

Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

PROPRIEDADES NEURAIS DAS CATEQUINAS ECG E EGCG:

Efeito Quelante de Metais e Genoproteção *in vitro* e Neuroproteção *in vivo* no

Modelo de Parkinson

RENATA TORRES ABIB

Orientadora: Prof. Dra. Carmem Gottfried

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Aos meus pais Maria Elza e Elbio Renato

“A dúvida é o princípio da sabedoria.”

Aristóteles

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RESUMO

O consumo do chá verde tem apresentado inúmeros benefícios à saúde humana, e estes efeitos têm sido atribuídos às catequinas (estruturas polifenólicas) que o constitui, especialmente à epicatequina galato (ECG) e à epigallocatequina galato (EGCG). A permeabilidade à barreira hemato-encefálica, o fácil acesso através da dieta e a baixa toxicidade das catequinas, apontam-nas como promissoras para prevenção e tratamento de doenças crônicas neurodegenerativas. Em função disso, esta tese visa à análise de catequinas em parâmetros *in vitro* (genoproteção e atividade mitocondrial) e *in vivo* no modelo de Parkinson. A fim de investigar a propriedade genoprotetora utilizamos ECG nas concentrações de 0,1, 1 e 10 μM em células astrogliais da linhagem C6 e concluímos que a ECG apresenta efeito tempo-dose-dependente, sendo genoprotetora em concentrações baixas e tempos curtos, e, embora não tenha ocorrido morte celular significativa, observou-se um efeito genotóxico quando em altas concentrações e tempos prolongados. Além disso, a ECG 0,1 e 1 μM foi capaz de proteger do dano ao DNA induzido por H_2O_2 – espécie reativa gerada principalmente pela mitocôndria. Sabendo do efeito neuroprotetor das catequinas, investigamos o efeito da EGCG *in vitro* e *in vivo*. Em frações ricas em mitocôndrias de encéfalos de ratos, a EGCG foi testada nas concentrações de 10, 50 e 100 μM durante 2 h na presença ou não de Cd^{2+} 200 μM – metal altamente tóxico - e concluímos que a EGCG não somente apresenta um efeito antioxidante importante, visto através da prevenção total da lipoperoxidação e da perda de viabilidade mitocondrial induzida por Cd^{2+} , como apresenta efeito quelante equimolar sobre este metal. Entretanto, a EGCG não preveniu a oxidação da glutathiona – importante defesa antioxidante do organismo - induzida por Cd^{2+} , isto sugere que a EGCG esteja agindo através da remoção das espécies reativas geradas pelo metal e/ou pela sua complexação ao cádmio, e não via aumento da glutathiona. Em modelo animal de Parkinson induzido por 6-OHDA, sugere-se que, a partir de dados preliminares, a EGCG tem efeito benéfico sobre a morte dos neurônios dopaminérgicos da substância negra, mas não sobre o comportamento motor. Estes achados sugerem que as catequinas em baixas concentrações - como as encontradas fisiologicamente (até 2 μM) - e em período relativamente curto - como as quais permanecem no organismo (até 8 h) após o consumo do chá, sejam benéficas em longo prazo.

ABSTRACT

Green tea consumption has shown numerous benefits to human health, and these effects have been attributed to catechins (polyphenolic structures) that constitute especially epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). Their blood-brain barrier permeability, easy access through the diet and low toxicity of catechins, show them as promising molecules for prevention and treatment of chronic neurodegenerative disorders. The aim of this thesis is analyze catechins *in vitro* parameters (genoprotection and mitochondrial activity) and *in vivo* using Parkinson's disease model. In order to investigate the genoprotective effects, we used ECG 0.1, 1 and 10 μM in C6 astroglial cells and we concluded that ECG shows dose-time-dependent effects, being genoprotective at low concentrations and short times, and presenting genotoxic effect at high concentrations and prolonged periods, although didn't occur significant cell death. Moreover, ECG 0.1 μM was able to protect DNA damage induced by H_2O_2 - reactive specie generated mainly by mitochondria. Knowing the neuroprotective effect of catechins, we investigated the effect of EGCG *in vitro* and *in vivo*. In isolated mitochondria from rat brain, EGCG 10, 50 and 100 μM was tested along 2 h in the presence or not of cadmium 200 μM – a high neurotoxic metal – and we concluded that EGCG presents not only an important antioxidant effect, as seen through the prevention of lipid peroxidation and total loss of mitochondrial activity induced by Cd^{2+} , but presented equimolar chelation effect on this metal. However, EGCG failed to prevent the oxidation of glutathione - an important antioxidant defense - induced by Cd^{2+} , suggesting that EGCG acting through the removal of reactive species generated by cadmium and by metal complexation, and these effects are not derived from an increasing glutathione levels. In an animal model of Parkinson's induced by 6-OHDA, EGCG had a beneficial effect on dopaminergic neurons death in substantia nigra, but not on motor behavior. These findings suggest that the catechins in low concentrations - such as those found physiologically (up to 2 μM) - and in relatively short period - like which remain in the body (up to 8 h) after tea consumption, are beneficial in long-term use.

Lista de Abreviaturas

6-OHDA = 6-hidroxi-dopamina;
ATP = adenosina trifosfato;
BHE = barreira hemato-encefálica;
CAT = catalase;
DA = dopamina;
DAT = transportador de dopamina;
DP = doença de Parkinson;
EC = epicatequina;
ECG = epicatequina-galato;
EGC = epigallocatequina;
EGCG = epigallocatequina-galato;
ERO = espécies reativas de oxigênio;
GP = globo pálido;
GPX = glutathione peroxidase;
GR = glutathione reductase;
GSH = glutathione reduzida;
IL-1 β = interleucina-1 β ;
IL-8 = interleucina-8;
MAO = monoamino oxidase;
MPTP = N-metil-4-fenil-1,2,3,6-tetrahidropiridina;
nNOS = óxido nítrico sintase neuronal;
NOS = óxido nítrico sintase;
Pgp = P-glicoproteína;
SN = substância negra;
SNC = sistema nervoso central;
SOD = superóxido dismutase;
STN = núcleo subtalâmico;
TH = tirosina hidroxilase;
TNF- α = fator de necrose tumoral alfa.

SUMÁRIO

1 INTRODUÇÃO	1
1.1 ESTRESSE OXIDATIVO, MITOCÔNDRIAS E MECANISMOS ANTIOXIDANTES	1
1.1.1 Espécies reativas de oxigênio.....	3
1.1.2 Estresse oxidativo e o sistema nervoso central.....	4
1.2 CHÁ VERDE	4
1.2.1 Flavonóides	4
1.2.2 Catequinas	5
1.2.3 Biodisponibilidade das catequinas.....	6
1.2.4 Mecanismos de ação da EGCG e da ECG.....	9
1.3 METAIS	10
1.4 DANO AO DNA	11
1.5 DANO MITOCONDRIAL.....	12
1.6 ASTRÓCITOS E LINHAGEM C6	12
1.7 DOENÇA DE PARKINSON.....	13
1.7.1 Modelos experimentais de Parkinson	15
1.7.1.1 Modelo de Parkinson induzido por 6-OHDA.....	15
1.7.2 Testes comportamentais	19
1.7.3 Evidências da EGCG sobre modelos de Parkinson	20
2 OBJETIVOS	21
3 RESULTADOS	22
CAPÍTULO I	22
CAPÍTULO II	28
CAPÍTULO III	59
4 DISCUSSÃO	68
5 CONCLUSÕES	81
6 PERSPECTIVAS	83
REFERÊNCIAS BIBLIOGRÁFICAS	84

1 INTRODUÇÃO

Com o aumento da expectativa de vida da população, observa-se um maior número de doenças neurodegenerativas diretamente relacionadas com a idade. As doenças degenerativas de Alzheimer e Parkinson parecem ter uma base comum, o estresse oxidativo, que por sua vez, pode estar diretamente relacionado à morte neuronal (Jenner, 2003; Brown *et al.*, 2005; Reynolds *et al.*, 2007).

Estudos epidemiológicos recentes demonstram que o consumo de dois copos/dia ou mais de chá verde, por mais de oito anos pode reduzir significativamente o risco de desenvolver a doença de Parkinson (Checkoway *et al.*, 2002; Tan *et al.*, 2003; Kuriyama *et al.*, 2006; Hu *et al.*, 2007; Kandinov *et al.*, 2009; Pereira and Garrett, 2010). Entretanto, os mecanismos moleculares que participam da ação neuroprotetora do chá ainda não estão bem estabelecidos.

1.1 ESTRESSE OXIDATIVO, MITOCÔNDRIAS E MECANISMOS ANTIOXIDANTES

As mitocôndrias são organelas responsáveis pela geração de energia, sustentação do metabolismo e da integridade celular, além disso, já está bem estabelecido que, em grande parte dos organismos, existe uma relação entre a disfunção mitocondrial e danos patológicos irreversíveis (Andreyev *et al.*, 2005). Em organismos aeróbicos, é na mitocôndria que 90% do oxigênio molecular é consumido e utilizado para gerar a maior parte da energia produzida durante o metabolismo oxidativo (Trushina and McMurray, 2007).

Os elétrons provenientes da oxidação dos nutrientes são coletados na forma de NADH e FADH₂, que são coenzimas que participam de uma série de reações redox e promovem o transporte de elétrons na mitocôndria através de uma série de complexos multiprotéicos até o complexo da citocromo C oxidase, que os transfere ao oxigênio

(Figura 1). Durante o processo, prótons são bombeados da matriz para o espaço intermembranas da mitocôndria e retornam gerando energia para a síntese de adenosina trifosfato (ATP). Em alguns sítios, como no complexo I e na coenzima Q, os elétrons podem escapar e reduzir o oxigênio molecular, formando ânion superóxido ($O_2^{\bullet-}$) - uma espécie reativa de oxigênio (ERO) (Adams and Turnbull, 1996; Indo *et al.*, 2007).

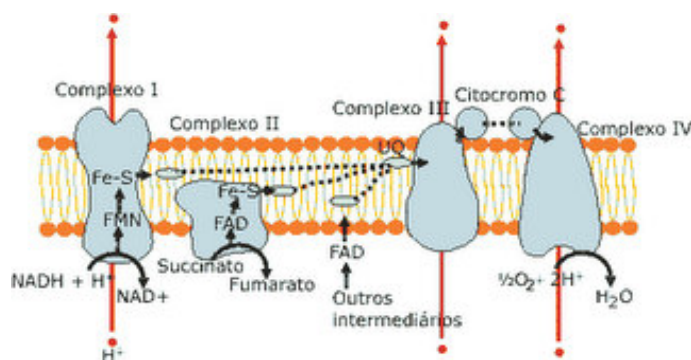


Figura 1: Cadeia de transporte de elétrons (<http://www.bioq.unb.br>, compilado em 21 de julho de 2011)

Para compensar essa formação de ERO através da cadeia de transporte de elétrons, há um sistema de antioxidantes formado pela superóxido dismutase (SOD), catalase (CAT), glutatona peroxidase (GPX), glutatona redutase (GR) e glutatona reduzida (GSH) (Kowaltowski and Vercesi, 1999).

Níveis aumentados de ERO podem ser resultantes não só do estresse oxidativo, mas de falhas nos sistemas de reparo desses danos (Halliwell, 2007a). O nível de GSH mitocondrial é crítico para regulação do estado redox (Kroemer *et al.*, 1998). Como antioxidante, a GSH efetivamente protege as células contra o dano causado pelo estresse oxidativo, sendo responsável pela remoção de peróxido de hidrogênio (H_2O_2), mediante ação da GPX (Teramoto *et al.*, 1999; Liddell *et al.*, 2004; Heverly-Coulson

and Boyd, 2010) e prevenção da formação de peróxidos lipídicos e a oxidação de moléculas (Heverly-Coulson and Boyd, 2010).

1.1.1 Espécies reativas de oxigênio

As ERO, tais como: radical hidroxila (OH^\bullet), $\text{O}_2^{\bullet-}$ e H_2O_2 , são produtos do metabolismo aeróbico normal e são consideradas importantes na sinalização celular, na expressão gênica, no crescimento e na sobrevivência celular (Leloup et al., 2010). Entretanto, o excesso de formação das ERO leva ao estresse oxidativo - desequilíbrio entre os sistemas oxidantes e as defesas antioxidantes - causando dano celular por oxidar estruturas macromoleculares como lipídios, proteínas e DNA, modificando suas funções biológicas (Droge, 2002).

O H_2O_2 é capaz de gerar OH^\bullet na presença de metais como ferro, através da reação de Fenton (Figura 2), sendo formado principalmente na matriz mitocondrial durante o processo de redução do oxigênio ou pela dismutação do radical superóxido, por ação da enzima SOD (Gutteridge, 1994). O H_2O_2 é capaz de: atravessar membranas (Sitaramam et al., 1989); causar dano mitocondrial (Dumont *et al.*, 1999; Viola *et al.*, 2007; Luo *et al.*, 2010); dar origem a novas ERO capazes de interagir com lipídios (Kose and Dogan, 1995) e proteínas; modular vias de sinalização celular, induzindo apoptose (Cerella *et al.*, 2009; Fabiani *et al.*, 2009; Nandi *et al.*, 2010) e gerar dano ao DNA (Kim *et al.*, 2000; Cai *et al.*, 2006; Kim and Kang, 2006; Ribeiro *et al.*, 2006; Nyaga *et al.*, 2007; Atilano *et al.*, 2009; Miranda-Vilela *et al.*, 2010).

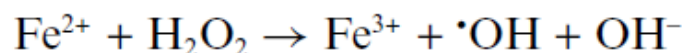


Figura 2: Reação de Fenton

1.1.2 Estresse oxidativo e o sistema nervoso central

O sistema nervoso central (SNC) é particularmente suscetível à lipoperoxidação devido ao seu alto conteúdo de ácidos graxos poliinsaturados e ao alto consumo de oxigênio – cerca de 20% do total consumido (Mariani *et al.*, 2005; Ferreira *et al.*, 2006; Lehtinen and Bonni, 2006). Esse dano aos lipídios pode ocasionar alterações funcionais e estruturais da membrana celular, além da inativação de enzimas (Cini and Moretti, 1995; Leutner *et al.*, 2001).

1.2 CHÁ VERDE

O chá verde, derivado da *Camellia sinensis*, é consumido há centenas de anos principalmente no leste asiático e o crescente interesse pela bebida se deve a evidências sobre a relação inversa existente entre seu consumo e o risco de doenças cardiovasculares (Batista Gde *et al.*, 2009; Kuriyama, 2010; Wang *et al.*, 2010), neurodegenerativas (Reznichenko *et al.*, 2006; Rezai-Zadeh *et al.*, 2008; Zhao, 2009) e câncer (Shimizu *et al.*, 2010; Tran *et al.*, 2010).

O tipo de processamento aplicado às folhas frescas da *Camellia sinensis*, determina o tipo do chá, por exemplo: o chá verde é pouco oxidado, o que lhe confere um alto poder antioxidante; o chá oolong passa pelo processo de semi-oxidação e o chá preto é totalmente oxidado (Koh *et al.*, 2010).

1.2.1 Flavonóides

Os polifenóis constituem uma classe das fitoalexinas encontradas nos tecidos de plantas. Embora existam mais de 10.000 polifenóis na natureza, apenas alguns alimentos comumente utilizados na dieta contém estes compostos, por exemplo: a casca da uva, as oleaginosas e o chá verde (Queen and Tollefsbol, 2010).

Os flavonóides, que são estruturas polifenólicas, começaram a ser estudados no início dos anos 80, quando se demonstrou a relação existente entre uma dieta rica em frutas e em vegetais com a redução do risco de doenças crônicas (Steinmetz and Potter, 1991). Pelo fato deste efeito não estar correlacionado aos nutrientes tradicionais, a atenção passou a ser direcionada a estes compostos potencialmente bioativos (Harnly et al, 2006). Os flavonóides mais abundantes do extrato do chá verde são as catequinas (Babu and Liu, 2008).

1.2.2 Catequinas

O típico chá verde, preparado pela infusão de 1 g de folhas da *Camellia sinensis* em 100 ml de água fervente por 3 minutos contém, em média, de 250 a 350 mg de matéria seca, composta por 30 a 42% de catequinas (aproximadamente 100 mg) e por 3 a 6 % de cafeína (Riemersma *et al.*, 2001; Babu and Liu, 2008).

As catequinas representam 80 - 90% dos flavonóides totais, sendo a (-)-epigallocatequina-3-galato (EGCG), a mais abundante (48-55%), seguida pela (-)-epicatequina-3-galato (ECG) (9-12%), (-)-epigallocatequina (EGC) (9-12%) e (-)-epicatequina (EC) (5 -7%) (Babu and Liu, 2008). Na Figura 3, estão representadas as estruturas das diferentes catequinas.

