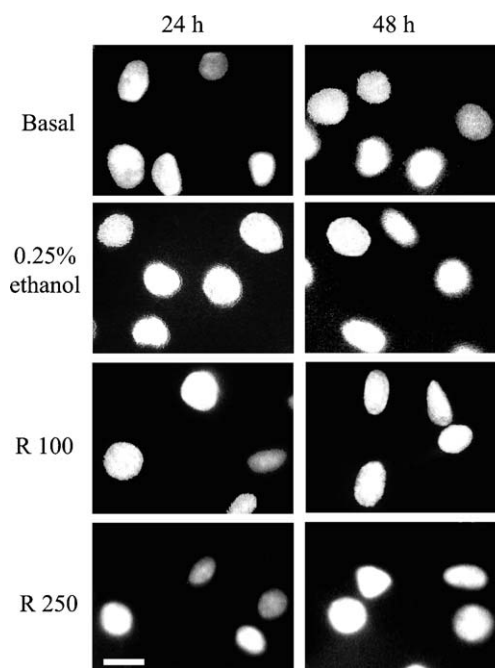


Fig. 4. Effect of resveratrol on nuclear morphology. DAPI staining was used to visualize nuclear morphology by fluorescence microscope after exposure to resveratrol at indicated concentrations for 24 and 48 h. Scale bar: 50  $\mu\text{m}$ .

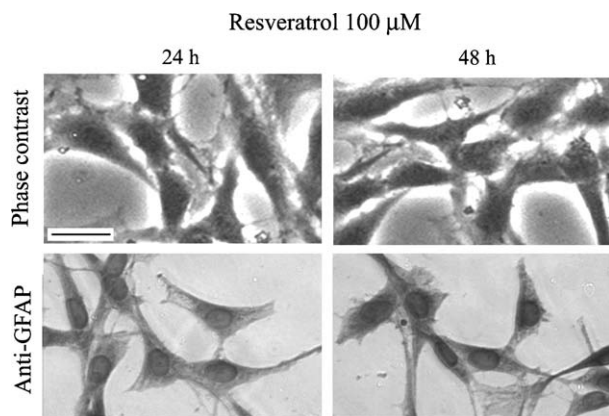


Fig. 5. Representative photomicrographs of C6 glioma cells exposed to resveratrol. Cells were cultured on circular glass and treated with or without 100  $\mu\text{M}$  resveratrol for 24 and 48 h. Cells were fixed and immunostained with anti-GFAP. Cells were viewed with a Nikon inverted microscope. Images from phase contrast and immuno-stained with anti-GFAP were transferred to computer with a digital camera (Sound Vision Inc., Wayland, MA). All images are representative fields from at least three experiments carried out in triplicate. Scale bar: 50  $\mu\text{m}$ .

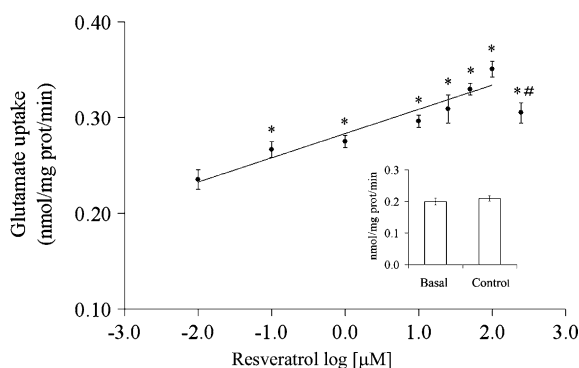


Fig. 6. Influence of resveratrol exposure on the glutamate uptake from C6 cells. Cells were treated with increasing concentrations (0.01, 0.1, 1, 10, 25, 50, 100 and 250  $\mu\text{M}$ ) of resveratrol for 1 h in DMEM without serum. After this time, the incubation medium was removed and glutamate uptake performed during 10 min as described in Materials and methods. The absence of ethanol effect (*per se*) was shown in the inset. The data represent the mean  $\pm$  SE values from four independent experiments performed in triplicate. Data analyses were performed by linear curve fitting and are expressed as the percentage change relative to control cells arbitrarily set at 100%. Significant difference from control (0.25% ethanol) values is indicated by \* $P < 0.05$ .

(0.36  $\mu\text{M}/\text{mg prot}/\text{h}$ ) was increased by resveratrol at concentrations of 10 and 100  $\mu\text{M}$  (Fig. 7). However, at 250  $\mu\text{M}$ , resveratrol was not different from the control. The basal activity of GS was also not affected by ethanol as well.

#### Release of S100B protein in C6 cells

Extracellular S100B content was measured at 24 and 48 h of resveratrol exposure. A significant increase in S100B was observed between 24 and 48 h, independently of resveratrol addition (Fig. 8). Moreover, a significant increment in this protein was observed after 48 h of resveratrol exposure at concentrations of 100 and 250  $\mu\text{M}$ .

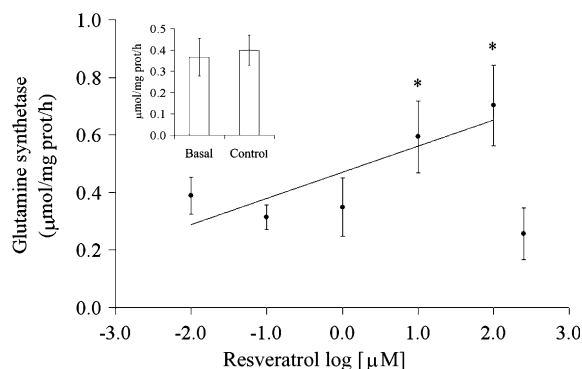


Fig. 7. Influence of resveratrol exposure on the GS activity from C6 cells. Cells were treated with increasing concentrations (0.01, 0.1, 1, 10, 100 and 250  $\mu\text{M}$ ) of resveratrol for 1 h in DMEM without serum. After this time, the incubation medium was removed and GS activity assay was performed as described in Materials and methods. The absence of ethanol effect (*per se*) was shown in the inset. The data represent the mean  $\pm$  SE values from four independent experiments performed in triplicate. Data analyses were performed by linear curve fitting and are expressed as the percentage change relative to control cells arbitrarily set at 100%. Significant difference from control (0.25% ethanol) values is indicated by \* $P < 0.05$ .

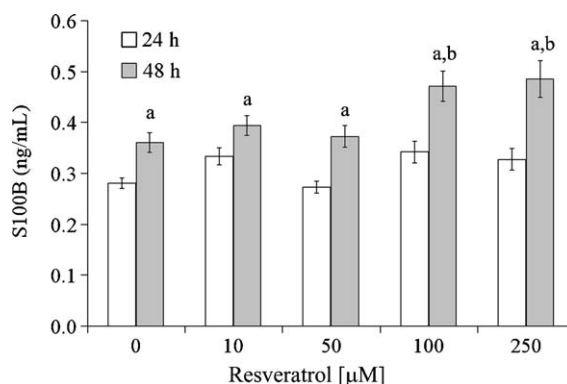


Fig. 8. Effects of resveratrol exposure on the S100B release into the medium in C6 cells. Cells were treated with resveratrol at indicated concentrations for 24 and 48 h in DMEM without serum. The data represent the mean  $\pm$  SE values from four independent experiments performed in triplicate. (a), Significantly different from respective sample at 24 h. (b), Significantly different from control (0.25% ethanol).  $P < 0.05$ .

#### Discussion

Resveratrol was shown to be effective against ischemic brain injury and kainic acid-induced seizures or neuronal cell damage [29] in rodents. The neuroprotection by resveratrol has been mainly attributed to its intrinsic antioxidant properties. However, their potential pharmacological or toxicological effects in brain need to be elucidated. In fact, little is known about the mechanism by which resveratrol acts on diverse cell types as well as its intracellular targets. Resveratrol has some structural resemblance with estrogens and this could explain its estrogenic effect as well as, at least in part, its anti-inflammatory activity, by binding to estrogen receptors [30]. Moreover, resveratrol has a modulatory activity on cyclooxygenases, lipoxygenases [3] and nitric

oxide synthase, which could mediate its anti-inflammatory and anti-cancer effect on specific cell types [31].

Cell proliferation can be modulated, among other factors, by alterations on the cytoskeleton and extracellular matrix [32]. In vivo, neuronal–glial interaction is facilitated by the characteristic morphology of astrocytes which in the intact brain are mainly process-bearing cells where processes typically capsule neuronal structures and the cerebral vasculature [33]. Several factors *in vitro* have been described regulating glial morphology and proliferation, including intracellular calcium [34], redox environment (C. Gottfried, unpublished results), acidification and substrate protein environment [24,35]. In our experimental conditions, resveratrol *per se*, did not affect C6 glioma cell morphology. However, we observed a reduced C6 glioma proliferation following 50 and 100  $\mu\text{M}$  resveratrol exposure for 48 h, which is consistent with the very well demonstrated anti-proliferative properties of resveratrol. The mechanism (s) for this growth inhibitory activity of resveratrol could be due to its ability to block ribonucleotide reductase [36] or to its antioxidant properties that have also been shown to inhibit proliferation of hepatic stellate cells [37]. Moreover, resveratrol at 250  $\mu\text{M}$  caused cell death probably via a necrotic process, as indicated by the absence of nuclear fragmentation in DAPI assay.

Regulation of the glutamate level in the synaptic cleft by glutamate transporters located mainly in astroglia is critical to avoid excitotoxic injury. In fact, a number of reports have shown increased extracellular levels of glutamate in certain neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, motor neuron disease and stroke [38]. It is also very well-known that, in these brain disorders, increases in ROS levels occur, which have been associated with increased release and decreased uptake of glutamate [39]. Here, we demonstrate that resveratrol is able to modulate glutamate uptake in C6 glioma cells. *in vitro* studies indicate that glutamate uptake by glial cells is regulated by the surrounding redox environment and that this uptake activity decreases under oxidizing conditions (see [40] for a review). Therefore, resveratrol could affect the redox environment of glutamate transporters and favor their activities, at least in a range from 0.1–100  $\mu\text{M}$ . Moreover, resveratrol could affect glutamate uptake independently of its antioxidant property [41]. Long and short-term changes in glutamate uptake activity are mediated by protein kinases [42] and resveratrol could affect such kinases. In support of this idea, an inhibitory effect of resveratrol on PKC and PKD and phorbol ester-responsive kinases has been described [43,44]. Moreover, it has been reported that phorbol 12-myristate 13-acetate quickly decreases GLT-1 activity, the main glutamate transporter found in C6 glioma, by inducing rapid transporter internalization [45]. Therefore, resveratrol could increase glutamate uptake by inhibition of phorbol ester-responsive kinases. However, the present study did not specifically address the question of the cellular mechanism through which glutamate

uptake is influenced by resveratrol; this aspect requires further investigation.

Interestingly, resveratrol at 100  $\mu\text{M}$  increased glutamate uptake by 50%, whilst at 250  $\mu\text{M}$  it only increased by 30%. This difference was significant, suggesting a biphasic effect. Accordingly, more recent data provide evidence that, depending upon its concentrations and cell type, resveratrol may have opposing effects in some biological parameters, presenting both anti and prooxidant activity and anti and proapoptotic effect [3]. In the same vein, we found that resveratrol (1–100  $\mu\text{M}$ ) increased GS activity in a concentration-dependent manner ( $R^2=0.95$ ), whilst 250  $\mu\text{M}$  resveratrol did not affect GS activity. It is important to mention that both glutamate uptake and GS assays were carried out 1 h after resveratrol exposure and, at this time, the decrease observed would not be due to cell death.

S100B is a calcium-binding protein that is primarily expressed in the central nervous system by astroglia. The protein belongs to the S100 family involved in the regulation of cell proliferation and morphology. Besides its intracellular role, S100B is secreted by a unknown mechanism and exerts regulatory effects on neighboring cells (astrocytes, neurons and microglia) depending on its concentration, being neurotrophic at nanomolar levels and apoptotic at micromolar levels [46,47]. Primary astrocytes and C6 glioma cell preparations have been used commonly and indistinctly to study S100B expression and secretion [48,49]. However, these cells have different regulatory mechanisms for the gene expression of the S100B [50]. We found that resveratrol exposure (at 100 and 250  $\mu\text{M}$ ) increased extracellular S100B content 48 h afterwards. Since no parallel loss of LDH from C6 cells was observed at 100  $\mu\text{M}$  resveratrol it would be reasonable to conceive that resveratrol affects the mechanism of S100B secretion. However, at 250  $\mu\text{M}$  resveratrol the membrane integrity is lost and therefore the extracellular S100B, at least in part, is due to cell leakage. In agreement, we found an increase of PI incorporation at this concentration of resveratrol.

Many extracellular roles for S100B have been proposed (see [46,47] for a review). This protein at nanomolar concentration *in vitro* stimulates neurite outgrowth and neuronal survival [51] and is able to protect hippocampal neurons against glutamate toxicity [52]. Extending our findings to astrocytes, it would be possible conceive that resveratrol stimulates S100B secretion that in turn stimulates neuronal survival and activity during brain injury and recovery.

It is important to mention some limitations and perspectives of this study. First, we have investigated resveratrol at concentration range from 0.01 to 250  $\mu\text{M}$ . These values were chosen based on many other recent works [53]. However, there is an apparent discrepancy between the concentrations used for *in vitro* resveratrol efficacy (commonly 5–100  $\mu\text{M}$ ) and the extracellular concentrations (less than 2  $\mu\text{M}$  in plasma) [54]. Low levels of resveratrol are due to its fast metabolism in liver and intestinal epithelial cells, mainly to resveratrol-glucuronide and resveratrol-sulfate

(see [31] for a review). These conjugations can be reversed in peripheral tissues and some polyphenols present in the diet (e.g. quercetin) can inhibit resveratrol glucuronidation making it difficult to estimate its bioavailability and final concentration in tissue targets. Second, cultured astrocytes and C6 glioma cells have been used almost equally to investigate astrocyte physiology because these cells exhibit many common biochemical properties [9,20–23]. However, further studies extending our findings will be necessary in primary cultures of astrocytes and in co-culture with neurons.

In summary, we have demonstrated for the first time, that resveratrol (at 50–100  $\mu$ M) improves important glial activities such as glutamate uptake, GS activity and S100B secretion. Changes in these parameters may contribute to the protective role of astrocytes in brain injury conditions, reinforcing the putative use of this compound in the therapeutic arsenal against neurodegenerative disorders and ischemia-reperfusion injury. In contrast, elevated concentrations of resveratrol may not have beneficial effects.

### Acknowledgments

This work was supported by the Brazilian funding agents; Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS) Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Pró-Reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (PROPESQ-UFRGS).