Estudos demonstram que as catequinas possuem muitos componentes estruturais que contribuem para suas propriedades antioxidantes. Algumas catequinas apresentam grupamentos hidroxilas no anel B e também na posição 5 e 7 no anel A (Figura 3). A EGCG e a ECG possuem o grupamento galato esterificado na 3ª posição do anel C, o que confere a estas duas um maior poder antioxidante em relação às outras (Guo *et al.*, 1996; Devika and Stanely Mainzen Prince, 2008). Alguns estudos demonstram que tanto os anéis fenólicos, como as hidroxilas livres das catequinas,

contribuem para que essas estruturas tenham propriedades quelante de metais (Zhang et al., 2008).

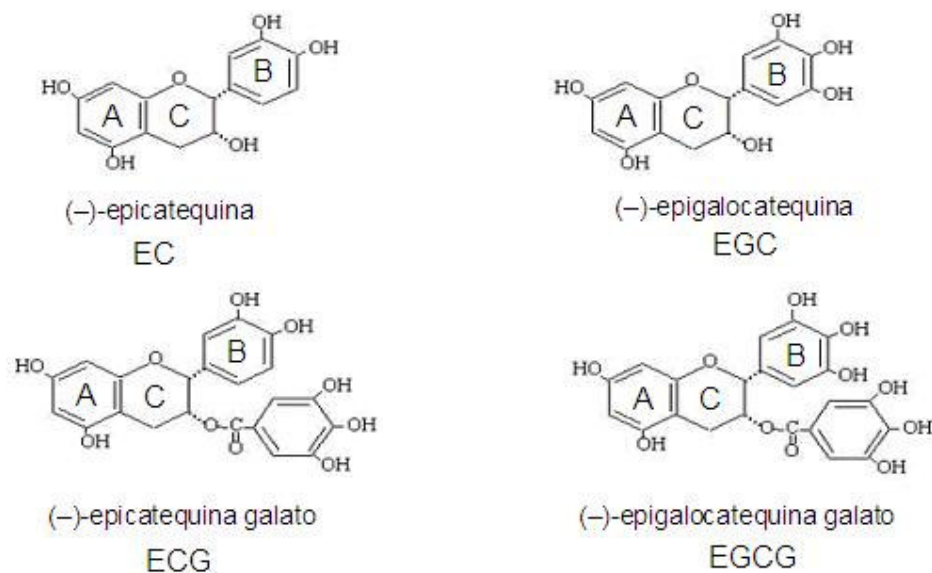


Figura 3: Estrutura química das catequinas

1.2.3 Biodisponibilidade das catequinas

Embora seja essencial para avaliação das suas funções biológicas, até o momento, pouco se sabe sobre a biodisponibilidade, o metabolismo e as biotransformações que sofrem as catequinas. Entretanto, sugere-se que muitos fatores possam interferir nos processos de absorção, distribuição e excreção.

Sabe-se que a biodisponibilidade dos polifenóis do chá é relativamente baixa (Henning et al., 2008) e a forma de administração parece ter grande influência. Na administração oral, deve-se levar em consideração que a absorção intestinal depende da flora bacteriana de cada indivíduo e é possível que a ingestão concomitante de outros alimentos interfira na absorção das catequinas (Warden et al., 2001). Além disso, o pH gástrico varia no jejum e no estado alimentado, alterando a estabilidade das catequinas (Chow et al., 2005).

Sessenta minutos após a administração oral de polifenóis do chá verde, baixas concentrações de EGCG já se encontram presentes no plasma, no fígado e no SNC (Nakagawa and Miyazawa, 1997b, a). Em humanos, o pico de concentração plasmática de catequinas é de aproximadamente 2 μ M, o que representa até 2% da quantidade ingerida (Pietta *et al.*, 1998; Yang *et al.*, 1998; Fu *et al.*, 2008a; Nakagawa *et al.*, 2009) e é atingido entre 1,5 – 2,5 h após o consumo (Nakagawa *et al.*, 1997; Riemersma *et al.*, 2001). O mesmo perfil de absorção é encontrado em ratos (Chen *et al.*, 1997; Tsang *et al.*, 2005). Já, na administração intravenosa ou intraperitoneal a mesma concentração plasmática é atingida com doses 100 vezes menores (Zhu *et al.*, 2000).

A EGCG apresenta a maior meia-vida em relação às outras catequinas (Chen *et al.*, 1997). Um estudo que utilizou a dose de 5 mg/Kg [H^3]EGCG em camundongos, mostrou que os animais que receberam uma segunda dose equivalente a primeira, após 6h, apresentavam 4 vezes mais [H^3]EGCG no SNC do que os camundongos que não receberam a segunda dose. Após 24 h, os animais que receberam as duas doses mantiveram níveis 3 vezes mais altos de [H^3]EGCG no SNC do que receberam apenas uma dose (Suganuma *et al.*, 1998). Estes resultados indicam que a ingestão crônica desta catequina pode ajudar na manutenção de níveis relativamente altos de EGCG no organismo.

As catequinas podem ser absorvidas no intestino por difusão passiva ou por transportadores (Vaidyanathan and Walle, 2001). Alguns transportadores presentes nas células epiteliais intestinais estão sendo estudados para melhor entender o processo de absorção e excreção das catequinas, são estes: P-glicoproteína (Pgp) e proteínas associadas à resistência de múltiplas drogas (MRP1 e MRP2) (Youdim *et al.*, 2003; Feng, 2006; Lambert *et al.*, 2007). A presença ou ausência destes transportadores nas células pode explicar a distribuição diferencial das catequinas

entre os tecidos (Suganuma et al., 1998), além disso, a presença do grupamento galato parece influenciar a ligação ao transportador (Kadowaki et al., 2008).

Já foi demonstrado que os flavonóides livres e também os conjugados, mesmo sendo moléculas polares, atravessam a barreira hemato-encefálica (BHE) (Youdim et al., 2004) e são capazes de exercer seus efeitos no SNC (Abd El Mohsen *et al.*, 2002; Youdim *et al.*, 2003). Embora o conhecimento sobre os mecanismos pelos quais estes compostos se difundem ainda seja limitado, já foi comprovada a presença de catequinas no SNC (<1% do total consumido em 24h) após sua administração em camundongos (Suganuma et al., 1998). Existem evidências do envolvimento da Pgp na permeabilidade do SNC aos flavonóides (Youdim *et al.*, 2003; Youdim *et al.*, 2004; Lin *et al.*, 2007).

Uma vez absorvidas pelas células epiteliais intestinais, as catequinas podem sofrer metilação, glicuronidação ou sulfatação (Vaidyanathan and Walle, 2002; Zhang *et al.*, 2004). Os metabólitos podem ser transportados para a circulação através dos MRPs, metabolizados no fígado e finalmente excretados pela urina (Yang et al., 1998). A excreção ocorre no máximo em 8 h após o consumo (Lee *et al.*, 1995; Lee *et al.*, 2002; Chow *et al.*, 2003).

A fim de quantificar os conjugados de catequinas, amostras plasmáticas de humanos, que receberam diferentes doses de EGCG, foram tratadas com sulfatases e glicuronidases, e não ocorreu aumento significativo dos níveis de EGCG, sugerindo que apenas uma pequena fração se apresenta conjugada ao ácido glicurônico e ao sulfato (Chow et al., 2003).

1.2.4 Mecanismos de ação da EGCG e da ECG

A importância das catequinas do chá verde em aumentar a resistência celular ao estresse oxidativo se deve a sua capacidade sequestradora de ERO e ao seu efeito quelante de metais. Assim, esses polifenóis são capazes de prevenir e/ou reduzir efeitos deletérios das ERO associados a doenças neurodegenerativas (Weinreb et al., 2007).

Pelo fato das catequinas poderem atravessar a BHE (Chu *et al.*, 2007; Lin *et al.*, 2007), tem-se sugerido um importante papel neuroprotetor para estes compostos. A EGCG é capaz de aumentar as defesas antioxidantes em estudos *in vivo* (Tuzcu *et al.*, 2008; Jeon *et al.*, 2010) e *in vitro* (Chen and Zhang, 2003), aumentando os níveis de GSH e a expressão de enzimas como a SOD, a GPX e a CAT (Fu *et al.*, 2008b; Dorchies *et al.*, 2009; Sahin *et al.*, 2010) (Figura 4).

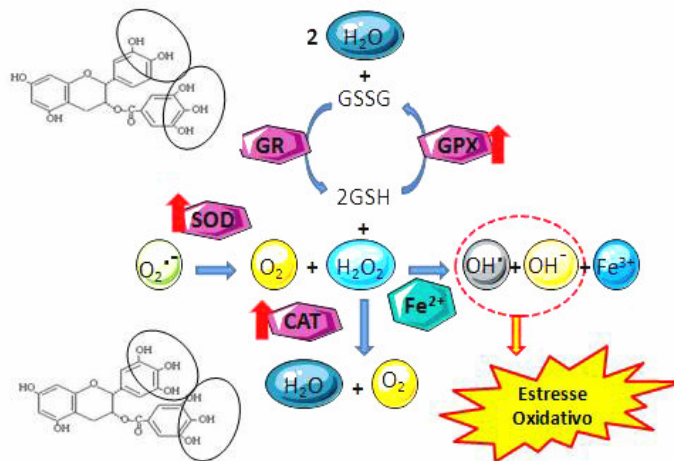


Figura 4: Efeitos da ECG e da EGCG sobre enzimas antioxidantes indicados com as setas vermelhas. GR: glutatona redutase; GPX: glutatona peroxidase; SOD: superóxido dismutase; CAT: catalase.

Sabe-se que a EGCG e a ECG podem formar complexos com o ferro, prevenindo assim a reação de Fenton (Ryan and Hynes, 2007), além disso, há

evidências de que a EGCG possa quelar manganês, zinco e cobre (Fernandez *et al.*, 2002; Esparza *et al.*, 2005; Navarro *et al.*, 2005). Entretanto, não se sabe seu efeito sobre o cádmio – importante metal associado ao desenvolvimento de doenças neurológicas (de Burbure *et al.*, 2006; Sinha *et al.*, 2008).

1.3 METAIS

Os metais pesados, por não serem biodegradáveis, permanecem no ambiente por um longo tempo e causam sérios problemas ecotoxicológicos. Homens, animais e plantas são expostos a uma variedade de metais através do ambiente, alimentos, água e solo (Rana, 2008).

A intoxicação por metais pesados particularmente neurotóxicos, genotóxicos e/ou carcinogênicos é amplamente discutida. Um fator comum na toxicidade desses metais é a geração de ERO, que podem levar a lipoperoxidação e modificações nas bases do DNA. Além disso, metais como o cádmio, mercúrio e níquel, possuem alta afinidade por grupamentos sulfidrilas presentes em proteínas e na GSH (Valko *et al.*, 2005; Flora *et al.*, 2008).

O Cd^{2+} é um poluente ambiental presente principalmente nas fumaças produzidas pela queima de cigarros, combustíveis fósseis e pelo refinamento de metais (Chen *et al.*, 2008). A exposição excessiva a metais pesados pode levar a consequências irreparáveis, sendo considerada uma fonte comum de neurotoxicidade (Wright and Baccarelli, 2007). Embora muitas pesquisas venham sendo feitas sobre este assunto, ainda são pouco conhecidos tratamentos eficazes para esse tipo de intoxicação (Flora *et al.*, 2008). Sabendo que a EGCG pode proteger células neuronais do estresse oxidativo e do dano mitocondrial induzido por chumbo (Chen *et al.*, 2003),

especula-se que a capacidade da EGCG de quelar metais possa ser um importante mecanismo pelo qual as catequinas possam mediar seus efeitos benéficos.

1.4 DANO AO DNA

O dano ao DNA está associado à carcinogênese, envelhecimento e doenças neurodegenerativas, como o Parkinson e o Alzheimer (Cozzi *et al.*, 1997; Bender *et al.*, 2006; Gackowski *et al.*, 2008; Wilson *et al.*, 2008). A etiologia do dano oxidativo ao DNA envolve uma série de alterações causadas por ERO (Aitken *et al.*, 2010) e pode ser induzido por maus hábitos alimentares e estilo de vida (Steinmetz and Potter, 1996; McCord and Edeas, 2005; Aggarwal *et al.*, 2009).

O dano produzido por oxidação do DNA é considerado o mais significativo dano oriundo do metabolismo celular. Estima-se que aproximadamente 2×10^4 lesões oxidativas ao DNA ocorram no genoma humano por dia (Ames and Shigenaga, 1992). Desta maneira, o reparo destas lesões possui um papel central na prevenção do aumento de mutações nos organismos vivos (Maluf, 2004).

Os nutracêuticos são alimentos ou substâncias constituintes de alimentos, cujo consumo garante benefícios a saúde (Zeisel, 1999), possuem características químicas diversas e podem atuar em diferentes rotas metabólicas (Gupta *et al.*, 2010). Durante a última década, vários nutracêuticos que colaboram na prevenção e reparo das lesões oxidativas ao DNA (Johnson and Loo, 2000) têm sido identificados, por exemplo: os vegetais crucíferos (Riso *et al.*, 2010), o café (Misik *et al.*, 2010), o resveratrol (Gatz *et al.*, 2008; Wong *et al.*, 2010) e a quercetina (Silva *et al.*, 2008), entretanto, pouco se sabe sobre o efeito genoprotetor das catequinas.

1.5 DANO MITOCONDRIAL

Um dos mecanismos de morte celular induzida por metais é a apoptose, um fenômeno de morte rigorosamente controlado. Sabe-se que as mitocôndrias desempenham um papel central na apoptose (Li et al., 2009) e na formação das ERO (Gobe and Crane, 2010). Metais pesados como o cádmio, prejudicam a funcionalidade mitocondrial, em parte por alterarem a regulação do estado redox (Smiri et al., 2010).

A EGCG é capaz de proteger os neurônios da apoptose induzida por estresse oxidativo mitocondrial e especula-se que este efeito seja em função do seu acúmulo neste compartimento celular, onde pode atuar localmente como sequestradora de ERO (Schroeder et al., 2009). Essa propriedade faz da EGCG uma promissora molécula na terapia para doenças que envolvem apoptose neuronal por estresse oxidativo.

1.6 ASTRÓCITOS E LINHAGEM C6

Os astrócitos são células gliais que interagem com outros tipos celulares (Figura 5) e exercem um papel fundamental na manutenção da biologia celular durante o desenvolvimento. Os astrócitos podem se tornar reativos em resposta a agentes tóxicos (ex: metais), infecções virais, hipóxia/isquemia e doenças neurodegenerativas. Esta reatividade pode ser benéfica para os neurônios, já que os astrócitos regulam processos biológicos, tais como: concentração de íons e de neurotransmissores extracelulares, secreção de fatores tróficos, controle da interface sangue-cérebro e defesa antioxidante (Takuma et al., 2004). Uma melhor compreensão das respostas gliais, particularmente dos astrócitos, nos possibilita desenvolver novas estratégias para tratar distúrbios neurológicos (Seifert et al., 2006).

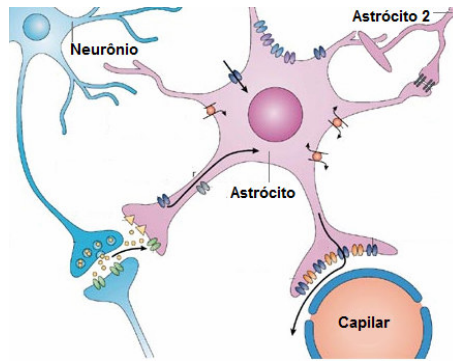


Figura 5: Interação dos astrócitos. Adaptado da literatura (Seifert et al., 2006).

A linhagem C6 foi estabelecida na década de 1960 e constitui-se numa grande fonte de estudos bioquímicos. Esta linhagem foi obtida após injeções do agente alquilante N-nitrosometiluréia em ratos (Benda et al., 1968) e é morfológicamente similar ao glioblastoma, quando injetada no encéfalo de ratos neonatos (Auer et al., 1981). A linhagem C6 é uma linhagem neural, podendo apresentar características de oligodendrócitos, astrócitos e neurônios, dependendo do número de passagens (Parker et al., 1980; Mangoura et al., 1989). A expressão das proteínas gliais GFAP e S100B atesta seu caráter astrocítico (Benda et al., 1971).

Por apresentar características de uma célula astrocítica, a linhagem C6 é utilizada para investigações bioquímicas e metabólicas que envolvam funções astrocíticas (Mangoura et al., 1989; Feng and Zhang, 2004).

1.7 DOENÇA DE PARKINSON

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum e afeta cerca de 1% da população com idade acima de 60 anos (Litvan et al., 2007a; Litvan et al., 2007b).

A DP, cuja origem é multifatorial, está associada à morte progressiva de neurônios dopaminérgicos na substância negra (SN), o que proporciona uma diminuição dos níveis de dopamina (DA) em diversas estruturas encefálicas (Pinard et al., 2008). Além disso, importantes fatores têm sido associados à etiologia da DP, como: inflamação, intoxicação e disfunção mitocondrial (Abou-Sleiman *et al.*, 2006; Jomova *et al.*, 2010). A presença de inclusões neuronais chamados corpos de Lewy, os quais são amplamente distribuídos no neocórtex, no hipocampo e nos núcleos da base, são especialmente prevalentes em casos de DP associados à demência (Gu et al., 2005).

Os sintomas da DP mais conhecidos são os motores, como tremor, rigidez (aumento do tônus muscular), bradicinesia (lentidão dos movimentos), acinesia (dificuldade em iniciar movimentos voluntários), alterações da postura e da marcha e micrografia. Entre os sintomas não motores, temos a depressão e déficits cognitivos. Estes sintomas ocorrem após a perda de aproximadamente 50% desses neurônios (Cardoso *et al.*, 2005), quando é possível fazer o diagnóstico.

As causas da morte e a progressão da perda dos neurônios dopaminérgicos ainda não são bem estabelecidas, mas parecem resultar de vários fatores, como disfunção mitocondrial, estresse oxidativo e nitrosativo, mau envelhecimento e acúmulo anormal de proteínas citoplasmáticas, disfunção no sistema de ubiquitinação e excitotoxicidade do glutamato (Beal, 2003; Moore *et al.*, 2005; Muqit *et al.*, 2006; Van Laar *et al.*, 2008; Caudle and Zhang, 2009).

O objetivo principal da maioria das terapias da DP consiste em aumentar os níveis de DA liberada no estriado. Isso geralmente é feito com a administração de agonistas dopaminérgicos, que cruzam a BHE e estimula a síntese de DA nas células que se mantêm vivas na substância negra, aliviando assim, alguns dos sintomas. Entretanto,

este tratamento não altera o curso da DP ou o ritmo pelo qual os neurônios degeneram (Ogawa *et al.*, 2005; Nagatsua and Sawadab, 2009).

1.7.1 Modelos experimentais de Parkinson

Em vista dos atuais tratamentos da DP não terem plena eficácia, modelos animais baseados em toxinas que mimetizam os seus sintomas são bastante empregados. A utilização de antioxidantes, principalmente da dieta, tem sido um foco de estudos a fim de diminuir o risco e/ou a velocidade de progressão dessa doença.

Algumas neurotoxinas utilizadas para induzir modelos experimentais de Parkinson são: N-metil-4-fenil-1,2,3,6-tetraidropiridina (MPTP) (Tetrud and Langston, 1989) e 6-hidroxidopamina (6-OHDA) (Simola *et al.*, 2007). O sítio de infusão, assim como o conhecimento sobre o mecanismo de ação e a diferença em relação à potência e à especificidade dessas toxinas determinam a fase e a característica da doença, possibilitando reproduzir um modelo animal que melhor se adapte ao estudo.

Estudos apontam que ratos são mais resistentes ao MPTP do que à 6-OHDA, indicando que o MPTP possa ter uma menor potência em lesar os neurônios (Giovanni *et al.*, 1994a; Giovanni *et al.*, 1994b; Blum *et al.*, 2001; Ferro *et al.*, 2005). Além de ser mais eficiente, a 6-OHDA (Figura 6) não atravessa a BHE, ao contrário do que acontece com o MPTP (Harik, 1992).

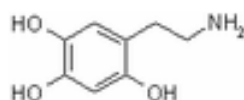


Figura 6: Estrutura química da 6-OHDA.

1.7.1.1 Modelo de Parkinson induzido por 6-OHDA

A 6-OHDA é tóxica tanto central quanto periféricamente. Entretanto, como esta neurotoxina é incapaz de atravessar a BHE, sua toxicidade no SNC se dá apenas

quando injetada diretamente no encéfalo (Simola et al., 2007). Os efeitos neurotóxicos da 6-OHDA ocorrem através de mecanismos envolvendo o acúmulo da toxina nos neurônios catecolaminérgicos, seguido de alteração na homeostase celular, ativação de células gliais e dano neuronal (Rodrigues *et al.*, 2004; Raicevic *et al.*, 2005).

O acúmulo intracelular da 6-OHDA é mediado principalmente pelos transportadores de membrana de DA (DAT), os quais reconhecem e captam 6-OHDA devido a sua similaridade estrutural com as catecolaminas endógenas. Bloqueios farmacológicos, assim como a inativação gênica desses transportadores previnem a toxicidade induzida por 6-OHDA, indicando que o seu acúmulo nos neurônios dopaminérgicos representa um ponto chave para seus efeitos neurotóxicos (Luthman *et al.*, 1997; Van Kampen *et al.*, 2000).

A 6-OHDA produz ERO tanto por mecanismos enzimáticos como não enzimáticos, os quais são amplificados por elementos intracelulares como o manganês e o ferro (Cadet and Brannock, 1998; Choi *et al.*, 1999; Lotharius *et al.*, 1999). A oxidação da 6-OHDA pela monoamina oxidase A (MAO-A) gera H_2O_2 , que é altamente citotóxico e pode levar a produção de outras ERO (Cohen et al., 1976). Além disso, a 6-OHDA pode se auto-oxidar, gerando também ERO e quinonas (Figura 7) (Padiglia *et al.*, 1997; Palumbo *et al.*, 1999; Soto-Otero *et al.*, 2000).

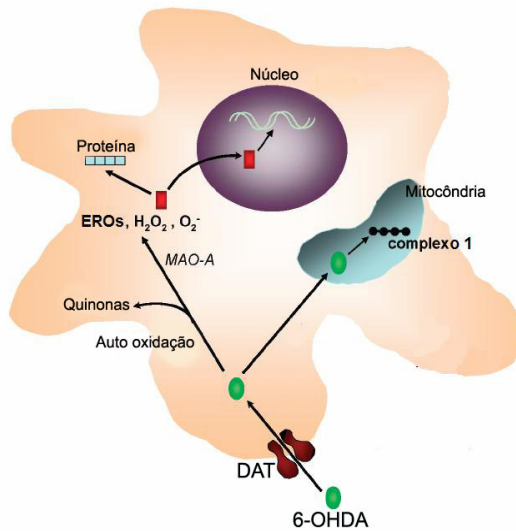


Figura 7: Neurotoxicidade da 6-OHDA. Adaptado da literatura (Simola et al., 2007).

O aumento nos níveis de ERO induzido pela 6-OHDA resulta na rápida depleção de enzimas antioxidantes, amplificando assim a neurotoxicidade e causando anormalidades na estrutura e no metabolismo celular, o que resulta em dano neuronal (Blum et al., 2001). A 6-OHDA pode também alterar a função mitocondrial, ligando-se diretamente ao complexo I e IV (Glinka and Youdim, 1995), sugerindo que o uso de agentes antioxidantes possa atenuar seus efeitos nocivos (Cadet *et al.*, 1989; Perumal *et al.*, 1989; Zbarsky *et al.*, 2005).

Uma vez que animais lesionados bilateralmente com 6-OHDA apresentam alta taxa de mortalidade em função do prejuízo motor dificultar sua alimentação, a infusão desta droga é feita, usualmente, de forma unilateral, a fim de possibilitar experimentos comportamentais e tratamentos crônicos (Ferro et al., 2005).

As estruturas-alvo da 6-OHDA são os núcleos da base, constituídos por: estriado (caudado e putâmen), globo pálido (GP), núcleo subtalâmico (STN) e a SN (Figura 8). Esses núcleos formam circuitos, onde lesões interferem em diversas funções, tais como o movimento coordenado. A SN, através da via nigroestriatal, libera DA no corpo

estriado, modulando a atividade dos outros núcleos da base (Packard and Knowlton, 2002; Pazo and Belforte, 2002).

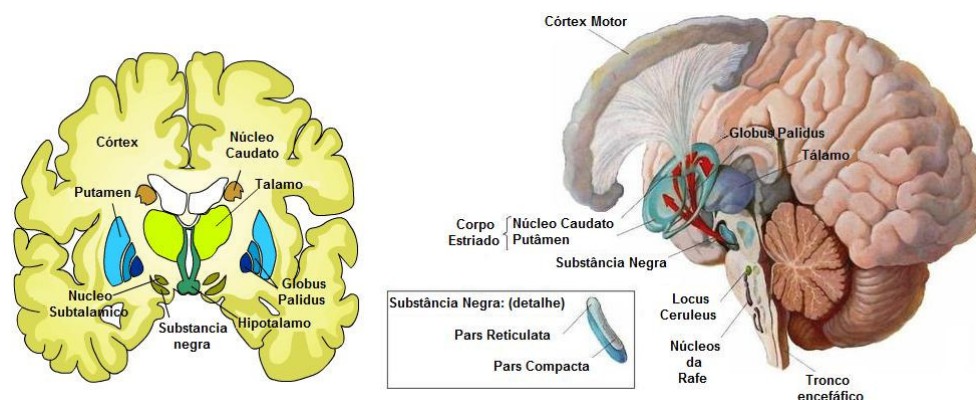


Figura 8: Regiões afetadas na doença de Parkinson. Adaptado da literatura (Brodsky and Nutt, 2010)

A DA é uma catecolamina sintetizada a partir da tirosina, por ação das enzimas tirosina hidroxilase (TH) e DOPA descarboxilase, como ilustrado na Figura 9. A quantificação da TH é uma técnica muito utilizada para evidenciar a presença de neurônios dopaminérgicos, sendo a diminuição do conteúdo desta enzima uma característica da DP (Fukuda *et al.*, 1999; Jollivet *et al.*, 2004).

A infusão da 6-OHDA na SN ou no estriado produz uma degeneração nos neurônios dopaminérgicos, além de déficits motores, os quais mimetizam a DP em humanos, o que torna a 6-OHDA uma das neurotoxinas mais usadas como modelo de Parkinson em animais. Quando infundida no estriado, a 6-OHDA induz uma gradual denervação dopaminérgica, que mimetiza os sintomas do estágio inicial da DP, sendo este sítio bastante utilizado em estudos comportamentais e bioquímicos (Liu *et al.*, 1999; Yamato *et al.*, 2001; Pierson *et al.*, 2005).

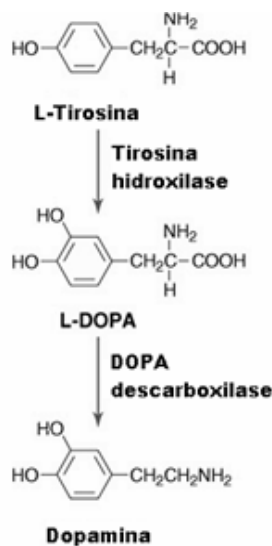


Figura 9: Formação da dopamina.

1.7.2 Testes comportamentais

A infusão de 6-OHDA unilateralmente produz um desbalanço funcional nos sistemas nigroestriatais dopaminérgicos, resultando em déficit motor - indicativo de acinesia - e no comportamento típico rotacional em resposta a agentes dopaminomiméticos (Simola et al., 2007).

A administração de drogas que estimulam os receptores dopaminérgicos, como L-DOPA e o agonista dopaminérgico apomorfina, induz rotações no sentido oposto em que a 6-OHDA foi infundida (giros contralaterais). Ao contrário, drogas que agem em terminais dopaminérgicos, aumentando os níveis de DA, como anfetamina, induzem rotações no mesmo sentido onde foi infundida (giros ipsilaterais) (Deumens et al., 2002).

Alguns testes comportamentais são apropriados para avaliar habilidades motoras e funções cognitivas em animais submetidos a 6-OHDA, por exemplo: avaliação da motricidade sobre grade (Silvestrin et al., 2009), as rotações induzidas por metilfenidato, além da avaliação de déficits proprioceptivos evidenciados pela

negligência sensorial das patas dianteiras no teste da fita adesiva (Castaneda et al., 2005).

1.7.3 Evidências da EGCG sobre modelos de Parkinson

Já foi demonstrado que a EGCG pode prevenir danos induzidos por 6-OHDA *in vitro* (Chao et al., 2010), e também a neurodegeneração induzida por MPTP, tanto em *in vivo* (Levites *et al.*, 2001; Mandel and Youdim, 2004), quanto *in vitro* (Li et al., 2006). Entretanto, mais estudos da EGCG sobre a neurotoxicidade induzida por 6-OHDA são necessários, principalmente *in vivo*, a fim de identificar o possível efeito benéfico dessa catequina sobre os efeitos induzidos por este modelo.

Desta forma, com base nas propriedades benéficas demonstradas pelo consumo de chá verde e nos importantes aspectos estruturais dos seus derivados, o estudo da resposta das principais catequinas que o compõem, como a EGCG e a ECG, sobre parâmetros envolvendo função glial e neuroproteção relacionados as suas capacidades antioxidante e quelante de metais, irá contribuir para a melhor compreensão dos efeitos destes polifenóis.

2 OBJETIVOS

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

Capítulo I

Investigar o efeito da ECG sobre dano ao DNA em linhagem celular C6, abordando os seguintes aspectos:

1. Viabilidade celular;
2. Genoproteção da ECG frente ao dano oxidativo induzido por H₂O₂.

Capítulo II

Investigar o efeito da EGCG contra o dano mitocondrial induzido por cádmio, através da avaliação de:

1. Viabilidade, conteúdo de GSH e lipoperoxidação;
2. Complexação EGCG x cádmio.

Capítulo III

Verificar a ação da EGCG no modelo de Parkinson induzido por 6-OHDA em ratos, através de:

1. Avaliação comportamental;
2. Quantificação da morte neuronal.

3 RESULTADOS

Capítulo I

*GENOPROTECTIVE EFFECTS OF THE GREEN TEA-DERIVED POLYPHENOL/
EPICATECHIN GALLATE IN C6 ASTROGLIAL CELLS*

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Genoprotective Effects of the Green Tea-Derived Polyphenol/Epicatechin Gallate in C6 Astroglial Cells

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ABSTRACT *In vitro* and *in vivo* studies have recently reported significant chemopreventive effects of green tea-derived polyphenols in different diseases. However, it remains unclear how such effects could be triggered. In order to elucidate the effects of epicatechin gallate (ECG) in C6 cells, both by itself and against H₂O₂-induced genotoxicity, measurements of DNA strand breaks and chromosome loss were performed. DNA damage was measured by comet and micronucleus assays. The present study shows for the first time how ECG, the major green tea-derived polyphenol, is able to exert dose-dependent genoprotective effects in an H₂O₂-induced toxicity model of C6 astroglial cells. We demonstrate that doses of ECG in a range from 0.1 to 1 μ M were able to completely prevent H₂O₂-induced genotoxicity *in vitro*. In contrast, considerably higher concentrations of ECG (10 μ M) were able to reverse previous positive effects in a dose- and time-dependent manner. The same results were confirmed by both comet ($F_{3,9} = 336,148$; $P < .001$) and micronucleus ($F_{3,9} = 23,228$; $P < .001$) methods. Together, our data show ECG as a dose-dependent genoprotective compound in C6 astroglial cells. This indicates that small doses of polyphenols included in our diet could have beneficial effects on neural cells, contributing to prevention of oxidative stress-associated brain pathologies. In addition, our data highlight the importance of strictly modulating doses and/or consumption of antioxidant-fortified foods or additional supplements containing such beneficial molecules.