### References

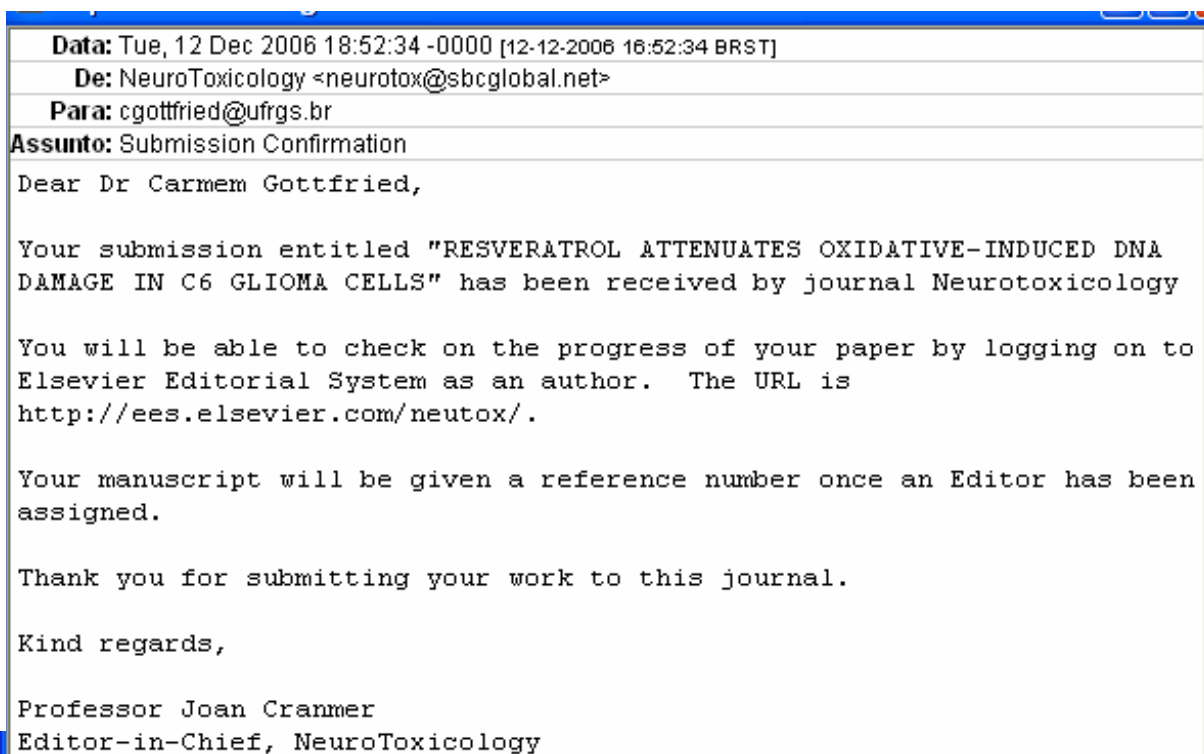
- [1] G.J. Soleas, E.P. Diamandis, D.M. Goldberg, *Clin. Biochem.* 30 (1997) 91–113.
- [2] L. Fremont, L. Belguendouz, S. Delpal, *Life Sci.* 64 (1999) 2511–2521.
- [3] S. Pervaiz, *Drug Resist. Updat.* 7 (2004) 333–344.
- [4] S. Renaud, M. de Lorgeril, *Lancet* 339 (1992) 1523–1526.
- [5] M. Sato, Y. Suzuki, T. Okuda, K. Yokotsuka, *Biosci. Biotechnol. Biochem.* 61 (1997) 1800–1805.
- [6] L.M. Hung, J.K. Chen, S.S. Huang, R.S. Lee, *Cardiovasc. Res.* 47 (2000) 549–555.
- [7] M. Miloso, A.A. Bertelli, G. Nicolini, G. Tredici, *Neurosci. Lett.* 264 (1999) 141–144.
- [8] V. Castagne, M. Gautschi, K. Lefevre, A. Posada, P.G. Clarke, *Prog. Neurobiol.* 59 (1999) 397–423.
- [9] A.C. Rego, C.R. Oliveira, *Neurochem. Res.* 28 (2003) 1563–1574.
- [10] K.G. Manton, S. Volovik, A. Kulminski, *Curr. Alzheimer Res.* 1 (2004) 277–293.
- [11] K. Takuma, A. Baba, T. Matsuda, *Prog. Neurobiol.* 72 (2004) 111–127.
- [12] S. Desagher, J. Glowinski, J. Premont, *J. Neurosci.* 16 (1996) 2553–2562.
- [13] S.D. Skaper, B. Ancona, L. Facci, D. Franceschini, P. Giusti, *FASEB J.* 12 (1998) 725–731.
- [14] J. Tanaka, K. Toku, B. Zhang, K. Ishihara, M. Sakanaka, N. Maeda, *Glia* 28 (1999) 85–96.
- [15] R. Dringen, J.M. Gutterer, J. Hirrlinger, *Eur. J. Biochem.* 267 (2000) 4912–4916.
- [16] M.J. Wang, H.M. Huang, S.J. Hsieh, K.C. Jeng, J.S. Kuo, *J. Neuroimmunol.* 112 (2001) 28–34.
- [17] Q. Wang, J. Xu, G.E. Rottinghaus, A. Simonyi, D. Lubahn, G.Y. Sun, A.Y. Sun, *Brain Res.* 958 (2002) 439–447.
- [18] P. Benda, J. Lightbody, G. Sato, L. Levine, W. Sweet, *Science* 161 (1968) 370–371.
- [19] K.K. Parker, M.D. Norenberg, A. Vernadakis, *Science* 208 (1980) 179–181.
- [20] D. Mangoura, N. Sakellaridis, J. Jones, A. Vernadakis, *Neurochem. Res.* 14 (1989) 941–947.
- [21] Z. Feng, J.T. Zhang, *Free Radic. Biol. Med.* 37 (2004) 1790–1801.
- [22] S.R. Cechin, P.R. Dunkley, R. Rodnight, *Neurochem. Res.* 30 (2005) 603–611.
- [23] C. Funchal, A.Q. Dos Santos, M.C. Jacques-Silva, A. Zamoner, C. Gottfried, M. Wajner, R. Pessoa-Pureur, *Metab. Brain Dis.* 20 (2005) 205–217.
- [24] C. Gottfried, S.R. Cechin, M.A. Gonzalez, T.S. Vaccaro, R. Rodnight, *Neuroscience* 121 (2003) 553–562.
- [25] C. Gottfried, F. Tramontina, D. Goncalves, C.A. Goncalves, E. Moriguchi, R.D. Dias, S.T. Wofchuk, D.O. Souza, *Mech. Ageing Dev.* 123 (2002) 1333–1340.
- [26] C.K. Petito, M.C. Chung, L.M. Verkhovskiy, A.J. Cooper, *Brain Res.* 569 (1992) 275–280.
- [27] F. Tramontina, J. Karl, C. Gottfried, A. Mendez, D. Goncalves, L.V. Portela, C.A. Goncalves, *Brain Res. Brain Res. Protoc.* 6 (2000) 86–90.
- [28] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, *J. Biol. Chem.* 193 (1951) 265–275.
- [29] M. Virgili, A. Contestabile, *Neurosci. Lett.* 281 (2000) 123–126.
- [30] B. Jannin, M. Menzel, J.P. Berlot, D. Delmas, A. Lancon, N. Latruffe, *Biochem. Pharmacol.* 68 (2004) 1113–1118.
- [31] P. Signorelli, R. Ghidoni, *J. Nutr. Biochem.* 16 (2005) 449–466.
- [32] M.A. Wozniak, K. Modzelewska, L. Kwong, P.J. Keely, *Biochim. Biophys. Acta* 1692 (1994) 103–119.
- [33] R. Ventura, K.M. Harris, *J. Neurosci.* 19 (1999) 6897–6906.
- [34] E. Alberdi, M.V. Sanchez-Gomez, C. Matute, *Cell Calcium* 38 (2005) 417–425.
- [35] S.R. Cechin, C. Gottfried, C.C. Prestes, L. Andrighetti, S.T. Wofchuk, R. Rodnight, *Brain Res.* 946 (2002) 12–23.
- [36] M. Fontecave, M. Lepoivre, E. Elleingand, C. Gerez, O. Guittet, *FEBS Lett.* 421 (1998) 277–279.
- [37] N. Kawada, S. Seki, M. Inoue, T. Kuroki, *Hepatology* 27 (1998) 1265–1274.
- [38] G. Campiani, C. Fattorusso, M. De Angelis, B. Catalanotti, S. Butini, R. Fattorusso, I. Fiorini, V. Nacci, E. Novellino, *Curr. Pharm. Des.* 9 (2003) 599–625.
- [39] A.C. Bowling, M.F. Beal, *Life Sci.* 56 (1995) 1151–1171.
- [40] D. Trotti, N.C. Danbolt, A. Volterra, *Trends Pharmacol. Sci.* 19 (1998) 328–334.
- [41] Z. Ovesna, K. Horvathova-Kozics, *Neoplasma* 52 (2005) 450–455.
- [42] B.A. Guillet, L.J. Velly, B. Canolle, F.M. Masméjean, A.L. Nieoullon, P. Pisano, *Neurochem. Int.* 46 (2005) 337–346.
- [43] S.J. Slater, A.C. Cook, J.L. Seiz, S.A. Malinowski, B.A. Stagliano, C.D. Stubbs, *Biochemistry* 42 (2003) 12105–12114.
- [44] J.R. Stewart, K.L. Christman, C.A. O'Brian, *Biochem. Pharmacol.* 60 (2000) 1355–1359.
- [45] A. Kalandadze, Y. Wu, M.B. Robinson, *J. Biol. Chem.* 277 (2002) 45741–45750.
- [46] R. Donato, *Int. J. Biochem. Cell Biol.* 33 (2001) 637–668.
- [47] L.J. Van Eldik, M.S. Wainwright, *Restor. Neurol. Neurosci.* 21 (2003) 97–108.
- [48] S.S. Pinto, C. Gottfried, A. Mendez, D. Goncalves, J. Karl, C.A. Goncalves, S. Wofchuk, R. Rodnight, *FEBS Lett.* 486 (2000) 203–207.
- [49] G.E. Davey, P. Murmann, C.W. Heizmann, *J. Biol. Chem.* 276 (2001) 30819–30826.
- [50] F. Castets, W.S. Griffin, A. Marks, L.J. Van Eldik, *Brain Res. Mol. Brain Res.* 46 (1997) 208–216.
- [51] F. Wittingham-Major, J.L. Staecker, S.W. Barger, S. Coats, L.J. Van Eldik, *Cell Biol.* 109 (1989) 3063–3071.
- [52] D. Kogel, M. Peters, H.G. König, S.M. Hashemi, N.T. Bui, V. Arolt, M. Rothermundt, J.H. Prehn, *Neuroscience* 127 (2004) 913–920.
- [53] F. Picard, M. Kurtev, N. Chung, A. Topark-Ngarm, T. Senawong, R. Machado De Oliveira, M. Leid, M.W. McBurney, L. Guarente, *Nature* 429 (2004) 771–776.
- [54] A.J. Gescher, W.P. Steward, *Cancer Epidemiol. Biomarkers Prev.* 12 (2003) 953–957.

## **CAPÍTULO II**

### **RESVERATROL ATTENUATES OXIDATIVE-INDUCED DNA DAMAGE IN C6 GLIOMA CELLS**

**SUBMETIDO AO PERIÓDICO NEUROTOXICOLOGY**

## CARTA DE SUBMISSÃO





**RESVERATROL ATTENUATES OXIDATIVE-INDUCED DNA DAMAGE IN C6  
GLIOMA CELLS**

ANDRÉ QUINCOZES-SANTOS, ANA CRISTINA ANDREAZZA, PATRICIA  
NARDIN, CLÁUDIA FUNCHAL, CARLOS-ALBERTO GONÇALVES, CARMEM  
GOTTFRIED

Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde,  
Departamento de Bioquímica, Rua Ramiro Barcelos 2600 anexo, 90035-003 Porto Alegre,  
RS, BRAZIL.

CORRESPONDENT AUTHOR: Dr. Carmem Gottfried, Universidade Federal do Rio  
Grande do Sul, Instituto de Ciências Básicas da Saúde, Departamento de Bioquímica, Rua  
Ramiro Barcelos 2600 anexo, 90035-003 Porto Alegre, RS, BRAZIL.

Fax: 5551 3316 5535 Tel: 5551 3316 5565 E-mail: [cgottfried@ufrgs.br](mailto:cgottfried@ufrgs.br)

## **Abstract**

The antioxidant compound, *trans*-resveratrol, is found in substantial amounts in several types of red wine and has been proposed to have beneficial effects in brain pathologies that may involve oxidative stress. The objective of the present study was to investigate the genoprotective effects of resveratrol under conditions of oxidative stress induced by hydrogen peroxide in C6 glioma cells. DNA damage was assessed by the alkaline single-cell gel electrophoresis assay or comet assay. In order to investigate the genoprotective effects of resveratrol against oxidative stress induced by hydrogen peroxide on DNA damage, two models of oxidative stress induction were utilized. I. 1 mM hydrogen peroxide for 0.5 h (10-250  $\mu$ M of resveratrol) and II. 0.1 or 0.5 mM hydrogen peroxide for 6 h (10-100  $\mu$ M of resveratrol). Resveratrol was able to prevent oxidative damage to cellular DNA, induced in model I, at all concentrations tested; however, at 6 h of incubation, resveratrol prevented DNA damage only partially. After 6 h of incubation (up to 48 h) resveratrol *per se* induced a slight time and dose-dependent DNA damage. In conclusion, these results provide evidence that resveratrol may act as a significantly bioactive compound, supporting the possibility that, due to its antioxidant properties, it may be important in health and disease for protecting against DNA damage through oxidative stress.

Keywords: Resveratrol, genotoxicity, neuroprotection, hydrogen peroxide, comet assay, C6 glioma.

## 1. Introduction

Resveratrol (3,5,4'-trihydroxy-stilbene, scheme 1), a polyphenol present in grapes and red wine, has antioxidant properties and a wide range of other biological effects (Fremont, 2000; Baur and Sinclair, 2006).