KEY WORDS: • antioxidant • astrocyte • DNA damage • epicatechin gallate • oxidative stress

INTRODUCTION

SINCE EARLY CIVILIZATION, people have been using plants as medicine, and their consumption has significantly increased nowadays.¹

Green tea, from *Camellia sinensis*, is obtained as a result of minimal oxidation during the production process. It is widely consumed throughout the world and has received a great deal of attention since tea polyphenols were shown to be strong antioxidants.²

Green tea-derived polyphenols, such as catechins, may be responsible for reactive oxygen species quenching.³ The chemopreventive effects of green tea have been attributed to biochemical activities induced by its polyphenolic constituents such as (-)-epicatechin gallate (ECG), (-)-epicatechin, (-)-epigallocatechin 3-gallate, and (-)-epigallocatechin.^{4,5}

The capability of flavonoids to act as an antioxidant depends on their molecular structure, the position of their

hydroxyl groups, and other possible chemical substitutions in their organic structure.⁶ The relative antioxidant activity of green tea-derived catechins is (-)-epigallocatechin 3-gallate = ECG > (-)-epigallocatechin > (-)-epicatechin.⁷ Some studies have demonstrated that excessive production of free radicals and reactive oxygen species, such as hydrogen peroxide (H₂O₂), is indeed harmful, promotes aging, and can strongly influence the development of many diseases such as cancer⁸ as well as cardiovascular and neurodegenerative disorders.^{9–11} Tea polyphenols are retained in the brain and may exert neuroprotective effects whenever their consumption is controlled.¹² In fact, prolonged green tea ingestion is able to significantly protect hippocampal proteins and lipids against oxidative damage.¹³

Previous works have demonstrated that intracellular reactive oxygen species and reactive nitrogen species such as superoxide anion, hydroxyl radicals, H₂O₂, lipid peroxyl radicals, nitric oxide, and peroxynitrite can lead to destruction of different cellular components, including lipids, proteins, and DNA.^{6,14} However, cells can still survive thanks to antioxidant-dependent repair mechanisms.¹⁵ Characteristic properties of polyphenols such as penetration in brain tissue as well as their antioxidant and iron-chelating capabilities may make such compounds putative natural

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drugs for the treatment of oxidative-related neurodegenerative diseases.¹⁶

Glial cells, particularly astrocytes, are known to extensively interact with neuronal elements in the brain, influencing their activity and exerting a prominent role in both protection and repair of nervous tissue after damage.¹⁷ The C6 cell line was originally derived from rat tumors induced by *N*-nitrosomethylurea,¹⁸ and it is widely used as an astrocyte-like cell line.^{19–25}

The main aims of our study were to elucidate the effects of ECG in C6 cells, both by itself and against H₂O₂-induced genotoxicity by measuring DNA strand breaks and chromosome loss.

EXPERIMENTAL PROCEDURES

Materials

Ethidium bromide, material for cell culture, and ECG were purchased from Sigma (St. Louis, MO, USA). 4',6'-Diamidino-2-phenylindole was from Calbiochem (La Jolla, CA, USA). Dulbecco's modified Eagle's medium was purchased from Gibco BRL (Carlsbad, CA, USA), and fetal bovine serum was purchased from Cultilab (Campinas, SP, Brazil). All other chemicals were obtained from regular commercial suppliers.

Cell culture

C6 astroglial cells were cultured as previously described.²⁵ In our preparations, more than 95% of the cells exhibited positive immunoreactivity to glial fibrillary acidic protein. Late passages of cells (100 passages, minimum) were seeded in flasks and cultured in Dulbecco's modified Eagle's medium (pH 7.4) supplemented with 5% fetal bovine serum, 2.5 mg/mL amphotericin B (Fungizone®, Bristol-Myers Squibb, Princeton, NJ, USA), and 100 U/L gentamicin. Exponentially growing cells were incubated for 1, 6, 12, and 24 hours at 37°C in an atmosphere of 5% CO₂/95% air in Dulbecco's modified Eagle's medium (pH 7.4) without serum in the absence or presence of ECG (0.1, 1, or 10 μM). The concentrations of ECG used in these experiments were obtained from previous determinations.²⁶

H₂O₂ treatment

In order to investigate the genoprotective effects of ECG against H₂O₂-induced oxidative stress, cells were pre-incubated with different concentrations of ECG (0.1, 1, and 10 μM) for 1 hour at 37°C in an atmosphere of 5% CO₂/95% air in Dulbecco's modified Eagle's medium (pH 7.4) without serum. After this time, the medium was maintained, and 1 mM H₂O₂ was added.²⁷ Cells were incubated in the same conditions for an additional 30 minutes.

Nuclear morphology assay

C6 cells were cultured on circular glass coverslips and treated with or without 1 and 10 μM ECG for 1, 6, 12, or 24 hours. Cells were fixed for 20 minutes with 4% parafor-

maldehyde in phosphate-buffered saline, stained with 0.2 μg/mL 4',6'-diamidino-2-phenylindole for 1 hour, and visualized under a fluorescent microscope (Nikon [Tokyo, Japan] inverted microscope using a TE-FM epi-fluorescence accessory). Apoptotic cells were morphologically identified by nuclear shrinkage and chromatin condensation and/or fragmentation.

Cytokinesis-block micronucleus assay

Micronuclei are DNA-containing structures that result from chromosomal loss during mitosis. They represent a subgroup of all chromosomal aberrations. This makes the micronucleus frequency test a widely accepted method for investigating both *in vitro* and *in vivo* genotoxicity in human biomonitoring studies.²⁸ The cytokinesis-block micronucleus technique was performed as previously described^{29,30} with minor modifications. After treatment, cells were incubated with 2 μg/mL cytochalasin B for 24 hours, fixed with chilled methanol/glacial acetic acid (3:1 vol/vol) for 5 minutes, and stained with Giemsa. Each data point represents the mean of eight independent experiments. In each experiment, 1,000 binucleated cells/sample were analyzed.

Comet assay

After different treatments as described above, C6 cells were detached by incubating in the presence of trypsin/EDTA (0.05%). During trypsinization, cells were carefully manipulated to avoid mechanical stress. Comet assay (single-cell gel electrophoresis) was performed as previously described.²⁷ In brief, slides were prepared by mixing 30 μL of C6 cell suspension with 70 μL of low-melting-point agarose (0.75%). Following electrophoresis, slides were incubated with 5 μg/mL ethidium bromide and left in the dark for 20 minutes to stain the DNA. 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. Therefore, the damage index scale could range from 0 (all nuclei without tail, 100 cells×0) to 400 (all nuclei with maximally elongated tails, 100 cells×4). Slides were viewed on a Nikon inverted microscope using a TE-FM epifluorescence accessory, and images were transferred to a computer with a digital camera (Sound Vision Inc., Wayland, MA, USA).

Statistical analysis

To verify the dose- and time course-dependent effect of ECG, we used one-way analysis of variance for repeated measures, followed by a *post hoc* analysis (Tukey's test). ECG effects×different doses of H₂O₂ were analyzed statistically by two-way analysis of variance followed by a *post hoc* analysis (Tukey's test). Data are mean ± SEM values. Values with *P* < .05 were considered to be significant. All analyses were carried out in a PC-compatible computer using the Statistical Package for Social Sciences (SPSS) software (SPSS Inc., Chicago, IL, USA).

TABLE 1. MICRONUCLEUS FREQUENCY IN C6 CELLS AFTER INCUBATION WITH EPICATECHIN GALLATE

Time (hours)	Micronucleus frequency among 1,000 binucleated cells at ECG (μM)			
	0	0.1	1	10
1	1.0 \pm 0.09 ^a	1.3 \pm 0.11 ^b	1.4 \pm 0.12 ^b	1.6 \pm 0.12 ^c
6	1.0 \pm 0.07 ^a	1.3 \pm 0.09 ^b	1.3 \pm 0.11 ^b	1.7 \pm 0.12 ^c
12	1.4 \pm 0.11 ^b	2.0 \pm 0.15 ^d	2.0 \pm 0.19 ^d	2.3 \pm 0.17 ^e
24	1.6 \pm 0.13 ^c	2.3 \pm 0.14 ^c	2.4 \pm 0.17 ^c	2.7 \pm 0.19 ^f

Cells were incubated with epicatechin gallate (ECG) (0.1, 1.0, and 10 μM) for 1, 6, 12, or 24 hours. Data are mean \pm SEM values for micronucleus frequency among 1,000 binucleated cells, from eight independent experiments performed in duplicate. To verify the main effect of time course and different doses of ECG, repeated-measures one-way analysis of variance was used, followed by *post hoc* analysis with Tukey's test.

^{abcdef}Values that do not share a common letter differ significantly at $P < .001$.

RESULTS

Effect of ECG on chromosome loss in C6 cells

T1 ▶ Micronucleus frequency (Table 1) was increased by ECG, compared to their respective control values ($F_{3,48} = 262,381$; $P < .001$). The analysis also indicate a significant interaction between concentration and time ($F_{3,9} = 23,228$; $P < .001$).

Effect of ECG on nuclear morphology and DNA strand breaks in C6 cells

To assess the direct effect of ECG on DNA integrity, cells were incubated in the presence of 0.1, 1, and 10 μM ECG for

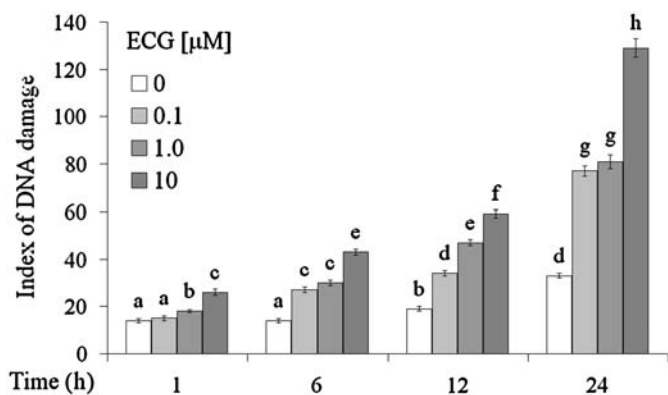


FIG. 1. *In vitro* time- and dose-dependent effects of ECG on DNA damage. C6 astroglial cells were exposed to different concentrations of ECG (0.1, 1, and 10 μM) for different time intervals (1–24 hours). The extent of damage to DNA was determined by the comet assay. The index of DNA damage was calculated as described in Materials and Methods. Data are mean \pm SEM values of 16 experimental determinations performed in duplicates. To verify the dose- and time course-dependent effect of ECG, repeated-measures analysis of variance was performed, followed by *post hoc* analysis with Tukey's test. ^{abcdef}Columns that do not share a common letter differ significantly at $P < .001$.

1, 6, 12, or 24 hours. As shown in Figure 1, ECG induced DNA damage ($F_{3,64} = 1946,747$; $P < .001$). This effect was concentration and time dependent ($F_{3,9} = 336,148$; $P < .001$). Only 2% of cells exposed to 10 μM ECG after 24 hours presented nuclear fragmentation by 4',6'-diamidino-2-phenylindole staining assay (data not shown).

Genoprotective effects of ECG against H₂O₂-induced DNA damage in C6 cells

To investigate the effect of ECG on DNA damage induced by H₂O₂, cells were preincubated with different concentrations of ECG (0.1, 1, and 10 μM) for 1 hour. After this time, 1 mM H₂O₂ was added and maintained for 30 minutes (Fig. 2A). Such an assay was performed at the 1-hour time point taking into account that at this time ECG by itself had minimal influence on DNA integrity (Fig. 1). ECG-induced genoprotective effects after treatment are shown in Figure 2B. The index of DNA damage observed when cells were incubated in the presence of H₂O₂ in the absence of ECG was 42.5 \pm 3.9. In this context, ECG at 0.1 and 1.0 μM (20.0 \pm 1.3 and 22.0 \pm 1.5, respectively) ($F = 47,529$; $P < .001$) was able to significantly prevent the H₂O₂-induced genotoxicity *in vitro*.

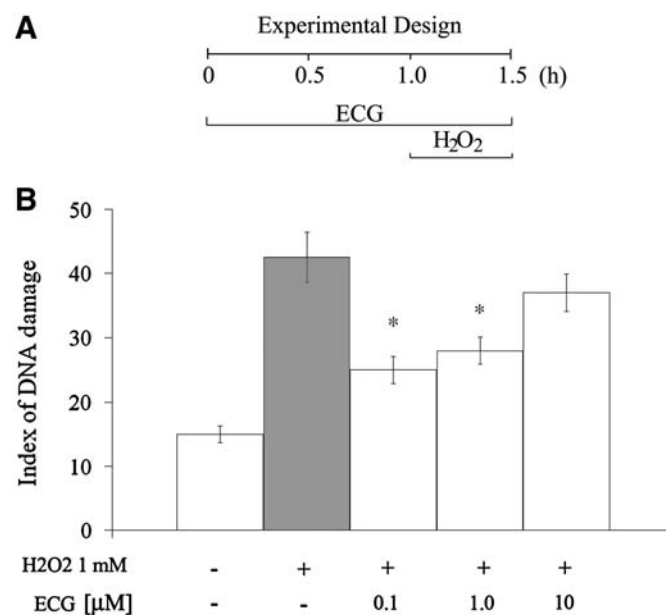


FIG. 2. Protective effect of ECG against H₂O₂-induced DNA damage in C6 astroglial cells. Cells were preincubated for 1 hour in the presence of ECG (0.1, 1, and 10 μM) before exposure to H₂O₂. Culture medium was maintained, and then 0.1 mM H₂O₂ was added. Cells were incubated in the same conditions for 30 minutes longer. The extent of damage to DNA was determined by the comet assay. The index of DNA damage was calculated as described in Materials and Methods. Data are mean \pm SEM values. Differences were statistically analyzed by two-way analysis of variance followed by Tukey's test. *Significant differences from control values ($P < .001$).

DISCUSSION

It is very well documented that several redox-active compounds could have a dualistic effect, either beneficial or toxic, depending on the concentration used.^{25,27,31,32} The ability of polyphenols to scavenge reactive oxygen species depends on their chemical structures.³³ In addition, antioxidant/pro-oxidant activity of different redox-active compounds, such as polyphenols, largely depends on the levels consumed within the diet and may potentially cause DNA damage,³⁴ probably triggered by direct binding of polyphenol to DNA.³⁵

As previously reported by our group,²⁶ 10 μ M ECG induces morphological alterations in the C6 astroglial cell line (process-bearing cells) with a small increase in propidium iodide incorporation after 24 hours of incubation (up to 4%). In the present work, we observed that, at least up to 24 hours, ECG did not induce any indicative alteration in nuclear morphology related to apoptotic process (data not shown). DNA strand breaks in individual cells result from events such as direct scission of the DNA backbone.

In the present *in vitro* experimental model (1 hour in the presence of ECG, before exposure to H₂O₂ for 30 minutes), ECG was not cytotoxic to C6 cells. However, it is important to mention that index values below 30 are obtained from less prominent DNA strand breaks. In such context, the first 6 hours of ECG incubation resulted in mild DNA damage. Thereafter, ECG clearly induced a time- and dose-dependent genotoxicity.

The degree of chromosome loss, analyzed by the micronucleus frequency test (Table 1), reflects the capacity of the cells to resist oxidative stress and repair single-strand breaks.³⁴ As ECG by itself induced DNA damage at higher doses, we decided to investigate if this genotoxic effect could impair DNA repair. Actually, micronuclei frequency values from ECG exposure were significantly higher than control values. The highest dose of ECG induced an increase (about 60%) in all incubation time points (Fig. 1). It indicates that ECG genotoxicity at higher doses affects the repair system, probably via a pro-oxidant effect.³²

In order to investigate possible genoprotective effects of ECG against DNA damage induced by oxidant conditions, we used an experimental procedure previously established in our group.²⁷ In this model, cells were preincubated with antioxidant for 1 hour before H₂O₂-induced insult. H₂O₂ is particularly attractive as an oxidant model because its cellular actions and fate are well understood. It readily crosses the cellular membrane and gives rise to highly reactive hydroxyl radicals, which have the ability to react with different macromolecules, including DNA, proteins, and lipids, and to ultimately damage a cell.³⁶

Following the observation that the 1-hour time point of ECG exposure did not induce genotoxic effects by itself in C6 cells, we decided to keep the same time point for ECG preincubation, just before the H₂O₂ pulse at 30 minutes, which is known to be sufficient and enough to induce genotoxic properties.²⁷ We found that H₂O₂-induced genotoxicity was significantly prevented by 0.1 and 1 μ M ECG,

suggesting that, at these concentrations, ECG plays a beneficial effect probably due to its antioxidant properties. The reaction between ECG and hydroxyl radicals is particularly important for preventing oxidative injury because hydroxyl radicals have been shown to be highly responsible for several pathogenesis related to a wide range of diseases.

Despite the fact that our *in vitro* data demonstrated a significant astroglial genoprotection upon ECG treatment, some limitations should be under consideration regarding the difficulty of correlating *in vitro* experiments with *in vivo* treatments. Plasma ECG concentrations in rats range from 0.2 to 135 nM after 7.3 mg/kg ECG administration via the tail vein.³⁷ The pharmacokinetics of catechins in rat brain fetuses after pregnant ingestion of green tea extract could result in a ECG range from 10 to 80 pmol/g.³⁸ Moreover, under *in vitro* experiments, drug concentrations need to be usually higher than chronic *in vivo* treatments in order to mimic long-term physiological cell activities and their functions.²⁷

CONCLUSIONS

Our results clearly demonstrated that ECG can affect DNA in our cell model, but, most importantly, it is able to protect against H₂O₂-induced DNA damage, showing a statistically significant dose-dependent genoprotective effect. This indicates that small doses of polyphenols included in our daily diet could play genoprotective effects in neural cells and, therefore, could ameliorate oxidative stress-associated brain pathologies. Nevertheless, the dualistic genotoxic effect exerted by high doses of ECG suggests that caution is recommended when large quantities of antioxidants and/or supplements in foods are consumed.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that no competing financial interests exist.

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Capítulo II

*EPIGALLOCATECHIN-3-GALLATE PROTECTS RAT BRAIN MITOCHONDRIA
AGAINST CADMIUM INDUCED DAMAGE*

Manuscrito aceito para publicação pela Food and Chemical Toxicology

Renata Torres Abib, Kaite Cristiane Peres, Anderson Barbosa, Tanara Peres, Angela Bernardes, Lizandra Zimmermann, André Quincozes-Santos, Haidi Fiedler, Rodrigo Bainy Leal, Marcelo Farina e Carmem Gottfried

**EPIGALLOCATECHIN-3-GALLATE PROTECTS RAT BRAIN
MITOCHONDRIA AGAINST CADMIUM-INDUCED DAMAGE**

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Highlights

This study mainly focuses on Epigallocatechin 3-gallate against Cd^{2+} -induced mitochondrial toxicity

Co-incubation of EGCG with Cd^{2+} prevented the Cd^{2+} -induced mitochondrial dysfunction

EGCG completely prevented mitochondrial lipid peroxidation induced by Cd^{2+}

Spectroscopic studies have shown EGCG able to form a chemical complex with Cd^{2+} , in an equimolar ratio

EPIGALLOCATECHIN-3-GALLATE PROTECTS RAT BRAIN

MITOCHONDRIA AGAINST CADMIUM-INDUCED DAMAGE

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RUNNING HEAD: Mitochondrial Effect of Epigallocatechin gallate.

WORD COUNTS: 5.570

FIGURES: 06

TABLES: 01

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ABSTRACT

1
2 Many health claims have been made about the medicinal benefits of drinking green tea,
3
4 including neuroprotection. This study mainly focuses on Epigallocatechin 3-gallate
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6 (EGCG), a potent antioxidant, which is abundantly found in green tea. Cadmium [Cd²⁺]
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8 is a toxic pollutant that leads to neurotoxicity in both animals and humans. Although the
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10 entrance of Cd²⁺ in the adult central nervous system is limited, developmental
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12 neurotoxicity has been evidenced as result of the blood-brain barrier (BBB) immaturity.
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14 Moreover, high Cd²⁺ levels are known to impair BBB function. Furthermore, the
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16 molecular mechanisms related to its neurotoxic properties remain unknown. This study
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18 evaluates the potential protective effect of the major green tea polyphenol, EGCG,
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20 against Cd²⁺-induced mitotoxicity under *in vitro* conditions, using mitochondrial-
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22 enriched fractions from rat brain. Co-incubation of EGCG with Cd²⁺ prevented the
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24 Cd²⁺-induced mitochondrial dysfunction (capacity to reduce MTT to formazan). In
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26 addition, EGCG completely prevented mitochondrial lipid peroxidation induced by Cd²⁺
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28 but did not affect non protein thiols levels. Spectroscopic studies have shown EGCG
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30 able to form a chemical complex with Cd²⁺, in an equimolar ratio. In this study we
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32 demonstrate EGCG effectiveness in protecting against Cd²⁺-induced mitochondrial
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34 dysfunction and lipid peroxidation probably due to its antioxidant and chelating effects.
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43 **Key Words:** Epigallocatechin-gallate, Cadmium ion, neurotoxicity, mitochondrial
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1. INTRODUCTION

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2 Many health claims have been made about the medicinal benefits of drinking green tea,
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4 including neuroprotection (Weinreb et al., 2009). This study mainly focuses on
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6 Epigallocatechin 3-gallate (EGCG), a potent antioxidant, which is abundantly found in
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8 green tea.
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11 The chemical structures of catechins contribute to their antioxidant properties. Some
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13 catechins, such as EGCG, have a gallate moiety esterified at the 3rd position of the C
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15 ring, the catechol group on the B ring and the hydroxyl groups at the 5th and 7th
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17 positions on the A ring (Figure1). The potent free radical scavenging activity of EGCG
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19 was attributed to the presence of the gallate group (Devika and Stanely Mainzen Prince,
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21 2008).
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25 It is important to mention that polyphenol compounds (including green tea catechins)
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27 have metal chelating properties (Hider et al., 2001, Fernandez et al., 2002), which have
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29 been attributed mainly to their gallate group (Kumamoto et al., 2001).
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33 Cadmium (Cd^{2+}), a highly toxic heavy metal, is mainly released from the burning of
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35 fossil fuels and municipal wastes, refining of metals and consumption of tobacco
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37 product, resulting in the pollution of water, air, and soil (Chen et al., 2008). Once it is
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39 widely distributed and extensively used in modern society, human exposure to this and
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41 other toxic metals is unavoidable (Pourahmad et al., 2003).
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46 Furthermore, natural systems are complex and many factors influence the
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48 bioavailability of Cd^{2+} . Changes in pH, temperature, nature and concentration of ligands
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50 and complex ions, effectively regulate the bioavailability of the metal ions in water, air
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52 and soils (Westrup et al., Zimmermann et al., Fiedler et al., 2004, Fritzen et al., 2006,
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54 Saenz et al., 2010);
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1 In biological systems, Cd²⁺ is able to stimulate the production of reactive oxygen
2 species (ROS) and reactive nitrogen species, which are responsible, at least in part, for
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4 its toxicity (Stohs et al., 2001).
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6 Cd²⁺ was shown to induce its toxic effects under *in vitro* conditions, on developing
7 cortical cells and on immature hippocampal slices (Ohtani-Kaneko et al., 2008, Rigon et
8 al., 2008), and *in vivo* on developing brain (Rai et al., 2010, Amara et al., 2011).
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13 Although the Cd²⁺ entrance into the adult central nervous system (CNS) is limited,
14 developmental neurotoxicity seems to occur, at least in part, as a result of blood-brain
15 barrier (BBB) immaturity (Choudhuri et al., 1996, Rai et al., 2010, Amara et al., 2011).
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19 Moreover, high Cd²⁺ levels are known to impair BBB function (Bar-Sela et al., 2001).
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22 Although Cd²⁺ leads to oxidative stress in the CNS (Sinha et al., 2008, Goncalves et al.,
23 2010) specially during early development (Rai et al., 2010), the complete understanding
24 about molecular mechanisms related to Cd²⁺-induced neurotoxicity remains unknown.
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29 Apoptosis is a dynamic multistep mechanism of cell death controlled by mitochondria
30 (Bayir and Kagan, 2008) - major site of ROS generation - and is related to the
31 cytotoxicity induced by metals (Pulido and Parrish, 2003, Belyaeva et al., 2008).
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36 CNS is especially susceptible to oxidative stress due to its high oxygen turnover, as well
37 as of its high amount of polyunsaturated fatty acids (Floyd and Hensley, 2002).
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41 Therapeutic use of antioxidants from diet appears to prevent a range of aging effects as
42 well as neurodegenerative diseases (Mandel and Youdim, 2004). Several studies have
43 suggested that antioxidant compounds, such as polyphenols, help in the treatment of
44 neurodegenerative diseases (Rajeswari and Sabesan, 2008, Ritz et al., 2008, Wang et al.,
45 2009). However, the beneficial effects of such compounds toward metal-induced
46 neurotoxicity are scarce.
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1 Considering (i) mitochondrial ROS generation an important event related to
2 neurodegenerative/neuropathological conditions, (ii) Cd²⁺-induced neurotoxicity is
3 related to oxidative stress generation (Chen et al., 2011) and (iii) polyphenol
4 compounds have presented significant beneficial effects in pathological conditions
5 related to oxidative stress, the aim of the present study was to investigate the effect of
6 EGCG against Cd²⁺-induced mitochondrial damage, using mitochondrial-enriched
7 fractions from rat brain. Biochemical parameters related to the mitochondrial function,
8 as well as oxidative stress related parameters, were evaluated. Moreover, the potential
9 chelating effects of EGCG toward Cd²⁺ were examined in order to elucidate potential
10 mechanisms of mitotoxicity.
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26 **2. MATERIAL AND METHODS**

27 **2.1. Material**

28 Cadmium chloride and AgNO₃ were purchase from Merk. EGCG, 2-thiobarbituric acid,
29 55'-dithiobis-(2-nitrobenzoic-acid) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl
30 tetrazolium bromide (MTT) were purchased from Sigma (St. Louis MO, USA). All
31 other chemicals were purchased from common commercial suppliers.
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41 **2.2 Animals**

42 Male 14-day-old Wistar rats were collected from the Federal University of Santa
43 Catarina (UFSC) breeding colony. Rats were maintained in an air-conditioned room
44 (22-25°C) on a 12 h light/dark cycle with water and food available *ad libitum*. They
45 were treated, manipulated and killed according to the “Principles of laboratory animal
46 care.” (NIH publication no 80-23, revised 1996) and approved by the local Ethical
47 Committee for Animal Research (CEUA/UFSC PP00345).
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58 **2.3 Preparation of mitochondrial Enriched Fractions**

1 Mitochondrial-enriched fractions were prepared as described previously (Whittaker,
2 1962), with minor modifications (Franco et al., 2007). Briefly, 14-day-old male Wistar
3 rats were killed by decapitation. The whole brains were removed and homogenized on
4 ice in 10 volumes of isolation medium (10 mM HEPES buffer pH 7.0 containing 220
5 mM mannitol, 68mM sucrose, 10 mM KCl and 0.1% serum albumin), and the
6 homogenate was centrifuged at 4°C for 10 min at 1000 x g. The supernatant was then
7 centrifuged at 11500 x g for 10 min at 4°C, resulting in a myelin-rich supernatant and a
8 pellet (P2) consisting of synaptosomes and free mitochondria. The supernatant was
9 discarded, and the pellet was resuspended in the isolation medium but without albumin.
10 The mitochondrial-enriched fractions were kept on ice for 10-15 min until the
11 experiments were performed. N ranges from 5 to 8 independent fractions as indicated in
12 the figure legends and each independent fraction was obtained from 4 brains
13 homogenates.
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31 **2.4 Incubations**

32 P2 (approximately 4 mg of protein) was incubated with different cadmium chloride
33 concentrations (100, 200 or 300 µM) and/or different EGCG concentrations (10, 50 or
34 100 µM) in a medium containing 10 mM HEPES buffer (pH 7.0), 220 mM mannitol, 68
35 mM sucrose and 10 mM KCl (total incubation volume = 300 µL). Incubations were
36 carried out for 2 h at 25°C. Epigallocatechin-3-gallate was dissolved in NaCl 0.9%.
37 After incubations, mitochondrial function, thiobarbituric acid reactive substances
38 (TBARS) or GSH content were determined. All assays were performed in triplicates.
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53 **2.5 Thiobarbituric acid reactive substances (TBARS) assay**

54 The thiobarbituric acid (TBA) assay was used to assess lipid peroxidation as previously
55 described (Ohkawa et al., 1979). Our main aim for using this assay was to evaluate the
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1 efficacy of EGCG to protect mitochondrial membranes against Cd²⁺-induced lipid
2 impairment. Briefly, samples were incubated in a reaction media containing 0.28 % 2-
3 thiobarbituric acid, 1.2 % SDS, and 0.45 M/0.12 M acetic acid/HCl buffer (pH 3.4).
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5 After incubation at 95°C for 60 min, TBARS were measured at 532 nm and compared
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7 to a standard curve of malondialdehyde.
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10 11 **2.6 Assessment of mitochondrial function**

12 Mitochondrial function was assessed using MTT reduction assay. MTT reduction was
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14 chosen as an indicator of primary events of concerning mitochondrial dysfunction, as
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16 inhibition of the dehydrogenases can lead to metabolic impairment, decreasing ATP
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18 levels and increasing ROS generation. Briefly, after the preincubation of P2 with Cd²⁺
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20 and/or EGCG (2 h at 25°C), the reaction medium (300 µL) was incubated with 300 µL
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22 of 1.2 mM MTT for 30 min at 25°C. The purple formazan crystals were pelleted by
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24 centrifugation, and the supernatant was discarded. The pellets were dissolved in DMSO
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26 and the formazan was quantified spectrophotometrically at 550 nm. Data were
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28 expressed as a percentage of control.
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36 **2.7 Assessment of glutathione content**

37 Glutathione content was measured as nonprotein thiols (NPSH) according to a method
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39 previously described (Ellman, 1959), with minor modifications. Briefly, after the
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41 preincubation of P2 with Cd²⁺ and/or EGCG (2 h at 25°C), 300 µL of trichloroacetic
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43 acid 10% was added to the reaction medium (300 µL). After centrifugation (4,000 x g at
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45 4°C for 10 min), the protein pellet was discarded and free thiol groups were determined
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47 in the clear supernatant (which was neutralized with 0.1 M NaOH).
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51 **2.8 Interaction of Cd²⁺ and EGCG**

52 A standard solution containing 0.0980 ± 0.0013 mol/L Cd²⁺ was prepared by dissolving
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54 CdCl₂ H₂O in 100 mL of water, and the concentration of Cd²⁺ determined by volumetric
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1 titration with AgNO₃. The ligand was EGCG and a standard solution 1.0 x 10⁻³ mol/L
2 was prepared in an aqueous solution containing NaCl 0.9% m/v. Calibration solutions
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4 were prepared daily by appropriate dilution of the metal stock solutions and all
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6 analytical procedures were carried out in a laboratory with the TROX® (class 100) air
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8 filtrating system provides control of particles: 0.3 to 0.5 µm/min. All other inorganic
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10 reagents were the best available analytical reagent grade.
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14 UV-visible spectrophotometric measurements were carried out at 24.0 ± 1.0 °C on a
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16 FEMTO 800 XI spectrophotometer equipped with deuterium and tungsten lamps and 1
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18 cm quartz cell, calibrated with NIST traceable UV/Vis reference materials. Doubly
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20 deionized water with less than 5.6 10⁻⁸ Ω⁻¹ cm⁻¹ conductivity and pH between 6.4 and
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22 6.8 from a NANO pure analytical deionization system (type D-4744) was used to
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24 prepare solutions of standards and reagents.
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28 The pH was measured with a Metrohm model 713 pH meter. The glass electrode was
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30 calibrated with standard buffers, in a thermostatic stirred vessel, at a temperature of 24±
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32 0.1 °C.
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36 Spectrophotometric measurements were carried out at 24.0 ± 1.0°C and the spectra were
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38 recorded in the region of 200 to 350 nm. Initially, solutions of catechin 50 µM in pH
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40 values 7.6, 8.3 and 8.6 were prepared and aliquots of 2 mL of these solutions were
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42 transferred into a quartz cuvette and sealed with a septum rubber. Then, aliquots of Cd²⁺
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44 (2 mM) were added. After each addition, the spectra were registered and the absorbance
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46 values at wavelengths of 272 and 318 nm.
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50 The absorbance versus [Cd²⁺] was modeled in terms of a complex formation reaction
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52 with a 1:1 stoichiometry of catechin to Cd²⁺. The binding constant for complex
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54 formation was calculated by the means of the non-linear regression analysis of the data
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56 using equation 1.
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$$Abs = [(Abs_{ini} + Abs_{fin} \cdot K \cdot [Cd^{2+}]) / (1 + K \cdot [Cd^{2+}])] \quad (1)$$

The terms *Abs* represent the experimental absorbance values for each addition of Cd^{2+} ; Abs_{ini} is the initial value of absorbance without Cd^{2+} ; Abs_{fin} is the final absorbance value obtained; the $[Cd^{2+}]$ are expressed in μM , and K (in units $mol^{-1}L$) corresponds to the binding constant for a 1:1 complex formation (Fiedler et al., 2004).

2.9 Statistical analysis

Data are presented as mean \pm S.E.M. Statistical significance was assessed by ANOVA followed by Tukey's test, when appropriate. A value of $p < 0.05$ was considered to be significant.

3. RESULTS

EGCG *per se* did not alter viability and NPSH levels in the mitochondrial-enriched fractions of rat brain (data not shown). Cd^{2+} caused a significant [$F_{(3,10)} = 69.97$; $P < 0.0001$] and dose-dependent (Pearson $r = -0.9477$; $P < 0.0001$) decrease in mitochondrial function (Figure 2). In addition, Cd^{2+} induced a slight but significant [$F_{(3,22)} = 5.73$; $P = 0.0047$] oxidation of the mitochondrial GSH levels (Figure 2).