*Scheme 1. Chemical structures of trans- and cis-resveratrol isomers.*

Many studies now attest the cardioprotective (Das et al, 2006; Stef et al,2006; Shen et al, 2006) and chemopreventive (Pervaiz, 2004; Signorelli and Ghidoni, 2005; Delmas et al., 2006) effects of this compound. In addition, epidemiological evidence shows that moderate consumption of red wine is inversely correlated with the incidence of dementia and Alzheimer's disease (Tredici et al., 1999; Marambaud et al., 2005). Furthermore, direct neuroprotective effects of resveratrol against oxidative stress have been demonstrated *in vitro* (Chanvitayapongs et al., 1997; Savaskan et al, 2003). The underlying mechanisms of the neuroprotective action of resveratrol, however, are not fully understood. We recently demonstrated that resveratrol is able to modulate glial parameters such as glutamate uptake, glutamine synthetase and S100B secretion in C6 glioma cells (Dos Santos et al., 2006) and in primary astrocyte cultures (L.M.V. Almeida – unpublished results). Brain tissue is particularly vulnerable to oxidative damage, possibly due to its high consumption of oxygen and the consequent generation of high quantities of reactive oxygen species (ROS) during oxidative phosphorylation (Castanet et al., 1999). Several regions of the brain are particularly rich in iron, which promotes the production of damaging oxygen free radical species such as  $^{\circ}\text{OH}$  radicals (Halliwell and Gutteridge 1989). Furthermore, the brain is relatively poorly endowed with protective antioxidant enzymes or antioxidant compounds. ROS formation has been implicated in damage to nervous tissue in several brain pathologies, such as ischemia-reperfusion injury and neurodegenerative disorders (Rego

and Oliveira, 2003; Manton et al., 2004). Excessive ROS (e.g, hydrogen peroxide - H<sub>2</sub>O<sub>2</sub>) can lead to lipid, protein and DNA oxidation, causing cell damage to all cellular constituents. Irreparable DNA damage is involved in carcinogenesis, aging and other degenerative diseases (Cozzi et al, 1997).

Several enzymes expressed in brain, including monoamine oxidase and tyrosine hydroxylase, lead to H<sub>2</sub>O<sub>2</sub> formation as a normal by-product of their activity. Putative signaling pathways, activated by resveratrol in neural cells, have now been demonstrated in neuroblastoma cell lines, where the phosphorylation of some key kinases has been shown to occur within minutes of exposure to resveratrol (Miloso et al., 1999). The actions of resveratrol on protein kinases appear to be complex: it activates extracellular regulated kinases ERK1 and ERK2 (Miloso et al., 1999; Alkhalaf and Jaffal, 2006), while inhibiting protein kinase C (PKC), a mediator of cancer cell proliferation (Atten et al., 2005).

The C6 glioma cell line was originally derived from rat brain tumors induced by *N*-nitrosomethylurea (Benda et al., 1968); these cells have oligodendrocytic, astrocytic, and neuronal properties (Parker et al., 1980) and are widely used as an astrocyte-like cell line (Mangoura et al., 1989; Feng and Zang, 2004; Cechin et al., 2005; Funchal et al., 2005; Dos Santos et al., 2006). In this study, the effects of resveratrol on H<sub>2</sub>O<sub>2</sub>-mediated DNA damage in C6 glioma cells were determined using alkaline single-cell gel electrophoresis (the comet assay).

## **2. Material and Methods**

**2.1. Materials:** *trans*-resveratrol, propidium iodide, ethidium bromide and material for cell culture were purchased from Sigma (St. Louis MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Carlsbad, CA, USA) and fetal

bovine serum (FBS) was from Cultilab (Campinas, SP, Brazil). All other chemicals were purchased from regular commercial suppliers.

## **2.2. C6 glioma culture and resveratrol treatment**

C6 glioma culture was performed essentially accordingly to the procedure previously described (Dos Santos et al., 2006). After cells reached confluence, the culture medium was removed by suction and the cells were incubated for 1, 6, 12, 24 or 48 hours at 37°C in an atmosphere of 5% CO<sub>2</sub> / 95% air in DMEM (pH 7.4) without serum in the absence or presence of resveratrol (10, 50, 100 or 250 µM), taking cells exposed to vehicle (0.25% ethanol) as controls. The concentrations of resveratrol used in these experiments were obtained from previous determinations (Dos Santos et al., 2006). Importantly, in all parameters analyzed, the results obtained with vehicle were not different from those obtained in basal conditions without ethanol.

## **2.3. Hydrogen peroxide treatment**

In order to investigate the protective effects of resveratrol against oxidative stress induced by H<sub>2</sub>O<sub>2</sub>, two models of oxidative stress induction were used. Before H<sub>2</sub>O<sub>2</sub> exposure, cells were pre-incubated with different concentrations of resveratrol (10, 50, 100 or 250 µM) for 1 h at 37°C in an atmosphere of 5% CO<sub>2</sub> / 95% air in DMEM (pH 7.4) without serum. After this, the medium was maintained and H<sub>2</sub>O<sub>2</sub> was added as follows: MODEL I - 1 mM H<sub>2</sub>O<sub>2</sub> for 0.5 h (in the presence or absence of 10, 50, 100 and 250 µM of resveratrol) and MODEL II - 0.1 or 0.5 mM H<sub>2</sub>O<sub>2</sub> for 6 h (in the presence or absence of 10, 50 and 100 µM of resveratrol). During incubations, cells were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> / 95% air.

## **2.4. Morphological studies and propidium iodide uptake in C6 glioma cells.**

During treatments, cells were analysed and photographed with a Nikon inverted microscope phase-contrast optics. Cellular damage was assessed by fluorescent image analysis using the propidium iodide (PI) method (Pringle et al., 1996), which measures the incorporation of PI by the cells. Cells were treated with 7.5  $\mu$ M PI concomitantly with H<sub>2</sub>O<sub>2</sub> addition and maintained until the end of incubation, after which cells were analysed and photographed with a Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory. Optical density was determined with the Optiquant version 02.00 software (Packard Instrument). Density values obtained were expressed as the density light unit (DLU).

### **2.5. Comet assay**

A standard protocol for comet assay preparation and analysis was adapted (Singh et al., 1988; Tice et al., 2000). After different treatments as described above, C6 glioma cells were detached by incubating in the presence of trypsin/EDTA 0.05%. During trypsinization, cells were carefully manipulated to avoid mechanical stress. Slides were prepared by mixing 50  $\mu$ l of C6 glioma suspension with 70  $\mu$ l of low melting point agarose (0.75%). The mixture (cells/agarose) was added to a fully frosted microscope slide coated with a layer of 300  $\mu$ l of normal melting agarose (1%). After solidification, the cover slip was gently removed, and the slides were placed in lysing solution (2.5 M NaCl, 100 mM disodium EDTA and 10 mM Tris, pH 10.0, with freshly added 1% Triton X-100 and 10% dimethyl sulfoxide) for up to 24 h. Subsequently, the slides were incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 10 min and electrophoresed. Following electrophoresis, slides were immersed in neutralizing buffer (0.4 M Tris-HCl, pH 7.5, 4°C) for 5 min, before finally applying 50  $\mu$ L of 5  $\mu$ g/mL ethidium bromide to them and leaving

in the dark for 20 min to stain the DNA. Negative and positive controls were used for each electrophoresis assay in order to ensure the reliability of the procedure. Images of 100 randomly selected nuclei (50 nuclei from two replicated slides) were analyzed for each treatment. Nuclei were scored visually for comet tail size based on an arbitrary scale of 0-4, i.e., ranging from no damage to extensive damage of DNA (Scheme 2). Thus, a group damage index could range from 0 (all nuclei without tail, 100 cells x 0) to 400 (all nuclei with maximally long tails, 100 cells x 4) (Collins et al. 1996; Collins et al. 1997).

*Scheme 2. Representative images of tail sizes indicating DNA fragmentation used to calculate index of DNA damage. During electrophoresis any relaxed or broken DNA fragments migrate further than supercoiled, undamaged DNA. Slides were viewed on a Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory and images were transferred to a computer with a digital camera (Sound Vision Inc. Wayland, MA).*

**2.6. Statistical analysis.** Data from the experiments are presented as mean  $\pm$  S.E.M. and were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey's test. Values of  $P < 0.05$  were considered to be significant. All analyses were carried out on an IBM compatible PC, using the Statistical Package for Social Sciences (SPSS) software.

### **3. Results**

#### **3.1. Effect of resveratrol on DNA damage in C6 glioma cells**

As shown in Fig. 1A, resveratrol *per se* induced slight DNA damage in a time and dose-dependent manner. Interestingly, the same profile was observed with regard to the percentage of nuclei with an index of damage 4, as shown in Fig. 1B (see the description of the comet assay and scheme 2 in the Material and Methods section for a better

understanding of the different degrees of DNA damage used to calculate the index of DNA damage). During the first six hours of resveratrol incubation, C6 glioma cells presented a very low index of DNA damage (up to  $42 \pm 1.25$  – Fig. 1A) and an insignificant percentage (up to 2% - Fig. 1B) of damage 4, the highest degree of DNA fragmentation. It should be pointed out that it was not possible to measure the index of DNA damage at 48 h with 250  $\mu$ M resveratrol due to the presence of cell death in agreement with our previous results (Dos Santos et al., 2006).

### **3.2. Cell morphology and membrane integrity evaluation under conditions of oxidative stress induced by H<sub>2</sub>O<sub>2</sub> in C6 glioma cells**

C6 cells were morphologically analysed by phase contrast microscopy after different exposure times of exposure to H<sub>2</sub>O<sub>2</sub>, as indicated in Fig. 2. Under basal culture conditions, as demonstrated previously (Funchal et al., 2006), C6 glioma cells presented a rounded appearance, and this characteristic shape was not altered even in control medium containing 0.25% ethanol vehicle (Fig. 2A panel d) or different times of exposure to resveratrol (Fig. 2A, panels a and g). Importantly, 0.25% ethanol did not alter basal morphology (data not shown) in model I of DNA oxidative damage. Fig. 2A, panels b, e, h show H<sub>2</sub>O<sub>2</sub>-induced morphological alterations in C6 cells, consisting of process-bearing cells, as indicated by the arrows, and resveratrol was not able to prevent this effect (Fig. 2A, panels c, f, i). In order to confirm the absence of cell death in all parameters investigated in both models of H<sub>2</sub>O<sub>2</sub>-induced DNA damage, cell integrity was evaluated by PI uptake. There was no cell integrity alteration, as observed by density values indicated in each panel (Fig 2B).

### **3.3. Genoprotective effects of resveratrol against H<sub>2</sub>O<sub>2</sub>-induced DNA damage in C6 glioma cells**



For model I of treatment, the index of DNA damage observed when C6 glioma cells were incubated in the presence of H<sub>2</sub>O<sub>2</sub> without resveratrol was  $39 \pm 2.05$  (Fig. 3A). In this model, resveratrol was able to almost totally prevent oxidative damage to cellular DNA induced by H<sub>2</sub>O<sub>2</sub> at all concentrations tested ( $P < 0.05$ ). However, for model II, the effective concentrations of resveratrol against oxidative 0.1 mM H<sub>2</sub>O<sub>2</sub>-induced DNA damage ( $73 \pm 2.56$ ) ranged from 50 to 100  $\mu$ M ( $29 \pm 1.88$  and  $31 \pm 1.99$ , respectively). However, in the presence of 0.5 mM H<sub>2</sub>O<sub>2</sub>, resveratrol presented a partial dose-dependent prevention of H<sub>2</sub>O<sub>2</sub>-induced DNA damage ( $89 \pm 2.89$ ) in C6 glioma cells (Fig. 3A). Interestingly, the percentage of nuclei containing damage 4 (Fig. 3B) showed the same profile as the DNA damage index determined in Fig. 3A. It should be pointed out that this comparative analysis was also described in Fig 1.

#### **4. Discussion**

The present results constitute, to our knowledge, the first evidence of a neuroprotective role of resveratrol against oxidative DNA damage evaluated by the comet assay. H<sub>2</sub>O<sub>2</sub> is the major mediator of oxidative stress and a potent mutagen. Large quantities of H<sub>2</sub>O<sub>2</sub> can be generated by the respiratory chain and several other metabolic pathways. Moreover, activated inflammatory cells (e.g. microglia) are able to produce this molecule via the oxidative burst mechanism. Although this peroxide is a weak oxidant, it can be converted, in the presence of reduced transition metals such as ferrous and cuprous ions, to highly reactive hydroxyl radicals that are believed to mediate the genotoxicity of this compound (Halliwell and Gutteridge 1989).

Model I of H<sub>2</sub>O<sub>2</sub> exposure was used to induce an intense (1 mM peroxide) and acute (30 min) damage. Cells pretreated with resveratrol (for 1 h) were fully protected from peroxide

damage. Model II of H<sub>2</sub>O<sub>2</sub> exposure was used to induce a less intense (0.1-0.5 mM peroxide), but lasting (6 h) damage. Pre-incubation with resveratrol at 10 μM was not able to prevent DNA damage induced by peroxide, but at 50-100 μM, resveratrol was protective. However, caution is necessary to interpret results of the comet assay. This assay is very sensitive for detecting low levels of DNA damage, but this damage is usually not necessarily correlated with cell viability (Brendler-Schaab et al, 2005). In fact, based on PI uptake, cell integrity was maintained under our models (I and II) after oxidative insult.

Our results support the idea that resveratrol has a potential role for neuroprotection *in vitro* and could be beneficial in brain disorders involving oxidative stress. In neuropathologies, as well as in the natural process of aging, the involvement of oxygen free radical overproduction is strongly suspected as a major factor related to the progressive derangement from normal function (Beckman and Ames, 1998) and could involve several mechanisms of cell signaling.

The effect of resveratrol *per se* on DNA damage was clear, depending on its concentration and time of incubation. Accordingly, many other data suggest that, depending upon its concentration and the cell type, resveratrol and other antioxidants in the diet may have opposing effects on some biological parameters, presenting both anti- and pro-oxidant activities and anti- and pro-apoptotic effects (Pervaiz, 2004). Recently, we showed that resveratrol was able to stimulate glutamate uptake activity in C6 glioma cells up to 100 μM, but it decreased this activity at 250 μM (Dos Santos et al., 2006).

Many protein targets of ROS are involved in the regulation of cell morphology, including G proteins and protein kinases (Zhu et al, 2005; Ben-Mahdi et al, 2000). Alterations in C6 glioma cell morphology were induced by H<sub>2</sub>O<sub>2</sub>; however, resveratrol (at

antioxidant concentrations) was not able to reverse the changes putatively caused by oxidative stress. Furthermore, resveratrol did not induce any morphological changes in C6 cells at pro-oxidant concentrations, suggesting that the effect observed on morphology is not exclusively due to changes in the redox state of these cells.