The potential protective effects of EGCG against Cd^{2+} -induced mitochondrial dysfunction are depicted in Figure 3. EGCG completely prevented the loss of mitochondrial function induced by Cd^{2+} [$F_{(3,9)} = 26.63$; $P < 0.0001$] (Figure 3A). Cd^{2+} 200 μM caused a significant increase in lipoperoxidation, that was completely prevented by EGCG 100 μM [$F_{(3,12)} = 28.08$; $P < 0.0001$] (Figure 3 C). Cd^{2+} caused a slight but significant decrease in GSH levels [$F_{(3,29)} = 4.62$; $P = 0.0092$] and EGCG did not prevent this event (Figure 3 B).

Figure 4 shows the changes in the UV-vis spectra for the Cd^{2+} and EGCG complex formation interaction at pH 8.3. Addition of Cd^{2+} causes a decrease on the absorbance at 318 nm with a subtle increase at 272 nm, suggesting formation of a complex between

1 Cd²⁺ and EGCG. The isosbestic point at 288 nm is a clear indication of a reversible
2 chemical equilibrium between the metal ion and the organic gallate.
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4 The insert in Figure 4 shows the absorbance as a function of added Cd²⁺ at 318 nm. The
5 data were calculated with equation 1 - consistent with a 1:1 stoichiometry between
6 catechin and Cd²⁺ - and resulted in an association constant $K = (3.5 \pm 0.3) \times 10^4 \text{ L mol}^{-1}$
7 ($r^2 = 0.994$). This result shows the formation of a cadmium-EGCG complex. The binding
8 constant for the complex formation were calculated by means of nonlinear regression analysis
9 of the absorbance changes observed upon addition of Cd²⁺.
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19 In addition, it was demonstrated that the affinity (in terms of changes in the binding constant) is
20 pH-dependent (Figure 5) indicate that the effect becomes significantly greater at higher
21 pH values. This increase in the association of EGCG with the metal ion is greater for
22 values above the pKa due to the presence of negatively charged phenolate groups.
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28 The binding constants obtained for different pH values are presented in table 1. The
29 results are fully consistent with the proposed 1:1 gallate:Cd²⁺ complex formation and
30 suggest that gallate ionization favors complex formation.
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36 **4. DISCUSSION**

37 Polyphenols, such as catechins, have shown neuroprotective effects in several
38 neuropathological conditions related to excitotoxicity and oxidative stress (Weinreb et
39 al., 2009). Previous studies from our group reported significant beneficial effects of
40 epicatechin-gallate against glutamate excitotoxicity (Abib et al., 2008) and DNA
41 damage (Abib et al., 2010).
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50 Only a few studies have been focused on beneficial effects of polyphenols against
51 metal-induced neurotoxicity (Mandel et al., 2004, Sethi et al., 2009) and there are not
52 any data or research development on the topic of the protective effects of EGCG against
53 Cd²⁺-induced mitotoxicity.
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1 Heavy metal-induced toxicity is a well known phenomenon, but effective treatments
2 remain unavailable. Given that the risk of human exposures to Cd²⁺ is constantly
3 increasing due to its widespread distribution in the environment and the lack of
4 biodegradable processes (Wright and Baccarelli, 2007), effective therapeutic for Cd²⁺
5 exposure/poisoning is required. Although there is no consensus on the effectiveness of
6 chelating therapy for Cd²⁺ toxicity, a significant amount of data in both animals
7 (Saljooghi and Fatemi, 2010, Jamilaldin Fatemi et al., 2011) and humans (Kelley, 1999,
8 Gil et al., 2011) have shown that Cd²⁺ chelation may increase the excretion of this
9 metal, reducing its toxic consequences. Interestingly, the co-administration of chelating
10 agents and antioxidants have been associated with better effects compared to
11 administration of each molecule individually (Gil et al., 2011).

12 We demonstrate a complex formation between EGCG and Cd²⁺ based on changes in the
13 UV-Vis spectrum. This result is different from that suggested by Yu and colleagues (Yu
14 et al., 2007), who have not found differences in the presence of EGCG and Cd²⁺ by this
15 method. This inconsistency may be justified by the different experimental conditions.

16 We used a four times lower EGCG concentration than the cited work, a condition that
17 can improve the visualization of complex formation. In addition, they used a lower pH
18 than us, which as demonstrated, can also influence the complex formation.

19 As previously discussed above, the chelating effect of EGCG observed in this study is
20 restricted to cadmium in mitochondria-enriched fractions and highlights the chelating
21 property of EGCG, featuring a new mechanism of action of this polyphenol in addition
22 to its classic antioxidant effect. On this topic, it is noteworthy that EGCG has
23 antioxidant and chelating properties therefore has great potential to protect against Cd²⁺-
24 induced toxicity under *in vivo* conditions, like intact brain cells model, that are quite

1 different from mitochondrial-enriched fractions model. However, additional studies are
2 necessary to confirm this hypothesis.
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4 Although there are limitations to extrapolate *in vitro* results to *in vivo* effects, since no
5 chelating agent should be still used in human Cd²⁺ poisonings (Andujar et al., 2010),
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7 our results suggest EGCG as a promising molecule for *in vivo* studies on Cd²⁺-induced
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9 neurotoxicity during development.
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14 Evidence indicates that the Cd²⁺-induced toxicity is related to oxidative stress
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16 (Goncalves et al., 2010) and leads to increased lipid peroxidation and DNA damage
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18 (Viaene et al., 1999, Jomova et al., 2010). It has been reported that this metal can cause
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20 dysfunction in mitochondria isolated from rat liver (Li et al., 2003), probably due to the
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22 production of H₂O₂ (Koizumi et al., 1996, Pourahmad et al., 2003) and also by
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24 inhibition of the respiratory chain (Wang et al., 2004) This ROS formation was
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26 prevented by uncouplers of the respiratory chain, suggesting that mitochondria is a
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28 critical organelle involved in this toxicity (Pourahmad et al., 2003).
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34 Furthermore, Cd²⁺ interacts with sulfhydryl (-SH) groups from proteins (altering the
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36 function of many thiol-containing proteins) and nonprotein molecules, such as GSH,
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38 harming the redox and antioxidant status (Leverrier et al., 2007, Thevenod, 2009).
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41 Besides, exposure to Cd²⁺ decrease the activity of ROS metabolizing enzymes, like
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43 catalase, glutathione reductase, glutathione peroxidase, glutathione-S-transferase and
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45 superoxide dismutase, that could lead to DNA damage, generation of oxidatively
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47 modified proteins (Thevenod, 2009) and lipoperoxidation (Mendez-Armenta et al.,
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49 2003, Lopez et al., 2006).
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53 Based on the antioxidant effects of EGCG, it is reasonable to justify, at least partially,
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55 the protective effects observed in our study. Indeed, the increase in mitochondrial lipid
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1 peroxidation induced by Cd²⁺ was completely blunted by EGCG co-exposure, which
2 likely influences the beneficial effects on mitochondrial function (MTT assay).
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4 It is well described that direct oxidative damage can affect the activity of
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6 dehydrogenases, proving the MTT assay as a useful methodology to evaluate
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8 mitochondrial function and Cd-induced mitotoxicity (Franco et al., 2007). On the other
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10 hand, it is reasonable to assume that Cd²⁺ interacts with sulfhydryl groups from
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12 mitochondrial proteins, such as succinate dehydrogenase (measured in the MTT assay),
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14 leading to mitochondrial dysfunction, increased ROS formation and lipid peroxidation.
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16 In addition, a tricky but interesting phenomenon observed in our study was the very
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18 small decrease of GSH levels after Cd²⁺ exposure, which was not affected by EGCG co-
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20 exposure. Such event indicates that Cd²⁺-induced mitochondrial dysfunction and lipid
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22 peroxidation do not necessarily represent a consequence of the direct interaction
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24 between Cd²⁺ and GSH. On the same note, both phenomena - mitochondrial
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26 dysfunction and lipid peroxidation - were prevented by EGCG.
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28 Catechins could undergo redox reactions and complexation with metal ions, changing
29
30 their bioavailability to interact with proteins and nonprotein biomolecules (Zhang et al.,
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32 2008). Corroborating these evidences, our results showed that EGCG was able to form a
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34 chemical complex with Cd²⁺ in an equimolar ratio. Thus, the protective effects of
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36 EGCG against Cd²⁺-induced mitochondrial dysfunction and lipoperoxidation might
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38 represent the consequence of at least two events: (i) the capacity of EGCG in
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40 scavenging ROS generated in the Cd²⁺-exposed mitochondria and (ii) their ability to
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42 chelate Cd²⁺ (scheme 1).
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53 It is reasonable to assume that daily consumption of small doses of polyphenols derived
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55 from green tea could be an interesting strategy to prevent the deleterious effects of
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1 heavy metals (particularly Cd²⁺) towards the CNS. However, additional studies are
2 necessary to confirm this idea.
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4 Some important issues concerning our experimental protocol must be mentioned:
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6
7 Firstly, because there is no information in the literature regarded to the EGCG and Cd²⁺
8 effect on mitochondrial enriched fraction, it was necessary to perform concentration
9 curves based on other studies that used cell culture models (Zhao et al., 2000, Jiao et al.,
10 2007, Crispo et al., 2010). Additionally, polyphenol concentrations used in *in vitro*
11 protocols (Crispo et al., 2010, Quincozes-Santos et al., 2007) are generally higher than
12 the levels found in plasma of animals and humans after ingestion of green tea due to the
13 short period of incubations characteristic of acute models. EGCG levels found in plasma
14 after 3 h intravenous administration of 100 mg/kg of tea polyphenols in rats are
15 approximately 2 μM (Fu et al., 2008).
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19 Concluding, this study highlights the protective effects of EGCG against Cd²⁺-induced
20 toxicity (metabolic dysfunction and lipoperoxidation) in rat brain mitochondria,
21 suggesting that this protection is related to the antioxidant and chelating properties of
22 the catechin. Although our *in vitro* results cannot be extrapolated to *in vivo* conditions,
23 they render EGCG a promising molecule for further pharmacological studies related to
24 metal-induced mitotoxicity at both peripheral and central levels.
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LEGENDS OF FIGURES

Figure 1. Chemical Structure of EGCG.

Figure 2. Effects of Cadmium on the mitochondrial function and glutathione levels in rat brain mitochondria. Glutathione levels were measured as nonprotein thiols

(NPSH). The mitochondrial function and NPSH are expressed as percent of control and represented as mean \pm standard error of mean. a) $p < 0.001$ and b) $p < 0.01$ when compared to the control group; c) $p < 0.05$ when compared to Cd 100 μM ; d) $p < 0.05$ when compared to Cd 200 μM by one-way analysis of variance (ANOVA), followed by Tukey's test. Experiments were performed in triplicate ($n=8$). Glutathione content in control condition was 1.0 ± 0.021 (mean \pm S.E.M) nmol/mg protein.

Figure 3. Effects of Cadmium and/or EGCG on mitochondrial function, glutathione levels and TBARS in rat brain mitochondria. Glutathione levels were measured as nonprotein thiols (NPSH). Data are expressed as percent of control and represented as mean \pm standard error of mean. a) $p < 0.05$, b) $p < 0.01$ and c) $p < 0.001$ when compared to the control group; d) $p < 0.05$ when compared to Cd 200 μM by one-way analysis of variance (ANOVA) followed by Tukey's test. Glutathione content in control condition was 1.0 ± 0.021 (mean \pm S.E.M) nmol/mg protein. Experiments were performed in triplicate ($n=5$). TBARS levels in control condition were 7.8 ± 0.14 (mean \pm S.E.M) nmol MDA/mg of protein.

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Figure 4. Uv-Vis spectra under the addition of increasing amounts of Cd²⁺ on EGCG (5.0×10⁻⁵ mol L⁻¹) in aqueous solution (pH = 8.3). The insert displays the corresponding absorbance variation at 318 nm as a function of the Cd²⁺ concentration.

Figure 5. Uv-Vis spectra under the addition of increasing amounts of Cd²⁺ on EGCG (5.0 ×10⁻⁵ mol L⁻¹) in aqueous solution (pH = 7.6). The final concentration of Cd²⁺ was 2.9×10⁻⁴ mol L⁻¹. The insert displays the effect on pH=8.6 and the final concentration of Cd²⁺ was 0.4 μM.

Binding constants of EGCG with Cd²⁺ in different pH values.

Conditions	Binding constant (1:1) $K / L \text{ mol}^{-1}$	r^2
pH 7.6	$(1.0 \pm 0.7) \times 10^3$	0.981
pH 8.3	$(3.5 \pm 0.3) \times 10^4$	0.994
pH 8.6	$(3.8 \pm 0.6) \times 10^4$	0.970

Binding constants were close to 8.6 and 8.3 with a significant difference in magnitude at pH 7.6. However, the best fit was provided at pH 8.3, according to information obtained by the correlation coefficient.

Figure 1
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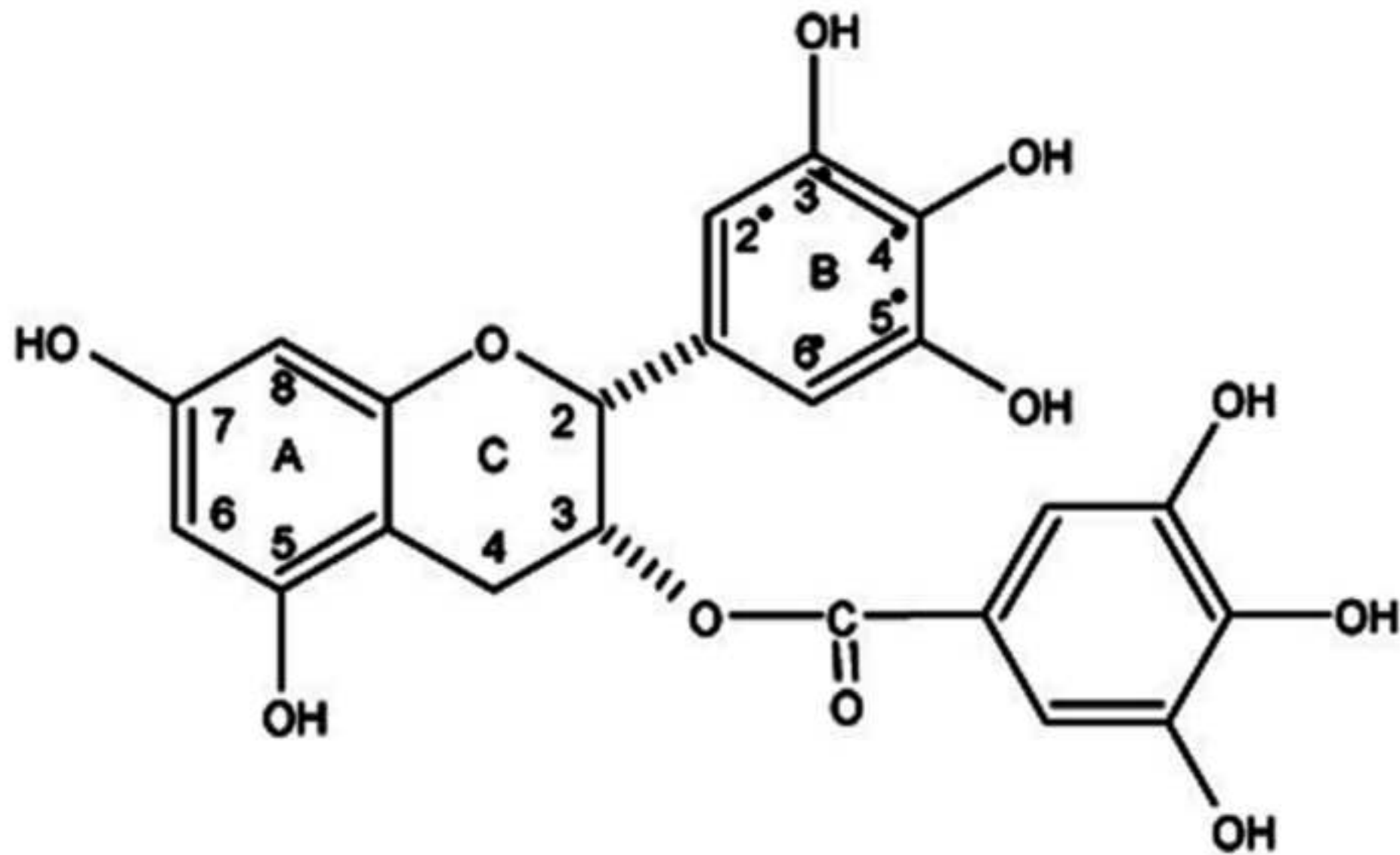