A recent study on astrocytes showed that  $H_2O_2$  triggers the phosphorylation of the p38 mitogen-activated protein kinase (MAPK) leading to cytoskeleton reorganization (Zhu et al, 2005). The signaling pathway involved in the cytoskeleton reorganization of C6 cells is not known, but if p38 is activated by  $H_2O_2$ , resveratrol may not be able to reverse this activation. Since resveratrol activates p38 MAPK, at least in cardiocytes (Das et al, 2006) and human malignant B cells (Shimizu et al, 2006), blockage of such alterations, induced by peroxide, that also involve the activation of this protein kinase would not be possible.

Another important signaling system is the protein kinase C (PKC) system, a family of 12 serine/threonine kinases, which play a central role in the growth factor signal transduction pathway and regulate a wide variety of cellular functions, including cell proliferation and cell death (Nishizuka, 1994; Koivunen et al., 2006). Neuroprotective abilities have been attributed to resveratrol, since protection against nitric oxide-related toxicity in cultured hippocampal neurons could involve PKC inactivation (Bastianetto et al., 2000). Several members of the PKC system serve as substrates for caspases and the PKC $\delta$  isozyme has been intimately associated with DNA damage-induced apoptosis. It has also been demonstrated that PKC $\delta$  is likely to be involved in resveratrol-mediated neuroprotection (Han et al., 2004). In addition, the function of PKC $\delta$  may be influenced by the presence of other PKC isozymes, such as PKC $\alpha$  (Matassa et al, 2003), and resveratrol appears to also exert an inhibitory effect on PKC $\alpha$  (Slater et al., 2003). Thus, this inhibitory

effect of resveratrol against DNA damage in C6 glioma cells could be explained by the antioxidant properties of resveratrol that probably inhibit the action of PKC $\delta$  and/or modulate the signaling processes of this enzyme. In this scenario, the inhibitory effects of resveratrol on PKC activities are likely to be important determinants in the mechanisms by which resveratrol exerts its beneficial effects on cellular function and deserves further investigations.

Two limitations of this study must be mentioned. First, we investigated resveratrol at a concentration range of 10 to 250  $\mu$ M based on other *in vitro* assays; however, plasmatic levels of this polyphenol are many times lower (Gescher and Steward, 2003). Secondly, although we found a promising benefit of resveratrol against oxidative damage in C6 cells, these results cannot be automatically extended to other neural cells, such as neurons; additional investigation into the neuroprotective activity of resveratrol against oxidative DNA damage in other experimental models and cell types is required.

In summary, our results from model I indicate an effective protective effect of resveratrol (at concentrations between 10 and 100  $\mu$ M) against oxidative DNA damage, induced by H<sub>2</sub>O<sub>2</sub>, in C6 glioma cells. Its protective effect was observed in two *in vitro* models involving different treatment with H<sub>2</sub>O<sub>2</sub>. Moreover, longer exposure (more than 12 h) of resveratrol at a higher concentration (250  $\mu$ M) caused an increase in DNA damage. This study contributes to further the understanding of the neuroprotective role of resveratrol, emphasizing the influence of this polyphenol and other antioxidants in the diet for human health, possibly in the prevention of brain disorders associated with oxidative stress.

**Acknowledgements:** This work was supported by the Brazilian funding agents; Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS) Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Pró-Reitoria de Pesquisa e Pós-Graduação da Universidade Federal do Rio Grande do Sul (PROPESq-UFRGS).

## 5. References

- Alkhalaf M, Jaffal S. Potent antiproliferative effects of resveratrol on human osteosarcoma SJSA1 cells: Novel cellular mechanisms involving the ERKs/p53 cascade. *Free Radic Biol Med* 2006; 41:318-325.
- Atten MJ, Godoy-Romero E, Attar BM, Milson T, Zopel M, Holian O. Resveratrol regulates cellular PKC alpha and delta to inhibit growth and induce apoptosis in gastric cancer cells. *Invest New Drugs* 2005; 23:111-119.
- Bastianetto S, Zheng WH, Quirion R. Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. *Br J Pharmacol* 2000; 131:711-720.
- Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov* 2006; 5:493-506.
- Beckman KB, Ames BN. The free radical theory of aging matures. *Physiol Rev* 1998; 78:547-581.
- Benda P, Lightbody J, Sato G, Levine L, Sweet W. Differentiated rat glial cell strain in tissue culture. *Science* 1968; 161:370-371.
- Ben-Mahdi MH, Gozin A, Driss F, Andrieu V, Christen MO, Pasquier C. Anethole dithiolethione regulates oxidant-induced tyrosine kinase activation in endothelial cells. *Antioxid Redox Signal* 2000; 2:789-799.
- Brendler-Schwaab S, Hartmann A, Pfuhler S, Speit G. The in vivo comet assay: use and status in genotoxicity testing. *Mutagenesis* 2005; 20:245-254.
- Castagne V, Gautschi M, Lefevre K, Posada A, Clarke PG. Relationships between neuronal death and the cellular redox status. Focus on the developing nervous system. *Prog Neurobiol* 1999; 59:397-423.

Cechin SR, Dunkley, PR, Rodnight R. Signal transduction mechanisms involved in the proliferation of C6 glioma cells induced by lysophosphatidic acid. *Neurochem Res* 2005; 30:603-611.

Chanvitayapongs S, Draczynska-Lusiak B, Sun AY. Amelioration of oxidative stress by antioxidants and resveratrol in PC12 cells. *NeuroReport* 1997; 8:1499-1502.

Collins AR, Dusinska M, Gedik CM, Stetina R. Oxidative damage to DNA: do we have a reliable biomarker? *Environ. Health Perspect* 1996; 104:465-469.

Collins AR, Dobson VL, Dusinska M, Kennedy G, Stetina R. The comet assay: what can it really tell us? *Mutat Res* 1997; 29:183-193.

Cozzi R, Ricordy R, Aglitti T, Gatta V, Perticone P, De Salvia R. Ascorbic acid and beta-carotene as modulators of oxidative damage. *Carcinogenesis* 1997; 18:223-228

Das S, Fraga CG, Das DK. Cardioprotective effect of resveratrol via HO-1 expression involves p38 map kinase and PI-3-kinase signaling, but does not involve NFkappaB. *Free Radic Res* 2006; 40:1066-1075

Delmas D, Lancon A, Colin D, Jannin B, Latruffe N. Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. *Curr Drug Targets* 2006; 7:423-442.

Dos Santos AQ, Nardin P, Funchal C, De Almeida LM, Jacques-Silva MC, Wofchuk ST, Gonçalves CA, Gottfried C. Resveratrol increases glutamate uptake and glutamine synthetase activity in C6 glioma cells *Arch Biochem Biophys* 2006; 453:161-167.

Feng Z, Zhang JT. Protective effect of melatonin on beta-amyloid-induced apoptosis in rat astrogloma C6 cells and its mechanism. *Free Radic Biol Med* 2004; 37:1790-1801.

Fremont L Biological effects of resveratrol. *Life Sci* 2000; 66:663-673.

Funchal C, Dos Santos AQ, Jacques-Silva MC, Zamoner A, Gottfried C, Wajner M, Pessoa-Pureur R. Branched-chain alpha-keto acids accumulating in maple syrup urine

- disease induce reorganization of phosphorylated GFAP in C6-glioma cells. *Metab Brain Dis* 2005; 20:205-217.
- Funchal C, Latini A, Jacques-Silva MC, Dos Santos AQ, Buzin L, Gottfried C, Wajner M, Pessoa-Pureur R. Morphological alterations and induction of oxidative stress in glial cells caused by the branched-chain alpha-keto acids accumulating in maple syrup urine disease. *Neurochem Int* 2006; 49:640-650.
- Gescher AJ, Steward WP. Relationship between mechanisms, bioavailability, and preclinical chemopreventive efficacy of resveratrol: a conundrum. *Cancer Epidemiol Biomarkers Prev* 2003; 12:953-957.
- Gutteridge JM, Halliwell B. Iron toxicity and oxygen radicals. *Baillieres Clin Haematol* 1989; 2:195-256.
- Halliwell B, Gutteridge JM Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990; 186:1-85.
- Han YS, Zheng WH, Bastianetto S, Chabot JG, Quirion R. Neuroprotective effects of resveratrol against beta-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C. *Br J Pharmacol* 2004; 141:997-1005.
- Koivunen J, Aaltonen V, Peltonen J. Protein kinase C (PKC) family in cancer progression. *Cancer Lett* 2006; 235:1-10.
- Mangoura D, Sakellaridis N, Jones J, Vernadakis A. Early and late passage C-6 glial cell growth: similarities with primary glial cells in culture. *Neurochem Res* 1989; 14:941-947.
- Manton KG, Volovik S, Kulminski A. ROS effects on neurodegeneration in Alzheimer's disease and related disorders: on environmental stresses of ionizing radiation. *Curr Alzheimer Res* 2004; 1:277-293.



Marambaud P, Zhao H, Davies P. Resveratrol promotes clearance of Alzheimer's disease amyloid-beta peptides. *J Biol Chem* 2005; 280:37377-37382.

Matassa AA, Kalkofen RL, Carpenter L, Biden TJ, Reyland ME. Inhibition of PKC $\alpha$  induces a PKC $\delta$ -dependent apoptotic program in salivary epithelial cells. *Cell Death Differ* 2003; 10:269-277.

Miloso M, Bertelli AA, Nicolini G, Tredici G. Resveratrol-induced activation of the mitogen-activated protein kinases, ERK1 and ERK2, in human neuroblastoma SH-SY5Y cells *Neurosci Lett* 1999; 264:141-144.

Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* 1995; 9:484-496.

Parker KK, Norenberg MD, Vernadakis A. "Transdifferentiation" of C6 glial cells in culture. *Science* 1980; 208:179-181.

Pervaiz S. Chemotherapeutic potential of the chemopreventive phytoalexin resveratrol. *Drug Resist Updat* 2004; 7:333-344.

Pringle AK, Benham CD, Sim L, Kennedy J, Iannotti F, Sundstrom LE. Selective N-type calcium channel antagonist omega conotoxin MVIIA is neuroprotective against hypoxic neurodegeneration in organotypic hippocampal-slice cultures. *Stroke* 1996; 27:2124-2130.

Rego AC, Oliveira CR. Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. *Neurochem Res* 2003; 28:1563-1574.

Savaskan E, Olivieri G, Meier F, Seifritz E, Wirz-Justice A, Muller-Spahn F. Red wine ingredient resveratrol protects from beta-amyloid neurotoxicity. *Gerontology* 2003; 49:380-383.

Shen M, Jia GL, Wang YM, Ma H. Cardioprotective effect of resveratrol pretreatment on myocardial ischemia-reperfusion induced injury in rats. *Vascul Pharmacol* 2006; 45:122-126.

Shimizu T, Nakazato T, Xian MJ, Sagawa M, Ikeda Y, Kizaki M. Resveratrol induces apoptosis of human malignant B cells by activation of caspase-3 and p38 MAP kinase pathways. *Biochem Pharmacol* 2006; 71:742-750.

Signorelli P, Ghidoni R. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *J Nutr Biochem* 2005; 16:449-466.

Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988; 175:184-191.

Slater SJ, Seiz JL, Cook AC, Stagliano BA, Buzas CJ. Inhibition of protein kinase C by resveratrol. *Biochim Biophys Acta* 2003; 1637:59-69.

Stef G, Csiszar A, Lerea K, Ungvari Z, Veress G. Resveratrol inhibits aggregation of platelets from high-risk cardiac patients with aspirin resistance. *J Cardiovasc Pharmacol* 2006; 48:1-5.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 2000; 35: 206-221.

Tredici G, Miloso M, Nicolini G, Galbiati S, Cavalletti G, Bertelli A. Resveratrol, map kinases and neuronal cells: might wine be a neuroprotectant? *Drugs Exp Clin Res* 1999; 25:99-103.

Zhu D, Tan KS, Zhang X, Sun AY, Sun, GY, Lee JC. Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes. *J Cell Sci* 2005; 118:3695-3703.

## LEGENDS

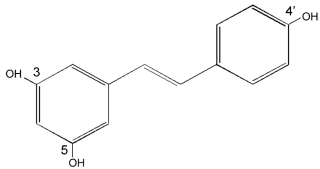
**Figure 1. Effect of different concentrations of resveratrol on DNA damage in C6 glioma cells.** Cells were incubated for different times (1-48 h) in the presence of different concentrations (10, 50, 100 and 250  $\mu$ M) of resveratrol. The extent of damage to DNA was assessed by the comet assay and the index of DNA damage (A) was calculated as described in Material and Methods. (B) Percentage of damage 4 in the same treatments shown in A. Data represent the mean  $\pm$  S.E.M. of the eight experimental determinations performed in duplicate. Significant differences from control (0.25% ethanol) values are indicated by  $*P < 0.05$ .

**Figure 2. Representative photomicrographs of C6 glioma cells exposed to resveratrol and H<sub>2</sub>O<sub>2</sub> and membrane integrity, measured by propidium iodide (PI) uptake.** Cells were pre-incubated for 1 h in the presence of different concentrations of resveratrol (as indicated above) before the addition of H<sub>2</sub>O<sub>2</sub>. Oxidative damage was induced with 1 mM H<sub>2</sub>O<sub>2</sub> for 0.5 h, co-incubated with 250  $\mu$ M resveratrol (Model I) or with 0.1 or 0.5 mM H<sub>2</sub>O<sub>2</sub> for 6 h, co-incubated with 100  $\mu$ M resveratrol (Model II). After incubation, cells were fixed and phase contrast images (A) were recorded as described in the Material and Methods section. Original images were adjusted by increasing the contrast. All images are representative fields from at least four experiments. Scale bar: 50  $\mu$ m. (B) Representative fluorescent images with PI in the same order as treatments shown in Fig. 2A. Values indicated in each panel are means  $\pm$  S.E.M from four independent experiments. Res, resveratrol. Scale bar: 50  $\mu$ m.

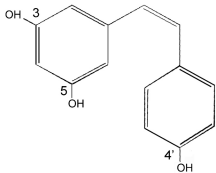
**Figure 3. Inhibitory effect of resveratrol on H<sub>2</sub>O<sub>2</sub>-induced DNA damage in C6 glioma cells.** Cells were pre-incubated for 1 h in the presence of different concentrations of

resveratrol (as indicated above) before the addition of H<sub>2</sub>O<sub>2</sub>. Oxidative damage was induced with 1 mM H<sub>2</sub>O<sub>2</sub> for 0.5 h, co-incubated with 10, 50, 100 or 250 μM resveratrol (Model I) or with 0.1 or 0.5 mM H<sub>2</sub>O<sub>2</sub> for 6 h, co-incubated with 10, 50 or 100 μM resveratrol (Model II). (A) Index of DNA damage. The extent of damage to DNA was assessed by the comet assay and calculated as described in Material and Methods. Data represent means ± S.E.M. of the eight experimental determinations performed in duplicate. a, significant differences from control (0.25% ethanol) values; b, significantly different from 50 μM resveratrol in the presence of 0.5 mM of H<sub>2</sub>O<sub>2</sub> (*P*<0.05). (B) Percentage of damage 4 in the same treatments shown in A. Lines indicate index of DNA damage in the control samples.

# Scheme 1

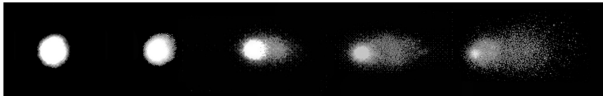


*trans*-resveratrol



*cis*-resveratrol

## Scheme 2



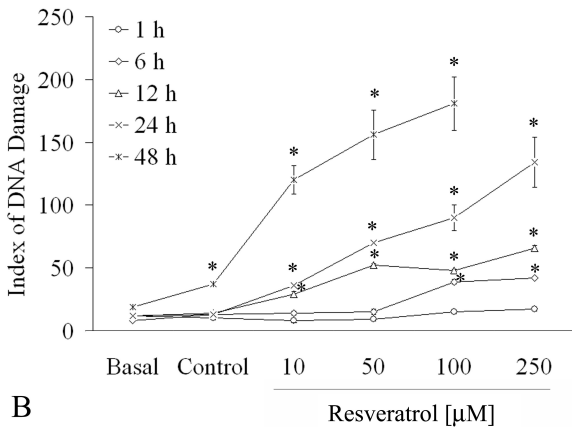
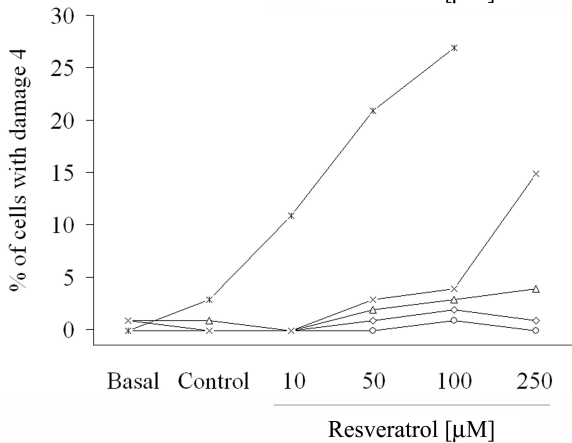
No damage

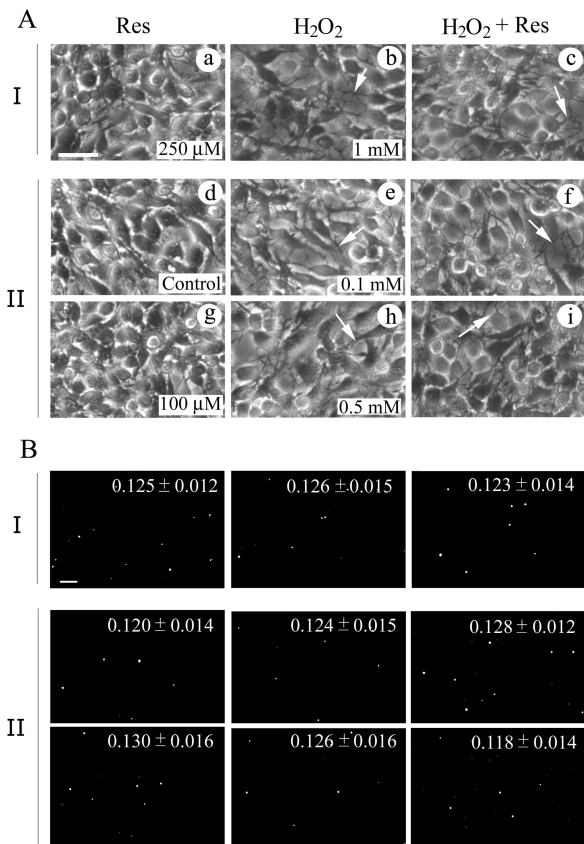
I

II

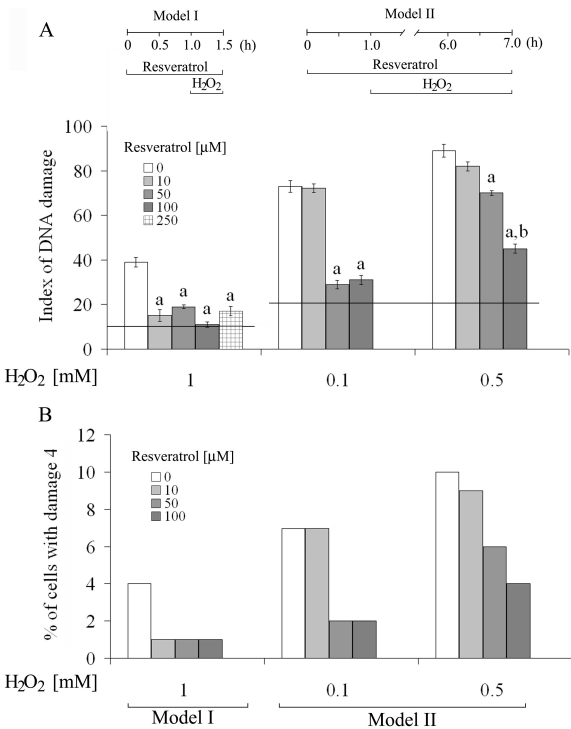
III

IV

**Figure 1****A****B**

**Figure 2**



**Figure 3**

## DISCUSSÃO

Nas duas últimas décadas foram estudadas muitas propriedades biológicas de produtos naturais. Conseqüentemente, muitos destes compostos apresentaram um excelente potencial terapêutico, entre eles o resveratrol. Entre os inúmeros efeitos biológicos do resveratrol, encontrado em uvas e “berries” e também no vinho tinto (Pervaiz, 2004), destacam-se as atividades antioxidante, antiinflamatória, cardioprotetora, antitumoral e neuroprotetora (Fremont et al, 1999; Pervaiz, 2004; Signorelli & Ghidoni, 2005; Baur & Sinclair, 2006).

A linhagem celular C6 tem sido amplamente utilizada em modelos de estudo para o melhor entendimento dos mecanismos fisiológicos e bioquímicos de diversas moléculas. Por ser considerada uma linhagem com características astrocíticas, foi utilizada neste trabalho como modelo de astrócito, sendo alvo do polifenol resveratrol. Dessa forma, analisamos o efeito *per se* do resveratrol, bem como parâmetros indicativos de atividade protetora neural.

Assim, esta dissertação está dividida em dois capítulos. O primeiro capítulo trata da ação do resveratrol sobre o glioma C6, avaliando parâmetros celulares, (como proliferação e morte celular) e gliais (como captação de glutamato, atividade da enzima GS e secreção de S100B). Já o segundo capítulo, aborda o papel protetor do resveratrol contra o dano oxidativo ao DNA induzido por H<sub>2</sub>O<sub>2</sub>, através do ensaio Cometa.

Numerosos estudos demonstram o efeito protetor do resveratrol contra isquemia cerebral, indução de dano por ácido caínico ou dano neuronal em roedores (Virgili & Contestabile, 2000; Ates et al, 2006). O efeito neuroprotetor do

resveratrol tem sido atribuído, principalmente, as suas propriedades antioxidantes. Entretanto, seu potencial papel farmacológico ou seus efeitos toxicológicos no cérebro necessitam mais esclarecimento. Pouco se sabe sobre o mecanismo pelo qual o resveratrol atua em diferentes tipos celulares, assim como seus diferentes alvos intracelulares. O resveratrol tem uma estrutura semelhante aos estrogênios, e isso pode explicar seu efeito estrogênico e em parte, sua atividade antiinflamatória pela ligação aos receptores estrogênicos (Jannin, et al, 2004). No entanto, o resveratrol modula a atividade das ciclooxigenases, lipooxigenases (Pervaiz, 2004) e oxido nítrico sintase, a qual pode mediar efeitos antiinflamatórios e antitumorais em alguns tipos celulares (Signorelli & Ghidoni, 2004).

A proliferação celular pode ser modulada entre outros fatores, por alterações no citoesqueleto e matriz extracelular (Wozniak et al, 1994). *In vivo*, a interação neurônio-glia é facilitada pelas características morfológicas dos astrócitos (Ventura & Harris, 1999) e *in vitro*, vários fatores têm sido descritos na regulação da morfologia e proliferação glial, incluindo o cálcio intracelular (Alberdi et al, 2005), o desequilíbrio redox (C. Gottfried, dados não publicados), a acidificação do pH intracelular e o tipo de substrato de adesão celular (Cechin et al, 2002; Gottfried et al, 2003). Em nossas condições experimentais, o resveratrol não afetou a morfologia celular do glioma C6, demonstrado através de imunocitoquímica para filamentos intermediários, onde nenhuma alteração de GFAP foi observada, após exposição de 100  $\mu$ M de resveratrol por 24 e 48 h. Entretanto, observou-se uma redução na proliferação celular, nas doses de 50 e 100  $\mu$ M de resveratrol após 48 horas de exposição (Figura 1, Capítulo I), o que é

consistente com as propriedades anti-proliferativas do resveratrol já descritas na literatura. A atividade inibitória do resveratrol sobre a proliferação celular pode ocorrer devido a sua habilidade em bloquear a ribonucleotídeo redutase<sup>7</sup> (Fontecave et al, 1998), ou pela sua propriedade antioxidante<sup>8</sup>, capaz de inibir a proliferação de, por exemplo, células hepáticas (Kawada et al, 1998). Já na concentração de 250  $\mu\text{M}$ , o resveratrol causou morte celular, provavelmente por um processo de necrose, uma vez que não houve irregularidade na morfologia nuclear, observada através do ensaio com o fluoróforo DAPI (Figura 4, Capítulo I).

A regulação dos níveis de glutamato na fenda sináptica, por transportadores de glutamato localizados principalmente nos astrócitos, é crítica para evitar excitotoxicidade neuronal. Vários artigos mostram um aumento extracelular nos níveis de glutamato em certas doenças neurodegenerativas, como Alzheimer, esclerose lateral amiotrófica e acidente vascular cerebral (Campiani et al, 2003). Sabe-se também que essas desordens cerebrais aumentam os níveis de ERO e isto tem sido associado ao aumento da liberação e queda na captação de glutamato (Bowling & Beal, 1995). O resveratrol foi capaz de modular a captação de glutamato em células de glioma C6, indicando que a captação de glutamato em células gliais pode ser regulada pelo papel antioxidante deste polifenol, uma vez que condições oxidantes podem induzir diminuição na captação de glutamato (Trotti et al, 1998). Assim, o resveratrol, nas concentrações entre 0,1 – 100  $\mu\text{M}$ , poderia estar modulando o estado redox dos transportadores de glutamato,

---

<sup>7</sup> O resveratrol seqüestra radical tirosil, que é essencial para a proteína ribonucleotídeo redutase, inibindo, dessa forma, a síntese de desoxiribonucleotídeos.

<sup>8</sup> O resveratrol inibe a proliferação de células hepáticas, alterando vias de transdução de sinal, como MAPK e reduzindo os níveis de ciclina D1, uma proteína do ciclo celular.

favorecendo sua atividade. Além disso, o resveratrol também poderia estar modulando a captação de glutamato independente de suas propriedades antioxidantes (Ovesna & Horvathoya-Kozics, 2005). Já é bem descrito que mudanças na captação de glutamato são mediadas por proteínas cinases (Guillet et al, 2005) e neste caso, o resveratrol poderia estar modulando essas cinases. Foi demonstrado por Slater e colaboradores (2003), que o resveratrol foi capaz de inibir PKC e PKD. Além disso, sabe-se que o forbol 12-miristoil 13-acetato, um ativador de PKC dependente de cálcio e diacilglicerol, diminuiu a atividade de GLT-1, um dos transportadores de glutamato encontrado em células C6, por induzir rapidamente a internalização do transportador, indicando que a PKC pode regular negativamente a captação de glutamato (Kalandadze et al, 2002). Dessa forma, o resveratrol poderia estar aumentando a captação de glutamato por inibir a PKC. O resveratrol aumentou a captação de glutamato em 50% na dose de 100  $\mu$ M, já com 250  $\mu$ M o aumento foi de apenas 30%. Essa diferença pode sugerir um efeito bifásico do resveratrol, dependendo da dose. Recentemente, alguns artigos têm mostrado que o efeito do resveratrol é dependente da concentração e tipo celular, podendo apresentar efeitos opostos em alguns parâmetros biológicos como atividade anti- e pró-oxidante e anti- e pró-apoptótica (Pervaiz, 2004).

O resveratrol também teve efeito sobre a enzima GS, aumentando sua atividade nas concentrações de 10 a 100  $\mu$ M, de maneira dose-dependente, enquanto que 250  $\mu$ M de resveratrol não afetou a atividade da GS (Figura 7, Capítulo I). É importante mencionar que ambos os ensaios de captação de glutamato e da atividade da GS foram feitos após 1 h de exposição ao resveratrol e neste período não houve morte celular em nenhuma das concentrações

testadas, portanto, o efeito negativo sobre estes dois parâmetros com a concentração de 250  $\mu\text{M}$  não se deve à morte celular.

A proteína S100B possui atividades intra e extracelulares, sendo sua atividade extracelular dependente da sua concentração. Em concentrações nM exerce papel neurotrófico e na ordem de  $\mu\text{M}$  apresenta efeito apoptótico (Donato, 2003). A exposição de resveratrol nas concentrações de 100 e 250  $\mu\text{M}$ , por 48 horas em células C6 aumentou o conteúdo extracelular de S100B (Figura 8, Capítulo I). Através da atividade intracelular da enzima lactato desidrogenase (LDH) observou-se que resveratrol somente alterou a integridade celular na concentração de 250  $\mu\text{M}$ , tanto em 24 quanto em 48 horas de exposição (Figura 2, Capítulo I). Assim, apenas a concentração de 100  $\mu\text{M}$  de resveratrol afetou o mecanismo de secreção de S100B, enquanto na concentração de 250  $\mu\text{M}$ , devido à perda da integridade da membrana o aumento na secreção de S100B foi devido ao extravasamento celular. Confirmando os dados obtidos pela medida do conteúdo intracelular de LDH, a marcação com iodeto de propídio mostrou morte celular somente na concentração de 250  $\mu\text{M}$  de resveratrol (Figura 3, Capítulo I). De acordo com dados de outros autores, a morte celular em C6 induzida por resveratrol ocorre por necrose (Michels et al, 2006). Portanto, os resultados obtidos com iodeto de propídeo, juntamente com o resultado obtido com DAPI, indicam mais uma vez que a morte celular induzida por resveratrol em C6 é por necrose e não por apoptose.