Figure 2
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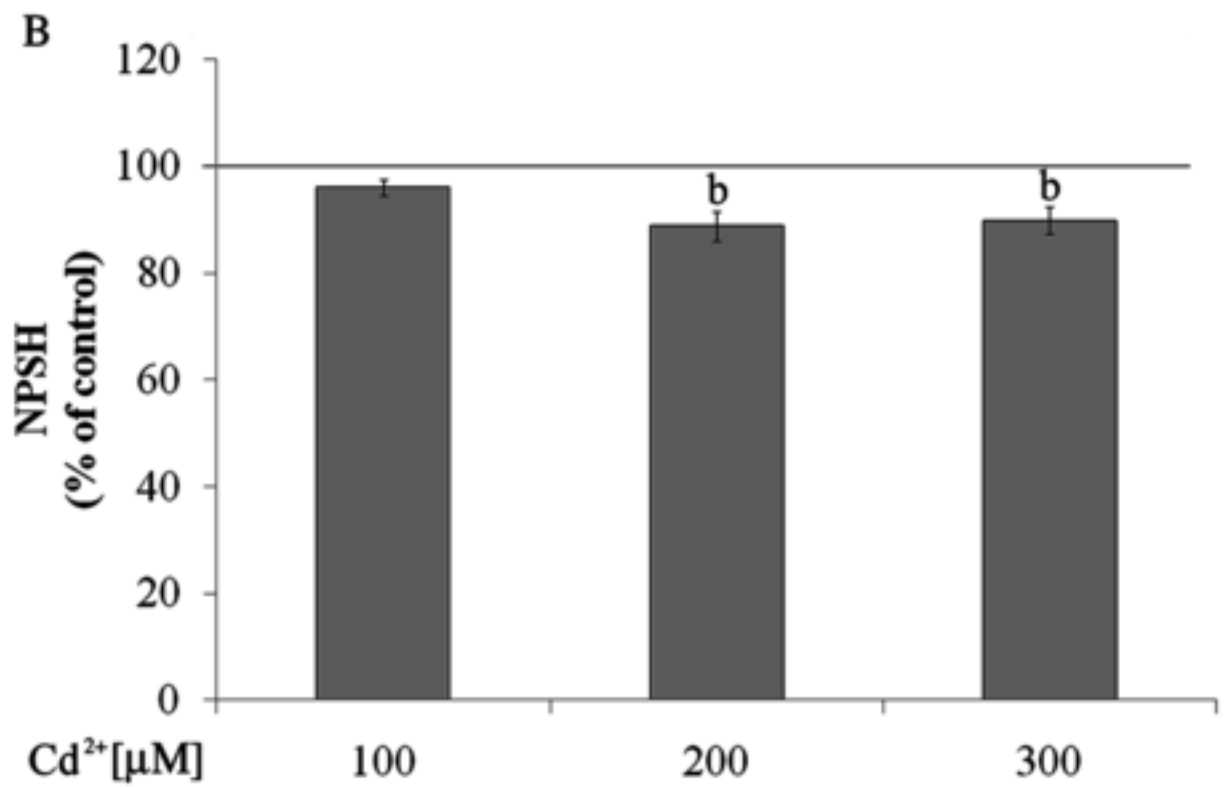
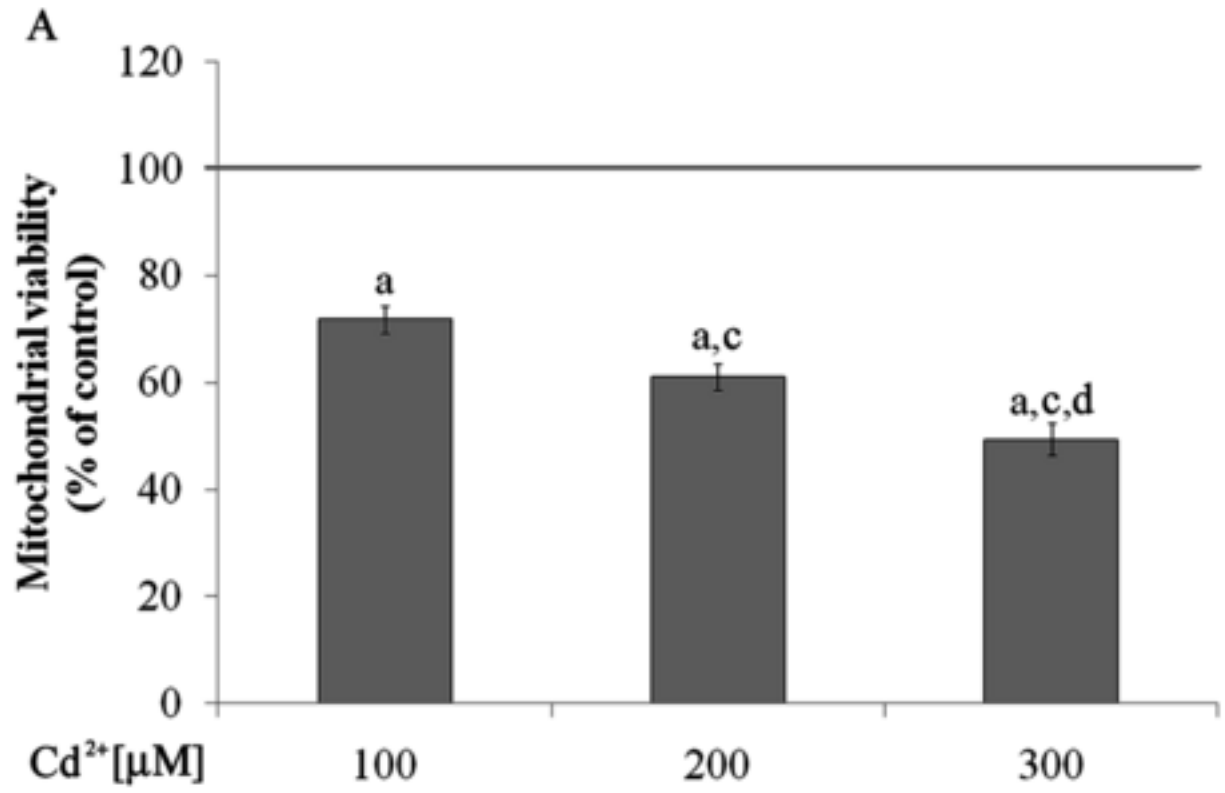


Figure 3
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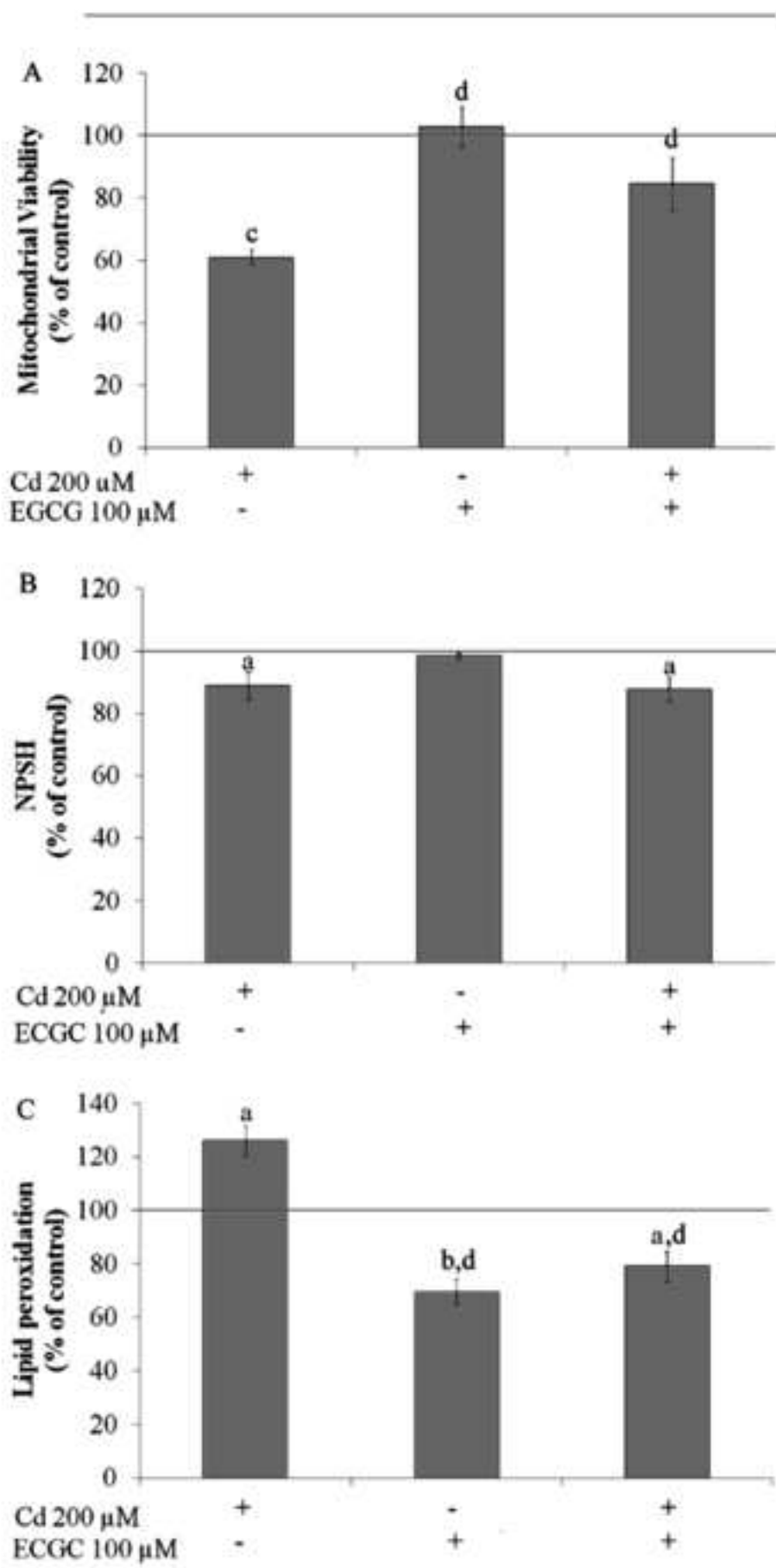


Figure 4
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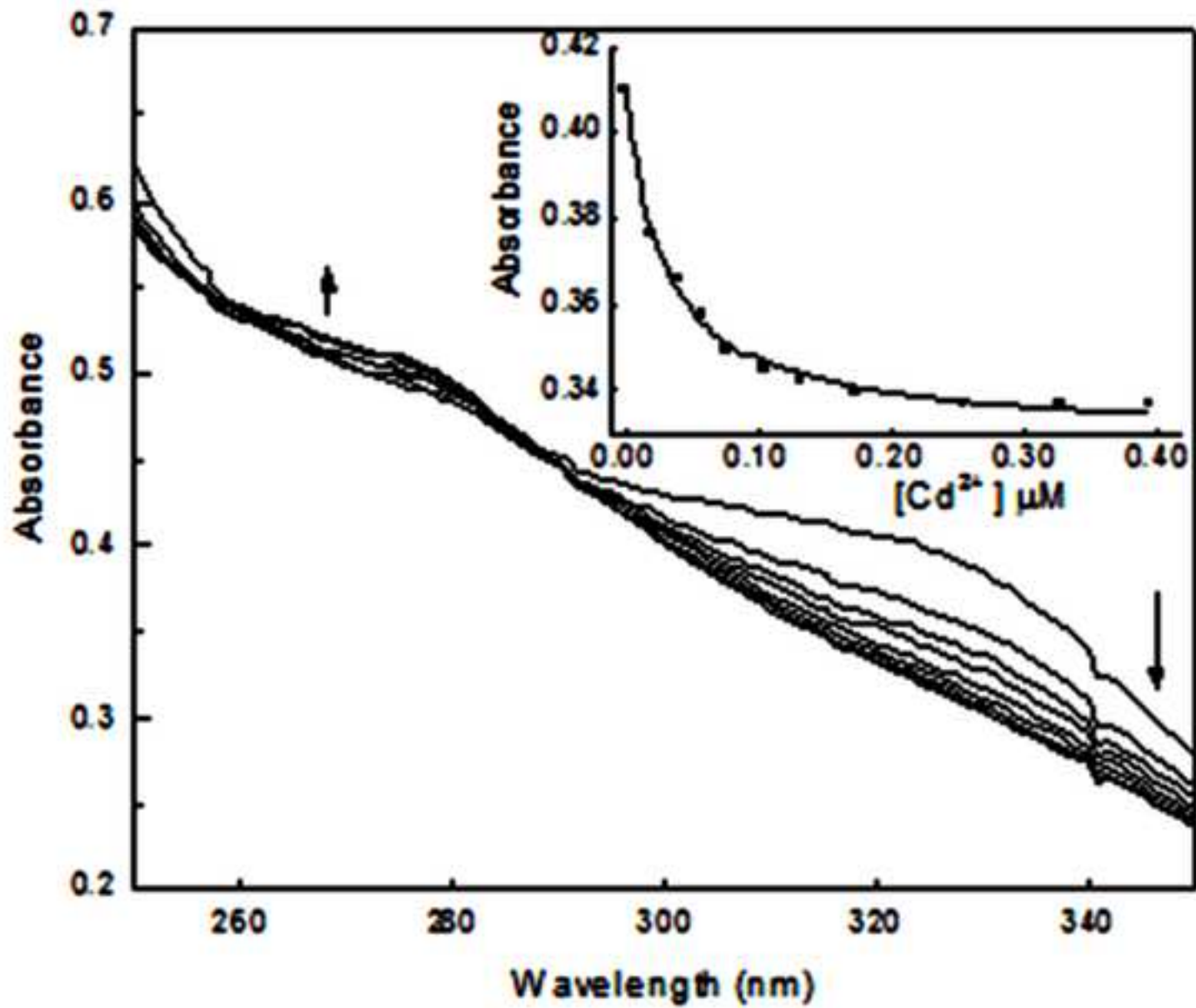