Conforme já descrito, concentrações nanomolares de S100B estimulam o crescimento de neuritos, a sobrevivência neuronal (Winningham et al, 1989) e a proteção contra a toxicidade induzida pelo glutamato em neurônios de hipocampo

(Kogel et al, 2004). Desta forma, nossos resultados inferem que o aumento de secreção de S100B (que foi na ordem de nanomolar), em glioma C6 exposto ao resveratrol, pode ser um indicativo de resposta glial para estimular a sobrevivência neuronal e funções cerebrais pós-injúria.

O estresse oxidativo está relacionado à patogênese de várias doenças do SNC, como acidentes vasculares isquêmicos e doenças neurodegenerativas, porém, o mecanismo molecular específico envolvido necessita melhor entendimento.

O  $H_2O_2$  é um dos maiores mediadores do estresse oxidativo e um importante agente mutagênico, que pode ser gerado em grandes quantidades pela cadeia respiratória e várias outras vias metabólicas. O cérebro expressa várias enzimas, incluindo monoamina oxidases e tirosina hidrolases que participam da formação normal de  $H_2O_2$  como produto de sua atividade. No entanto, células com atividade antiinflamatória (ex. microglia) são capazes de produzi-lo via mecanismo de “explosão” oxidativa. Embora o  $H_2O_2$  seja um forte agente oxidante, ele pode ser convertido, na presença de metais de transição, como ferro e cobre, em radical hidroxil, um ERO que pode mediar a genotoxicidade deste composto (Halliwell & Gutteridge, 1990). O cérebro é rico em ferro, sendo capaz de produzir ERO, mas pobre em enzimas e compostos antioxidantes (Manton et al, 2004). A formação de ERO tem sido relacionada com o dano ao tecido cerebral presente em várias neuropatologias, como isquemia e doenças neurodegenerativas, assim como no processo natural de envelhecimento (Beckman & Ames, 1998; Rego & Oliveira, 2003; Manton et al, 2004) e isto pode envolver muitos mecanismos de sinalização celular.

Para investigar o efeito neuroprotetor do resveratrol sobre o dano oxidativo ao DNA através do ensaio do cometa, foram estabelecidos dois modelos de indução de estresse oxidativo. No modelo I, utilizou-se 1 mM de H<sub>2</sub>O<sub>2</sub> por 30 minutos, com concentrações de resveratrol variando de 10 a 250 µM e no modelo II, 0,1 e 0,5 mM de H<sub>2</sub>O<sub>2</sub> por 6 h, com 10, 50 e 100 µM de resveratrol. Antes de incubar com H<sub>2</sub>O<sub>2</sub>, em ambos modelos, as células C6 foram pré-tratadas com resveratrol nas respectivas concentrações.

O ensaio Cometa é um método simples e rápido para avaliar dano recente ao DNA (Maluf & Erdtmann, 2000). Para realizar essa técnica é necessário utilizar células integras e pode ser aplicado a diversos tipos celulares (Maluf, 2004). Singh e colaboradores (1988) modificaram o método inicialmente descrito por Ostling & Johanson (1984), permitindo avaliar dano ao DNA em células individuais, sob condições alcalinas. Primeiramente as células C6 foram submetidas a pH alcalino, ocorrendo rompimento das membranas celular e nuclear, bem como rompimento em pequenos fragmentos de DNA, quando danos simples e duplos estiverem presentes. O dano ao DNA é quantificado através da análise de 100 células que recebem um escore que varia de 0 (célula sem dano) à 4 (dano máximo) de acordo com a intensidade da cauda (Esquema 2, Capítulo II). Portanto, quanto maior a cauda, mais dano ao DNA (Maluf, 2004).

O ensaio Cometa é muito sensível para detecção de baixos níveis de dano ao DNA, e este dano não está necessariamente correlacionado com a viabilidade celular (Brendler-Schaab et al, 2005). Através do ensaio com iodeto de propídio (marcador de morte celular e indicativo de necrose), observou-se que a



integridade celular foi mantida em nossas condições de insulto oxidante. Também é importante salientar que resultados recentes, que não fazem parte desta dissertação, indicaram aumento de ERO nos dois modelos de indução ao estresse oxidativo, através do ensaio de tert-butil hidroperóxido, feito segundo Flecha e colaboradores (1991).

Assim, os resultados obtidos *in vitro* com C6, sugerem que o resveratrol pode ser benéfico nas desordens cerebrais envolvendo estresse oxidativo. O efeito do resveratrol *per se* sobre o dano ao DNA também mostrou ser dependente da concentração e tempo de incubação. As concentrações de resveratrol variaram de 10 a 250  $\mu\text{M}$  e os tempos de incubação de 1 a 48 h. Nas primeiras 6 horas de incubação com resveratrol, o índice de dano não foi superior a  $42 \pm 1,25$  (Figura 1A, Capítulo II) e mostrou também baixa percentagem de dano 4, conforme Figura 1B, Capítulo II. Entretanto, após 12 h e nas concentrações mais elevadas de resveratrol houve aumento do índice de dano ao DNA e maior porcentagem de dano 4, sendo que com 250  $\mu\text{M}$  de resveratrol, devido a presença de morte celular, demonstrada através do ensaio com iodeto de propídio (Figura 3, Capítulo I), não foi possível avaliar dano ao DNA.

Nos dois modelos de indução de estresse oxidativo estudados, o resveratrol foi capaz de prevenir o dano ao DNA. No modelo I houve prevenção quase total de dano oxidativo ao DNA celular. Já no modelo II, o efeito foi mais significativo na presença de 0,5 mM de  $\text{H}_2\text{O}_2$ , comportando-se parcialmente de maneira dose-dependente e com 0,1 mM de  $\text{H}_2\text{O}_2$  houve efeito apenas nas concentrações de 50

e 100  $\mu$ M de resveratrol ( $29 \pm 1.88$  e  $31 \pm 1.99$ , respectivamente; Figura 3A, capítulo II).

O tratamento com  $H_2O_2$  também induziu alterações na morfologia celular de glioma C6 e o resveratrol não foi capaz de prevenir estas alterações morfológicas. Muitas proteínas alvos de ERO estão envolvidas na regulação da morfologia celular, incluindo proteínas G e proteínas cinases (Bem-Mahdi et al, 2000; Zhu et al, 2005), como a ROCK, que está envolvida na manutenção de fibras de actina, através da cascata da proteína G monomérica RhoA (Ongusaha et al., 2006). Considerando o duplo efeito do resveratrol (podendo ser anti e pró-oxidante), para nossa surpresa, o resveratrol não induziu nenhuma alteração morfológica em células C6, mesmo em concentrações mais altas, sugerindo que o efeito sobre a morfologia não seja apenas dependente do estado redox dessas células. É importante salientar que o resveratrol também não alterou a organização de filamentos intermediários, visto na Figura 5, Capítulo I.

Um estudo recente em astrócitos mostrou que o  $H_2O_2$  estimulou a fosforilação de p38 MAPK (Proteína cinase ativada por mitógenos), levando a reorganização do citoesqueleto (Zhu et al, 2005). A via de sinalização envolvida na reorganização do citoesqueleto de células C6 não é conhecida, mas se a p38 é ativada por  $H_2O_2$ , o resveratrol pode não ser capaz de reverter essa ativação, apesar de já ter sido demonstrado em outras células (como células cardíacas e células B) que o resveratrol pode ativar p38 MAPK (Shimizu et al, 2006).

Um importante sistema de sinalização é a PKC, uma família de 12 serina/treonina cinases que tem um papel central nas vias de transdução de sinal

e uma grande variedade de funções celulares, incluindo a proliferação e morte celular (Nishizuka, 1994; Koivunen et al, 2006). A inativação da PKC está relacionada ao papel neuroprotetor do resveratrol contra toxicidade induzida por óxido nítrico em cultura de neurônios hipocámpais (Bastianetto et al, 2000). Algumas isoformas da PKC servem como substrato para caspases e a isoenzima PKC  $\delta$  tem sido associada com a indução de dano ao DNA por apoptose. Demonstrou-se também que a PKC  $\delta$  está envolvida na neuroproteção mediada por resveratrol (Han et al, 2004). Porém, a PKC  $\delta$  pode ser influenciada pela presença de outras isoenzimas da PKC, como a PKC  $\alpha$  (Slater et al, 2003). Assim, este efeito inibitório do resveratrol contra o dano ao DNA em células de glioma C6 pode ser explicado pelas propriedades antioxidantes do resveratrol, que provavelmente inibem a ação da PKC  $\alpha$ . Neste cenário, o efeito inibitório do resveratrol sobre a PKC provavelmente é importante para determinar o mecanismo pelo qual o resveratrol exerce seu efeito benéfico sobre as funções celulares.

Conforme abordado até o momento, o resveratrol exerce seus efeitos biológicos por diferentes mecanismos, muitos não totalmente esclarecidos. A maior parte destes efeitos biológicos é mostrada em tratamentos *in vitro*. Uma das maiores limitações dos trabalhos *in vitro* são as altas concentrações de resveratrol utilizadas, pois se sabe, a partir de estudos farmacocinéticos, que o resveratrol é rapidamente metabolizado pelo fígado e por células intestinais (sofrendo reabsorção) e seus principais metabólitos são resveratrol-glicuronídeo e resveratrol-sulfato (Wenzel & Somoza, 2005). Alguns polifenóis presentes na dieta

(ex. quercetina) podem inibir a glicuronidação, dificultando a biodisponibilidade e concentração final dos metabólitos nos tecidos alvos.

A concentração plasmática de resveratrol em humanos é ao redor de 2  $\mu\text{M}$  e no cérebro ao redor de 0,4  $\mu\text{M}$  (Gescher & Steward, 2003). Nossas concentrações de estudos *in vitro* variaram de 0,01 a 250  $\mu\text{M}$  de resveratrol, e estão de acordo com as concentrações utilizadas na literatura recente (Picard et al, 2004), que demonstram melhores resultados *in vitro* entre as concentrações de 10 – 100  $\mu\text{M}$ .

De acordo com suas características de solubilidade, o resveratrol pode ser solubilizado em DMSO (dimetil-sulfóxido) ou etanol, sendo que nosso grupo optou por solubilizar em etanol, para aproximar nossos resultados de uma dieta baseada no consumo de vinho, visando estudos *in vivo*, administrando resveratrol em ratos, salientando que a presença de álcool não é necessária para a absorção do resveratrol (Goldberg et al, 2003).

Assim, a análise de todos os dados mostra o efeito benéfico do resveratrol sobre culturas de gliomas C6, sendo estas utilizadas como equivalente de cultura de astrócitos, para estudos sobre a fisiologia e bioquímica dos mesmos (Mangoura et al, 1989; Feng et al, 2004; Cechin et al, 2005; Funchal et al, 2005). Nossos resultados demonstram que o resveratrol, nas concentrações de 50 e 100  $\mu\text{M}$  aumentou os três parâmetros gliais analisados, captação de glutamato, atividade da GS e secreção de S100B. Essas alterações, nestes parâmetros, podem contribuir para o papel protetor dos astrócitos em condições de injúria cerebral, reforçando o uso deste composto como potencial terapêutico para doenças neurodegenerativas. Em contraste, concentrações elevadas de resveratrol podem

não apresentar efeitos benéficos. Nós também demonstramos o efeito protetor do resveratrol nas concentrações entre 10 e 250  $\mu\text{M}$  contra o dano oxidativo ao DNA induzido por  $\text{H}_2\text{O}_2$  nos dois modelos de insulto estudados. O efeito *per se* do resveratrol revelou que exposição maior que 12 h e concentração superior a 250  $\mu\text{M}$  aumentou o índice de dano ao DNA. Este estudo é mais uma contribuição do papel protetor do resveratrol, enfatizando a importância deste polifenol e de outros antioxidantes na dieta, possibilitando a prevenção de desordens cerebrais associadas ao estresse oxidativo.

## **CONCLUSÕES**

### **1. Conclusão Geral**

Os dados obtidos com a linhagem C6 nos permitem concluir que o resveratrol regula parâmetros celulares, exercendo efeito benéfico em relação às funções gliais (captação de glutamato, atividade da glutamina sintetase e secreção de S100B), bem como efeito protetor contra dano induzido por H<sub>2</sub>O<sub>2</sub>.

### **2. Conclusões específicas**

#### **Capítulo I**

- 1) O resveratrol foi capaz de inibir a proliferação celular do glioma C6 após 24 e 48 horas de incubação;
- 2) Não houve alteração da integridade celular, conforme medida do conteúdo intracelular de LDH;
- 3) A dose de 250  $\mu$ M de resveratrol induziu morte celular em glioma C6, evidenciando talvez, o efeito pró-oxidante do resveratrol;
- 4) A morte celular observada provavelmente é do tipo necrose, uma vez que não houve irregularidade na morfologia nuclear, típica de apoptose;
- 5) O citoesqueleto de filamentos intermediários (GFAP) não foi alterado por resveratrol;
- 6) O resveratrol aumentou a captação de glutamato e a atividade da enzima GS, sendo o aumento de captação de forma linear.

- 7) A secreção de S100B em gliomas C6 foi aumentada por resveratrol, evidenciando um possível papel trófico deste composto.

## **Capítulo II**

- 1) O resveratrol induziu dano ao DNA em células C6 de maneira dose-dependente;
- 2) O H<sub>2</sub>O<sub>2</sub> alterou a morfologia celular do glioma C6 e o resveratrol não foi capaz de prevenir estas alterações;
- 3) Nos dois modelos de indução de estresse oxidativo (modelo I e II) não se observou morte celular;
- 4) Resveratrol protegeu a linhagem celular C6 do dano ao DNA induzido por H<sub>2</sub>O<sub>2</sub>.

## PERSPECTIVAS

- Avaliar o potencial antioxidante do resveratrol em nossos modelos de indução de estresse oxidativo.
- Avaliar os efeitos do resveratrol em culturas primárias de astrócito e neurônio, bem como o efeito do meio condicionado resultante do tratamento com resveratrol.
- Verificar a ação do resveratrol *in vivo*, através da administração de doses diárias de resveratrol (VO e/ou IP) em ratos Wistar, avaliando diferentes parâmetros bioquímicos e histoquímicos.



## REFERÊNCIAS BIBLIOGRÁFICAS

AHLEMEYER B, BEIER H, SEMKOVA I, SCHAPER CE, KRIEGLSTEIN J (2000) S100 $\beta$  protects cultured neurons against glutamate- and staurosporine- induced damage and is involved in the antiapoptotic action of the 5-HT<sub>1A</sub>- receptor agonist, Bay x 3702. Brain Res. 858, 121-128.

ALBERDI E, SANCHEZ-GOMEZ MV, MATUTE C (2005) Calcium and glial cell death. Cell Calcium. 38, 417-25.

ALBERTS B, JOHNSON A, LEWIS J, RAFF M, ROBERTS K, WALTER P (2002) The cytoskeleton. *In: Molecular Biology of the Cell*, editado por Garland Publishing, New York, p. 780-820.

AMARA S & FONTANA A (2002) Excitatory amino acid transporters: keeping up with glutamate. Neurochem. Int. 41, 313-318.

AMES BN & SHIGENAGA MK (1992) Oxidants are a major contributor to aging. Ann N Y Acad Sci. 663, 85-96.

AMERICAN TYPE CULTURE COLLECTION. [www.atcc.org](http://www.atcc.org)

ANDERSON CM & SWANSON RA (2000) Astrocyte glutamate transport: review of

properties, regulation and functions physiological. *Glia* 32, 1-14.

ARAYA J, RODRIGO R, ORELLANA M, RIVERA G (2001) Red wine raises plasma HDL and preserves long-chain polyunsaturated fatty acids in rat kidney and erythrocytes. *Br J Nutr.* 86, 189-95.

ATES O, CAYLI S, ALTINOZ E, GURSES I, YUCEL N, SENER M, KOCAK A, YOLOGLU S (2006) Neuroprotection by resveratrol against traumatic brain injury in rats. *Mol Cell Biochem.* (Epub ahead of print)

AUER RN, RICE GP, HINTON GG, AMACHER AL, GILBERT JJ (1981) Cerebellar astrocytoma with benign histology and malignant clinical course. Case report. *J Neurosurg.* 54, 128-32.

BASTIANETTO S, ZHENG WH, QUIRION R (2000) Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. *Br. J. Pharmacol.* 131, 711-720.

BAUR JA & SINCLAIR DA (2006) Therapeutic potential of resveratrol: the in vivo evidence. *Nat. Rev. Drug Discov.* 5, 493-506.

BAYDAS G, NEDZVETSKII VS, TUZCU M, YASAR A, KIRICHENKO SV (2003) Increase of glial fibrillary acidic protein and S-100B in hippocampus and cortex of

diabetic rats: effects of vitamin Eur. J. Pharmacol., 462, 67-71.

BECKMAN KB & AMES BN (1998) The free radical theory of aging matures. *Physiol. Rev.* 78, 547-581.

BENDA P, LIGHTBODY J, SATO G, LEVINE L, SWEET W (1968) Differentiated rat glial cell strain in tissue culture. *Science* 161, 370-371.

BENDA P, SOMEDA K, MESSER J, SWEET W (1971) Morphological and immunochemical studies of rat glial tumors and clonal strains propagated in culture. *J. Neurosurg* 34, 310-323.

BEN-MAHDI MH, GOZIN A, DRISS F, ANDRIEU V, CHRISTEN MO, PASQUIER C (2000) Anethole dithiolethione regulates oxidant-induced tyrosine kinase activation in endothelial cells. *Antioxid. Redox Signal.* 2, 789-799.

BOWLING AC & BEAL MF (1995) Bioenergetic and oxidative stress in neurodegenerative diseases. *Life Sci.* 5, 1151-71

BRENDLER-SCHWAAB S, HARTMANN A, PFUHLER S, SPEIT G (2005) The in vivo comet assay: use and status in genotoxicity testing. *Mutagenesis* 20, 245-254.

CAMPIANI G, FATTORUSSO C, DE ANGELIS M, CATALANOTTI B, BUTINI S, FATTORUSSO R, FIORINI I, NACCI V, NOVELLINO E (2003) Neuronal high-affinity sodium-dependent glutamate transporters (EAATs): targets for the development of novel therapeutics against neurodegenerative diseases. *Curr Pharm Des.* 9, 599-625.

CARMIGNOTO G (2000) Reciprocal communication system between astrocytes and neurones. *Progress in Neurobiology* 62, 561-581.

CASTETS F, GRIFFIN WST, MARKS A, VAN ELDIK LJ (1997) Transcriptional regulation of the human S100 $\beta$  gene. *Mol. Brain Res.* 46, 208-216.

CECHIN SR, DUNKLEY PR, RODNIGHT R (2005) Signal transduction mechanisms involved in the proliferation of C6 glioma cells induced by lysophosphatidic acid. *Neurochem. Res.* 30, 603-611.

CECHIN SR, GOTTFRIED C, PRESTES CC, ANDRIGHETTI L, WOFCHUK ST, RODNIGHT R (2002) Astrocyte stellation in saline media lacking bicarbonate: possible relation to intracellular pH and tyrosine phosphorylation. *Brain Res.* 946, 12-23.

CHANVITAYAPONGS S, DRACZYNSKA-LUSIAK B, SUN AY (1997) Amelioration of oxidative stress by antioxidants and resveratrol in PC12 cells. *NeuroReport* 8, 1499-1502.

CHEN G, SHAN W, WU Y, REN L, DONG J, JI Z (2005) Synthesis and anti-inflammatory activity of resveratrol analogs. *Chem Pharm Bull* 53, 1587-90.

COZZI R, RICORDY R, AGLITTI T, GATTA V, PERTICONE P, DE SALVIA R (1997) Ascorbic acid and beta-carotene as modulators of oxidative damage. *Carcinogenesis*. 18, 223-8.

DAI C & HOLLAND EC (2001) Glioma models. *Biochim Biophys Acta* 1551, M19-M27.

DANBOLDT NC (2001) Glutamate uptake. *Prog. Neurobiol.* 65, 1-105.

DAS S, FRAGA CG, DAS DK (2006) Cardioprotective effect of resveratrol via HO-1 expression involves p38 map kinase and PI-3-kinase signaling, but does not involve NFkappaB. *Free Radic. Res.* 40, 1066-1075.

DAVEY GE, MURMANN P, HEIZMANN CW (2001) Intracellular  $Ca^{2+}$  and  $Zn^{2+}$  levels regulate the alternative cell density-dependent secretion of S100B in human glioblastoma cells. *J. Biol. Chem.* 276, 30819-30826.

DEMROW HS, SLANE PR, FOLTS JD (1995) Administration of wine and grape juice inhibits in vivo platelet activity and thrombosis in stenosed canine coronary arteries. *Circulation*. 91, 1182-8.

DONATO R (2003) Intracellular and extracellular roles of S100 proteins. *Microsc Res Tech* 60, 540-551.

DORÉ S (2005) Unique properties of polyphenol stilbenes in the brain: more than direct antioxidant actions; gene/protein regulatory activity. *Neurosignals* 14, 61-70.

DRACZYNSKA-LUSIAK B, DOUNG A, SUN AY (1998) Oxidized lipoproteins may play a role in neuronal cell death in Alzheimer disease. *Mol Chem Neuropathol*. 33, 139-48.

EMANUELLI T, PAGEL FW, PORCIUNCULA LO, SOUZA DO (2003) Effects of 5-aminolevulinic acid on the glutamatergic neurotransmission. *Neurochem Int*. 42, 115-21.

ENG LF, GHIRNIKAR RS, LEE YL (2000) Glial Fibrillary Acidic Protein: GFAP- Thirty- One Years (1969-2000). *Neurochem. Res*. 25, 1439-1451.

FAUST F, KASSIE F, KNASMÜLLER S, BOEDECKER RH, MANN M, MERSCH-SUNDERMANN V (2004) The use of the alkaline comet assay with lymphocytes in human biomonitoring studies. *Mutation Research* 566, 209-229.

FENG Z & ZHANG JT (2004) Protective effect of melatonin on beta-amyloid-induced apoptosis in rat astrogloma C6 cells and its mechanism. *Free Radic. Biol. Med.* 37, 1790-1801.

FLECHA BG, LLESUY S, BOVERIS A (1991) Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver, and muscle. *Free Radic Biol Med* 10, 93-100.

FONTECAVE M, LEPOIVRE M, ELLEINGAND E, GEREZ C, GUITTET O (1998) Resveratrol, a remarkable inhibitor of ribonucleotide reductase. *FEBS Lett.* 421, 277-9.

FREMONT L (2000) Biological effects of resveratrol. *Life Sci.* 66, 663-673.

FRIDOVICH I (1998) The trail to superoxide dismutase. *Protein Sci.* 7, 2688-90.

FRIZZO JK, TRAMONTINA F, BORTOLI E, GOTTFRIED C, LEAL RB, LENGYEL I, DONATO R, DUNKLEY PR, GONÇALVES CA (2004) S100B-mediated inhibition of the phosphorylation of GFAP is prevented by TRTK-12. *Neurochem. Res.*, 29, 735-740.

FUNCHAL C, DOS SANTOS AQ, JACQUES-SILVA MC, ZAMONER A, GOTTFRIED C, WAJNER M, PESSOA-PUREUR R (2005) Branched-chain alpha-

keto acids accumulating in maple syrup urine disease induce reorganization of phosphorylated GFAP in C6-glioma cells. *Metab. Brain Dis.* 20, 205-217.

GALLO V & GHIANI CA (2000) Glutamate receptors in glia: new cells, new inputs and new functions. *Trends Pharmacol. Sci.* 21, 252-258.

GEE JR & KELLER JN (2005) Astrocytes: regulation of brain homeostasis via apolipoprotein. *Eur. Int. J. Biochem. Cell Biol.* 37, 1145-1150.

GESCHER AJ & STEWARD WP (2003) Relationship between mechanisms, bioavailability, and preclinical chemopreventive efficacy of resveratrol: a conundrum. *Cancer Epidemiol. Biomarkers Prev.* 12, 953-957.

GOLDBERG DM, YAN J, SOLEAS GJ (2003) Absorption of three wine-related polyphenols in three different matrices by healthy subjects. *Clin Biochem.* 36, 79-87.

GOMES FCA, PAULIN D, MOURA NETO V (1999) Glial fibrillary acidic protein (GFAP) modulation by growth factors and its implication in astrocyte differentiation. *Braz. J. Med. Biol. Res.* 32, 619-631.

GOTTFRIED C, CECHIN SR, GONZALEZ MA, VACCARO TS, RODNIGHT R (2003) The influence of the extracellular matrix on the morphology and intracellular



pH of cultured astrocytes exposed to media lacking bicarbonate. *Neuroscience*. 121, 553-62.

GOTTFRIED C, TRAMONTINA F, GONCALVES D, GONCALVES CA, MORIGUCHI E, DIAS RD, WOFCHUK ST, SOUZA DO (2002) Glutamate uptake in cultured astrocytes depends on age: a study about the effect of guanosine and the sensitivity to oxidative stress induced by H<sub>2</sub>O<sub>2</sub>. *Mech Ageing Dev*. 123, 1333-40.

GROBEN B, DE DEYN PP, SLEGGERS H (2002) Rat C6 glioma as experimental model system for the study of glioblastoma growth and invasion. *Cell Tissue Res*. 310, 257-70.

GUILLET BA, VELLY LJ, CANOLLE B, MASMEJEAN FM, NIEOULLON AL, PISANO P (2005) Differential regulation by protein kinases of activity and cell surface expression of glutamate transporters in neuron-enriched cultures. *Neurochem Int*. 46, 337-44.

HALLIWELL B & GUTTERIDGE JM (1990) Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol*. 186, 1-85.

HALLIWELL B. & GUTERIDGE (2000) *Free radicals in biology and medicine*, 3ed. Clarendon, Oxford.

HAN YS, ZHENG WH, BASTIANETTO S, CHABOT JG, QUIRION R (2004) Neuroprotective effects of resveratrol against beta-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C. *Br. J. Pharmacol.* 141, 997-1005.

INOUE H, JIANG XF, KATAYAMA T, OSADA S, UMESONO K, NAMURA S (2003) Brain protection by resveratrol and fenofibrate against stroke requires peroxisome proliferator-activated receptor alpha in mice. *Neurosci Lett.* 352, 203-6.

IZQUIERDO I & MEDINA JH (1997) Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiol. Learn and Mem.* 68, 285-316.

JACOBSON M (1991) Neuroglial Ontogeny. In: *Developmental Neurobiology* 3 ed. New York, Plenum Press.

JANG M, CAI L, UDEANI GO, SLOWING KV, THOMAS CF, BEECHER CW, FONG HH, FARNSWORTH NR, KINGHORN AD, MEHTA RG, MOON RC, PEZZUTO JM (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 275, 218-20.

JANNIN B, MENZEL M, BERLOT JP, DELMAS D, LANCON A, LATRUFFE N (2004) Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: plasmatic protein binding and cell uptake. *Biochem Pharmacol.* 68, 1113-8.

JEANDET P, BESSIS R, GAUTHERON B (1991) The production of resveratrol (3,5,4'-trihydroxystilbene) by grapes berries in different developmental stages. *Am J Enol Vitic.* 42, 41-46.

JESSEN KR (2004) Glial cells. *Int. J. Biochem. Cell Biol.*, 36, 1861-1867.

JOHNSON H (1999) *A História do Vinho*, São Paulo – SP, Companhia das Letras, 1ª edição, 546 p.

KALANDADZE A, WU Y, ROBINSON MB (2002) Protein kinase C activation decreases cell surface expression of the GLT-1 subtype of glutamate transporter. Requirement of a carboxyl-terminal domain and partial dependence on serine 486. *J Biol Chem.* 277, 45741-50.

KAWADA N, SEKI S, INOUE M, KUROKI T (1998) Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. *Hepatology* 27, 1265-1274.

KIM YJ, HWANG SY, OH ES, OH S, HAN IO (2006) IL-1beta, an immediate early protein secreted by activated microglia, induces iNOS/NO in C6 astrocytoma cells through p38 MAPK and NF-kappaB pathways. *J Neurosci Res.* 84, 1037-46.

KIMELBERG HK, SANKAR P, JALONEN T, GODERIE SK (1992) Functional consequences of astrocytic swelling. *Progress in Brain Research* 94, 57-68.

KLEIHUES P & CAVENEE WK (2000) *Pathology and genetics of tumors of the nervous system*. IARC Press, Lyon.

KÖGEL D, PETERS M, KONIG HG, HASHEMI SM, BUI NT, AROLT V, ROTHERMUNDT M, PREHN JH (2004) S100B potently activates p65/c-Rel transcriptional complexes in hippocampal neurons: Clinical implications for the role of S100B in excitotoxic brain injury. *Neuroscience* 127, 913-920.

KOIVUNEN J, AALTONEN V, PELTONEN J (2006) Protein kinase C (PKC) family in cancer progression. *Cancer Lett.* 235, 1-10.

KONOPKA G & BONNI A (2003) Signaling pathways regulating gliomagenesis. *Curr Mol Med.* 3, 73-84.

LAMUELA-RAVENTOS RM & DE LA TORRE-BORONAT MC (1999) Beneficial effects of White wines. *Drugs under Exp Clin Res* 25, 121-124.

LANGEAKE P, CORNFORD CA, PRYCE RJ (1979) Identification of pterostilbene as a phytoalexin from *Vitis Vinifera* leaves. *Phytochem* 18, 1025-1027.

LANGKAGE P & PRYCE CA (1976) The production of resveratrol by *Vitis vinifera* and other members of Vitaceae as a response to infection or injury. *Physiol Plant Path* 9, 77-85.

LEGER AS, COCHRANE AL, MOORE F (1979) Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. *Lancet* 12, 1017-1020.

LEITE MC, BROLESE G, DE ALMEIDA LM, PINERO CC, GOTTFRIED C, GONCALVES CA (2006) Ammonia-induced alteration in S100B secretion in astrocytes is not reverted by creatine addition. *Brain Res Bull.* 70, 179-85.  
*Life Sci.* 56, 1151-71.

LOUREIRO SO, DE LIMA PELAEZ P, HEIMFARTH L, SOUZA DO, WAJNER M, PESSOA-PUREUR R (2005) Propionic and methylmalonic acids increase cAMP levels in slices of cerebral cortex of young rats via adrenergic and glutamatergic mechanisms. *Biochim Biophys Acta.* 1740, 460-6.

MAGISTRETTI PJ, PELLERIN L (1999) Astrocytes Couple Synaptic Activity to Glucose Utilization in the Brain. *News Physiol Sci.* 14, 177-182.

MALUF SW & ERDTMANN B (2000) Follow-up study of the genetic damage in lymphocytes of pharmacists and nurses handling antineoplastic drugs evaluated by

cytokinesis-block micronuclei analysis and single cell gel electrophoresis assay. Mutation Research 471, 21-27.

MALUF SW (2004) Monitoring DNA damage following radiation exposure using cytokinesis-block micronucleus method and alkaline single-cell gel electrophoresis. Clin Chim Acta. 347, 15-24.

MANGOURA D, SAKELLARIDIS N, JONES J, VERNADAKIS A (1989) Early and late passage C-6 glial cell growth: similarities with primary glial cells in culture. Neurochem. Res. 14, 941-947.

MANTON KG, VOLOVIK S, KULMINSKI A (2004) ROS effects on neurodegeneration in Alzheimer's disease and related disorders: on environmental stresses of ionizing radiation. Curr. Alzheimer Res. 1, 277-293.

MARTINI LH, CERESER V, JUNIOR IZ, JARDIM FM, VENDITE DA, FRIZZO ME, YUNES RA, CALIXTO JB, WOFCHUK S, SOUZA DO (2006) The Sesquiterpenes Polygodial and Drimaniol in vitro Affect Glutamatergic Transport in Rat Brain. Neurochem Res. 31, 431-8.

MATTHEWS GD, GOULD RM, VARDIMON L (2005) A single glutamine synthetase gene produces tissue-specific subcellular localization by alternative splicing. FEBS Lett. 579, 5527-34.

MCCORD JM & EDEAS MA (2005) SOD, oxidative stress and human pathologies: a brief history and a future vision. *Biomed Pharmacother.* 59, 139-42.

MCDONALD J & JOHNSON M (1990) Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. *Prog. in Neurobiol.* 54, 581-618.

MILLER NJ & RICE-EVANS CA (1995) Antioxidant activity of resveratrol In red wine. *Clin Chem* 41, 1789.

MICHELS G, WÄTJEN W, WEBER N, NIERING P, CHOVOLOU Y, KAMPKÖTTER A, PROKSCH P, KAHL R (2006) Resveratrol induces apoptotic cell death in rat H4IIE hepatoma cells but necrosis in C6 glioma cells. *Toxicology* 225, 173-182.

MILOSO M, BERTELLI AA, NICOLINI G, TREDICI G (1999) Resveratrol-induced activation of the mitogen-activated protein kinases, ERK1 and ERK2, in human neuroblastoma SH-SY5Y cells. *Neurosci Lett.* 264, 141-4.

MOORE BW (1965) A soluble protein characteristic of the nervous system. *Biochem. Biophys. Res. Commun.* 19, 739-744.

MOURAD PD, FARRELL L, STAMPS LD, CHICOINE MR, SILBERGELD DL (2005) Why are systemic glioblastoma metastases rare? Systemic and cerebral growth of mouse glioblastoma. *Surg Neurol.* 63, 511-9.

NAGANISHI S (1992) Molecular diversity of glutamate receptors and implications for brain functions. *Science* 258, 597-603.

NAKANISHI S, NAKAJIMA Y, MASSU M, UEDA Y, NAKAHARA K, WATANABE D, YAMAGUCHI S, KAWABATA S, OKADA M (1998) Glutamate receptors: brain function and signal transduction. *Brain Res. Rev.* 26, 230-235

NISHIZUKA Y (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* 9, 484-496.

OSTLING O & JOHANSON KJ (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun.* 123, 291-8.

OVESNA Z & HORVATHOVA-KOZICS K (2005) Structure-activity relationship of trans-resveratrol and its analogues. *Neoplasma.* 52, 450-5.

OZAWA S, KAMYIA H, TSUKI K (1998) Glutamate receptors in mammalian central nervous system. *Prog. Neurobiol.* 54, 581-618.



PARKER JC JR, PARRIS EL, REYES PF (1980) Clinicomorphologic markers for predicting behavior and guiding therapy for brain tumors. *J Surg Oncol.* 14, 21-34.

PEKNY M & PEKNA M (2004) Astrocyte intermediate filaments in CNS pathologies and regeneration. *J. Pathol.* 204, 428-437.

PERVAIZ S (2004) Chemotherapeutic potential of the chemopreventive phytoalexin resveratrol. *Drug Resist Updat.* 7, 333-344.

PICARD F, KURTEV M, CHUNG N, TOPARK-NGARM A, SENAWONG T, MACHADO DE OLIVEIRA R, LEID M, MCBURNEY MW, GUARENTE L (2004) Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature* 429, 771-6.

PINTO S, GOTTFRIED C, MENDEZ A, GONÇALVES D, KARL J, GONÇALVES CA, WOFCHUK S, RODNIGHT R. (2000) Immunocytometry and secretion of S100B in astrocyte cultures from different brain regions in relation to morphology. *Febs Lett.* 486, 23-207.

PORCIUNCULA LO, ROCHA JB, GHISLENI G, TAVARES RG, SOUZA DO (2004) The effects of ebselen on [3H]glutamate uptake by synaptic vesicles from rat brain. *Brain Res.* 1027, 192-5.

REGO AC & OLIVEIRA CR (2003) Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. *Neurochem. Res.* 28, 1563-1574.

RENAUD S & LORGERIL M (1992) Wine, alcohol, platelets, and the French Paradox for coronary heart disease. *Lancet* 339, 1523-1526.

ROBINSON MB & DOWN LA (1997) Heterogeneity and functional properties of subtypes of sodium-dependent glutamate transporters in mammalian central nervous system. *Adv. Pharmacol.* 37, 69-115.

RODNIGHT R, GONÇALVES CA, WOFCHUK ST, LEAL R (1997) Control of the phosphorylation of the astrocyte marker glial fibrillary acidic protein (GFAP) in the immature rat hippocampus by glutamate and calcium ions: possible key factor in astrocytic plasticity. *Braz. J. Med. Biol. Res.* 30, 325-338.

SALVADOR M & HENRIQUES JAP (2004) Radicais livres e a resposta celular ao estresse oxidativo. Ed. Ulbra, 204p.

SCANNEVIN RH, HUGANIR RL (2000) Postsynaptic organization and regulation of excitatory synapses. *Nat. Neurosci. Rev.* 1, 133-141.

SEGOVIA G, PORRAS A, DEL ARCO A, MORA F (2001) Glutamatergic neurotransmission in aging: a critical perspective. *Mech. of Aging and Dev.* 122,

1-29.

SHAO Y & McCARTHY KD (1994) Plasticity of astrocytes. *Glia* 11, 147-155.

SHIMIZU T, NAKAZATO T, XIAN MJ, SAGAWA M, IKEDA Y, KIZAKI M (2006) Resveratrol induces apoptosis of human malignant B cells by activation of caspase-3 and p38 MAP kinase pathways. *Biochem. Pharmacol.* 71, 742-750.

SIEMANN EH & CREASY LL (1992) Concentration of the phytoalexin resveratrol in wine. *Am J Enol Vitic* 43, 49-52.

SIGNORELLI P & GHIDONI R (2005) Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *J. Nutr. Biochem.* 16, 449-466.

SINGH NP, MCCOY MT, TICE RR, SCHNEIDER EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res.* 175, 184-91.

SINHA K, CHAUDHARY G, GUPTA YK (2002) Protective effect of resveratrol against oxidative stress in middle cerebral artery occlusion model of stroke in rats. *Life Sci* 71, 655-65.

SLATER SJ, SEIZ JL, COOK AC, STAGLIANO BA, BUZAS CJ (2003) Inhibition of protein kinase C by resveratrol. *Biochim. Biophys. Acta.* 1637, 59-69.

SOARES DE MOURA R, MIRANDA DZ, PINTO ACA, SICCA RF, SOUZA MAV, RUBENICH LMS, CARVALHO LCRM, RANGEL BM, TANO T, MADEIRA SVF, RESENDE AC (2004) Mechanism of the endothelium-dependent vasodilation and the antihypertensive of Brazilian red wine. *J Cardiovasc Pharmacol* 44, 302-309.

SOLEAS GJ, DIAMANDIS EP, GOLDBERG DM (1997) Wine as a biological fluid: history, production, and role in disease prevention. *Clin Lab Anal.* 11, 287-313.

SOUTO AA, CARNEIRO MC, SEFERIN M, SENNA MJH, CONZ A, OBBI K (2001) Determination of trans-resveratrol concentrations in Brazilian red wines by HPLC. *J Food Comp Anal* 14, 441-445.

STANIMIROVIC DB, BALL R, SMALL DL, MURUGANANDAM A (1999) Developmental regulation of glutamate transporters and glutamine synthetase activity in astrocyte cultures differentiated in vitro. *Int J Dev Neurosci.* 17, 173-84.

TAKANO T, LIN JH, ARCUINO G, GAO Q, YANG J, NADERGAARD M (2001) Glutamate release promotes growth of malignant gliomas. *Nature Medicine.* 7, 1010-1015.

TANAKA K (2000) Functions of glutamate transporters in the brain. *Neurosci. Res.* 37, 15-19.

TAPHOORN MJB & KLEIN M (2004) Cognitive deficits in adult patients with brain tumours. *The Lancet Neurology* 3, 159-168.

TRAMONTINA F, CONTE S, GONÇALVES D, GOTTFRIED C, PORTELA LV, VINADE L, SALBEGO C, GONÇALVES CA (2002) Developmental changes in S100B content in brain tissue, cerebrospinal fluid, and astrocyte cultures of rats. *Cell Mol. Neurobiol.* 22, 373-378.

TRAMONTINA F, LEITE MC, GONCALVES D, TRAMONTINA AC, SOUZA DF, FRIZZO JK, NARDIN P, GOTTFRIED C, WOFCHUK ST, GONCALVES CA (2006) High glutamate decreases S100B secretion by a mechanism dependent on the glutamate transporter. *Neurochem Res.* 31, 815-20.

TREDICI G, MILOSO M, NICOLINI G, GALBIATI S, CAVALLETTI G, BERTELLI A (1999) Resveratrol, map kinases and neuronal cells: might wine be a neuroprotectant? *Drugs Exp. Clin. Res.* 25, 99-103.

TROTTI D, DANBOLT NC, VOLTERRA A (1998) Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? *Trends Pharmacol Sci.* 19, 328-34.

VAN ELDIK LJ, WAINWRIGHT MS (2003) The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain. *Restor Neurol Neurosci.* 21, 97-108.

VAN ELDIK LJ, ZIMMER DB (1987) Secretion of S-100 from rat C6 glioma cells. Brain Res. 436, 367-70.

VENTURA R & HARRIS KM (1999) Three-dimensional relationships between hippocampal synapses and astrocytes. J Neurosci. 19, 6897-906.

VIRGILI M & CONTESTABILE A (2000) Partial neuroprotection of in vivo excitotoxic brain damage by chronic administration of the red wine antioxidant agent, trans-resveratrol in rats. Neurosci Lett. 281, 123-6.

WENZEL E & SOMOZA V (2005) Metabolism and bioavailability of trans-resveratrol. Mol Nutr Food Res. 49, 472-81.

WOZNIAK MA, MODZELEWSKA K, KWONG L, KEELY PJ (2004) Focal adhesion regulation of cell behavior. Biochim Biophys Acta. 1692, 103-19.

YE Z, ROTHSTEIN JD, SONTHEIMER H (1999) Compromised glutamate transport in human glioma cells: reduction-mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine-glutamate exchange. J. Neurosci. 19, 10767-10777.

YOUNG JZ (1991) The concept of neuroglia. Annals of the New York Academy of Sciences 633,1-18.

ZHU D, TAN KS, ZHANG X, SUN AY, SUN GY, LEE JC (2005) Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes. *J. Cell Sci.* 118, 3695-3703.

ZIMMER DB, VAN ELDIK LJ (1988) Levels and distribution of the calcium-modulated proteins S100 and calmodulin in rat C6 glioma cells. *J Neurochem.* 50, 572-9.

## ANEXO I

### LISTA DE FIGURAS

INTRODUÇÃO	PÁGINA
<b>Figura 1.</b> Trans-resveratrol.....	01
<b>Figura 2.</b> Isomerização do resveratrol.....	02
<b>Figura 3.</b> Citações do resveratrol no PubMed em função do tempo.....	04
<b>Figura 4.</b> Representação esquemática da formação de filamento intermediário...10	
<b>Figura 5.</b> Reação catalisada pela enzima Glutamina Sintetase.....	14
<b>Figura 6.</b> Representação esquemática de uma Sinapse Glutamatérgica.....	15
<b>Figura 7.</b> Reação de Fenton.....	17