Figure 5
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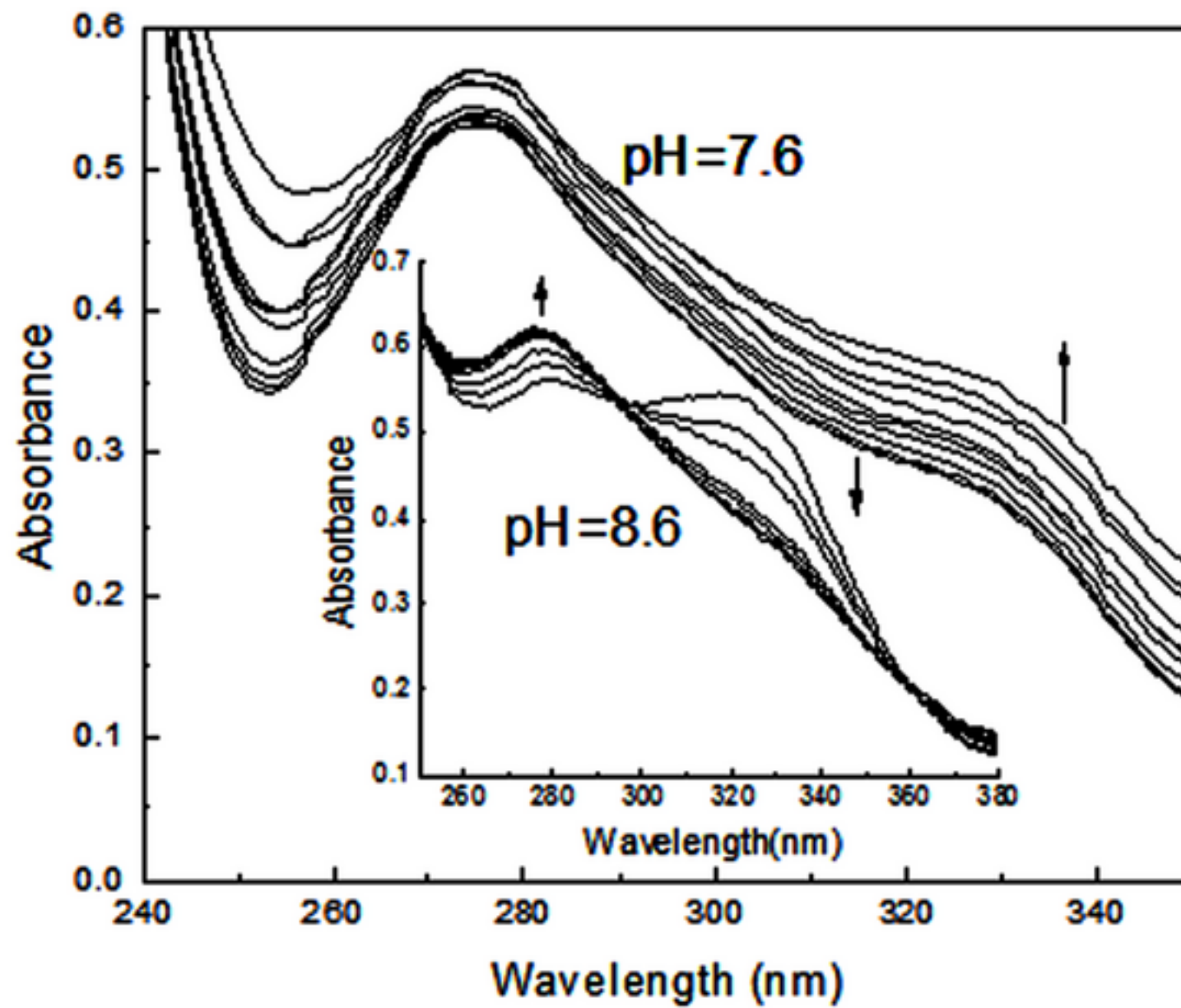
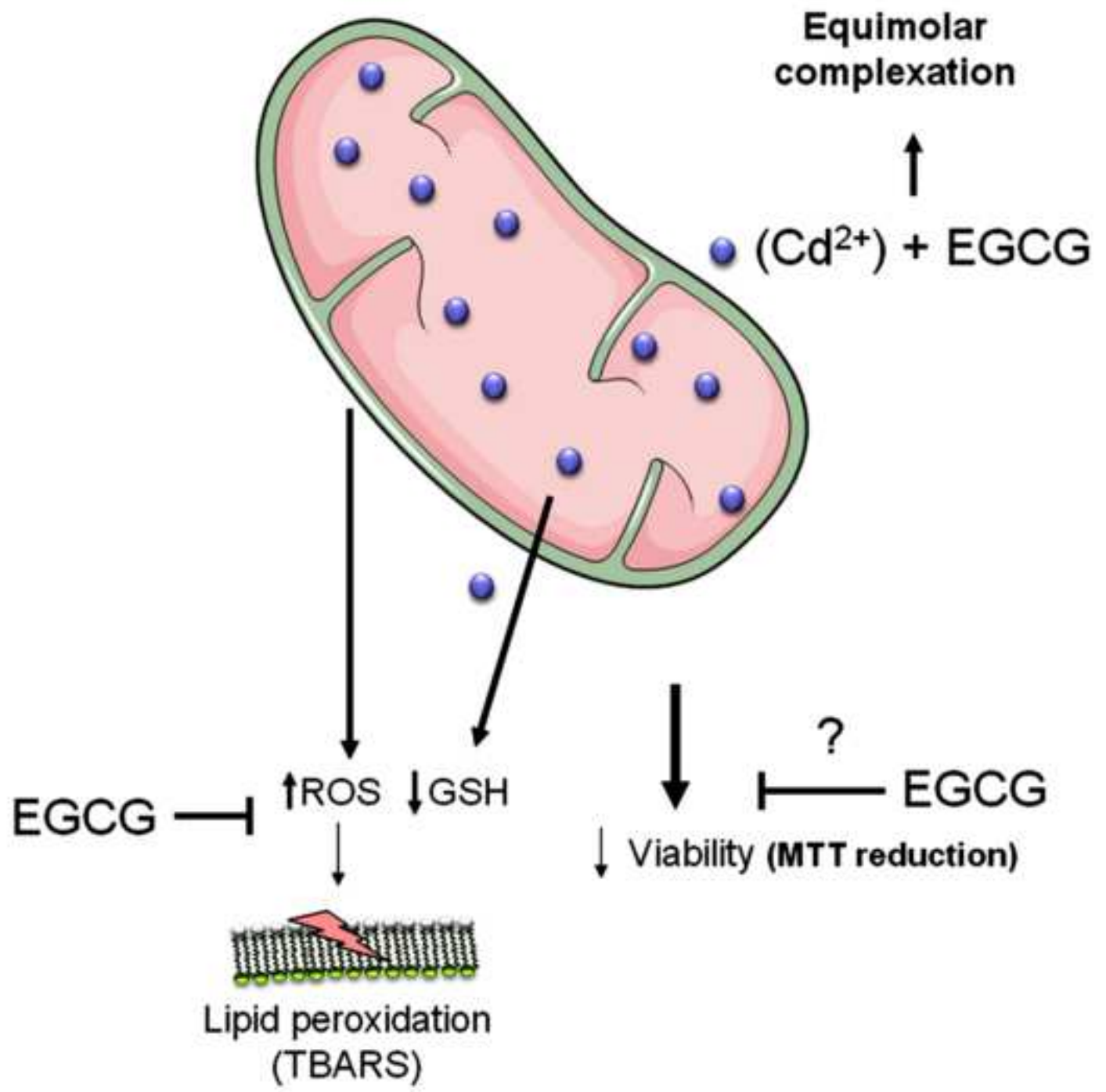


Figure 6
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4 DISCUSSÃO

Considerando que os astrócitos são importantes para a manutenção e preservação dos neurônios, é importante que estas células sejam estudadas sob diferentes estímulos. Para isso, a linhagem C6 é uma boa ferramenta a ser utilizada como modelo astrocítico. Anteriormente, nosso grupo demonstrou que a ECG na maior concentração testada (10 μ M), embora tenha promovido alterações morfológicas em células C6, não causou morte celular, pelo menos até o maior tempo avaliado (24 h). Além disso, a ECG foi capaz de modular positivamente a captação de glutamato e a secreção de S100B (Abib et al., 2008), funções características dos astrócitos, sugerindo que esta catequina possa exercer funções benéficas sobre este tipo celular *in vivo*.

No primeiro capítulo desta tese, demonstramos, em células C6, o aparecimento de micronúcleos ao longo do tempo de cultura (1 a 24 h), mesmo em condições basais. A partir de 12 h, a frequência de micronúcleos aumentou significativamente, chegando a aproximadamente 60% após 24 h de cultura, indicando que o tempo de cultura, por si só, pode ser um fator indutor da formação de micronúcleos. Isso pode ser devido às condições de manipulação do cultivo celular e/ou pela ausência de soro (condição necessária ao tratamento). Diferente do esperado, a ECG induziu formação de micronúcleos de forma dose-tempo-dependente, quando comparado ao controle, podendo-se concluir que a catequina esteja afetando diretamente as bases do DNA e/ou o sistema de reparo celular.

Entretanto, através da análise da morfologia nuclear, verificamos que ECG não ocasionou alteração na morfologia nuclear indicativa de apoptose em nenhuma das concentrações e tempos testados, o que está de acordo com nossos achados prévios (Abib et al., 2008). A partir destes resultados, podemos concluir que, embora a ECG

em altas concentrações possa ser genotóxica, este efeito não afeta a sobrevivência celular em 24 h.

Além disso, verificamos o efeito da ECG sobre o dano ao DNA pela técnica do cometa e comprovamos que a ECG *per se* também foi capaz de afetar o DNA de forma dose-tempo-dependente. Entretanto, em 1 h, na menor concentração testada de ECG não houve diferença em relação ao basal.

Por isso, o tempo de 1 h foi escolhido para testar a capacidade protetora da ECG frente ao dano com H_2O_2 , assim como demonstrado na literatura com resveratrol e quercetina (Li *et al.*, 2000; Sierens *et al.*, 2002; Yen *et al.*, 2003; Raschke *et al.*, 2006; Min and Ebeler, 2009; Moreira *et al.*, 2009; Makpol *et al.*, 2010). Assim, a ECG nas concentrações de 0,1 e 1 μM mostraram-se genoprotetoras frente ao estímulo por H_2O_2 , sugerindo que esta catequina possa estar exercendo seu papel antioxidante. Entretanto, como o H_2O_2 foi simplesmente adicionado ao meio que já continha ECG, pode ter ocorrido interação direta da ECG com o H_2O_2 , evitando que esta espécie reativa realmente exercesse seus efeitos tóxicos para as células, e desta forma a ECG estaria protegendo do dano celular.

Outra hipótese seria que o H_2O_2 , como é rapidamente metabolizado por este tipo celular, esteja agindo de forma tóxica, gerando novas ERO ou interagindo diretamente com as bases do DNA, e a ECG esteja protegendo as células destas conseqüências danosas da exposição ao H_2O_2 , por remover as novas ERO geradas, caracterizando seu efeito antioxidante, o que corrobora com efeitos encontrados com outros polifenóis em células PC12 (Silva *et al.*, 2008). Pode também a ECG estar atenuando este dano ao DNA através de sua interação com o ferro (Perron *et al.*, 2010), impossibilitando a reação de Fenton, e desta forma, amenizando os efeitos deletérios do H_2O_2 (Mello

Filho *et al.*, 1984; Luo *et al.*, 1994; Henle and Linn, 1997; Linn, 1998; Barbouti *et al.*, 2001).

O fato da ECG 10 μM não ter sido eficiente na proteção contra o dano ao DNA induzido por H_2O_2 pode ser explicado pelo efeito duplo observado também com outros polifenóis na literatura (Halliwell, 1996; Young and Lowe, 2001; Lambert and Elias, 2010; Riabchenko *et al.*, 2010), que em altas concentrações podem se tornar pró-oxidantes (Ohshima *et al.*, 1998; Halliwell, 2007b; Rowe *et al.*, 2008; Katsurahara *et al.*, 2009). Contudo, traçando-se um paralelo com um suposto efeito *in vivo*, pode-se sugerir que este polifenol seja benéfico em condições fisiológicas, pois dificilmente atingir-se-ia via oral uma concentração como a mais alta avaliada neste estudo *in vitro*, ainda mais se tratando de células do sistema nervoso central, onde apenas uma pequena fração do que é absorvido atravessa a BHE. Além disso, o tempo que os polifenóis do chá se mantêm no organismo é inferior a 8 h, sugerindo que essa condição também seja favorável ao efeito genoprotetor das catequinas. Embora não se possam aplicar estes resultados diretamente em humanos, estudos *in vitro* são essenciais para evidenciar possíveis rotas e mecanismos de ações de moléculas e dar o primeiro passo para orientar futuros estudos *in vivo*.

Ainda com a intenção de avaliar efeitos *in vitro* das catequinas, porém saindo do núcleo celular como foco, elegeram-se e utilizou-se uma importante organela responsável por diversos mecanismos essenciais para a sobrevivência celular, a mitocôndria. Por isso e por ser a principal geradora de ERO na célula, este compartimento celular é alvo de agentes neurotóxicos como os metais pesados. Sabendo que a EGCG pode atenuar os efeitos nocivos de metais (Hider *et al.*, 2001), utilizamos a EGCG frente ao dano induzido por cádmio em mitocôndrias isoladas de encéfalos de ratos.

O que observamos no segundo capítulo desta tese é que a EGCG em todas as concentrações testadas (10, 50 e 100 μM) não alterou viabilidade mitocondrial, tampouco os níveis de tióis não protéicos presentes na mitocôndria, indicando que, *per se*, esta catequina não é tóxica à mitocôndria, ou seja, não interfere diretamente com as funções biológicas desta organela, como esperado. Já o cádmio, diminuiu significativamente a viabilidade mitocondrial e os níveis de tióis não protéicos na mitocôndria, resultados que corroboram com dados de outros metais na literatura (Franco et al., 2007), sugerindo que esta perda esteja associada à oxidação de GSH e que essa possa ser uma das formas pela qual este metal prejudica o estado redox celular.

Durante a co-incubação com EGCG e cádmio por 2 h, observou-se que a catequina pode proteger totalmente a mitocôndria da perda de viabilidade e da lipoperoxidação induzida por cádmio, entretanto, não foi capaz de manter os níveis de tióis não protéicos na mitocôndria. Estes resultados nos sugerem que os efeitos protetores observados pelas catequinas possam envolver este compartimento celular.

Especulando estes resultados, podem-se inferir algumas possíveis rotas de ação prováveis deste mecanismo protetor da EGCG contra o dano induzido por cádmio, tais como: a) o cádmio pode estar inibindo o complexo III da cadeia respiratória, gerando ERO, que através da lipoperoxidação, afetam a integridade da membrana mitocondrial, acarretando em perda de viabilidade, enquanto a EGCG pode estar removendo essas ERO ou ligando-se diretamente ao metal, evitando seus efeitos; b) A EGCG pode não ter sido eficiente em manter os níveis de GSH, pelo fato do cádmio ter uma maior afinidade por grupamentos sulfidríla do que pela catequina, e por isso, mesmo ela tendo a capacidade de quelá-lo, não foi capaz de prevenir este dano, indicando também que o efeito benéfico da EGCG pode ocorrer independentemente de GSH,

visto que EGCG *per se* não foi capaz de aumentar os níveis de GSH, como observado na literatura em microglia (Kim et al., 2009), e isso pode ser explicado pelo fato da síntese de GSH ocorrer fora da mitocôndria; c) o cádmio pode estar se ligando diretamente com a GSH, e por diminuir essa defesa antioxidante, causaria estresse oxidativo, e conseqüentemente lipoperoxidação e perda de viabilidade mitocondrial, enquanto a EGCG poderia estar removendo as ERO, evitando o estresse oxidativo, ou então, ligando-se diretamente ao cádmio, e por fim d) o cádmio pode estar bloqueando a cadeia respiratória, gerando ERO, sendo estas removidas pela GSH, acarretando na diminuição desta defesa antioxidante.

A fim de esclarecer o possível efeito quelante da catequina sobre o cádmio, investigou-se sua interação e evidenciou-se pela primeira vez que existe a possibilidade de complexação EGCG-cádmio de forma equimolar. Desta forma, podemos especular que os efeitos benéficos encontrados se devem também a sua capacidade quelante. Entretanto, não podemos inferir que seja apenas por esta propriedade, visto que a EGCG *per se* foi capaz de diminuir a lipoperoxidação em relação ao controle, evidenciando seu caráter antioxidante. Além disso, como o efeito quelante é equimolar e ocorreu uma proteção total no que diz respeito à lipoperoxidação e à viabilidade mitocondrial, mesmo a EGCG estando em uma concentração menor que o cádmio, pode-se deduzir que exista também a capacidade antioxidante envolvida nesta proteção.

Estes resultados importantes sugerem que a EGCG seja uma molécula promissora no desenvolvimento de tratamentos para intoxicação por cádmio, ainda incipientes, e sua utilização em longo prazo pode atuar na prevenção dos efeitos nocivos da exposição a este metal.

Tanto o efeito genoprotetor observado com a EGCG, quanto o efeito antioxidante e quelante da EGCG, nos levam a crer que estas catequinas, derivadas do chá verde, possam ter efeitos benéficos que contribuam para retardar o desenvolvimento e a progressão de doenças neurodegenerativas, visto que estas doenças têm sido associadas ao estresse oxidativo, dano ao DNA e exposição crônica a metais.

Ainda que o detalhamento dos mecanismos de ação das catequinas seja melhor compreendido em estudos *in vitro*, modelos animais, os quais incluem diferentes tipos celulares e tecidos integrados, possibilitam o estudo da ação fisiológica e efeitos periféricos de drogas para o estudo de doenças neurodegenerativas. A fim de testar a capacidade neuroprotetora da EGCG, utilizou-se o modelo de DP induzido por 6-OHDA no terceiro capítulo desta tese.

A partir dos resultados preliminares obtidos pela administração de EGCG durante sete dias na concentração de 10 mg/Kg i.p., observou-se que EGCG pode ser capaz de proteger da morte os neurônios dopaminérgicos da substância negra – região encefálica atingida nesta doença. Esses resultados ainda serão ampliados para confirmar o efeito neuroprotetor da EGCG encontrado neste trabalho.

Dados da literatura mostram que a EGCG (2 mg/Kg via oral) foi neuroprotetora frente a droga MPTP *in vivo*, em camundongos (Levites et al., 2001). Entretanto, outro estudo que realizou pré-tratamento com EGCG (1 e 2 mg/Kg via oral) por 14 dias não confirmou efeito protetor aos neurônios dopaminérgicos lesionados com 6-OHDA na substância negra, embora tenha atenuado efeitos motores e cognitivos de forma dose dependente (Leaver et al., 2009). Esses estudos salientam a diferença de resposta entre os modelos MPTP e 6-OHDA, além da eficiência da EGCG em atenuar efeitos motores.

Os possíveis efeitos neuroprotetores da EGCG que sugerimos podem envolver diversos processos, que incluem: modulação da sinalização celular, efeitos anti-inflamatórios e/ou antioxidantes. Cabe ainda mencionar que um estudo *in vitro* mostrou que a utilização de antagonistas glutamatérgicos diminui significativamente a morte neuronal por 6-OHDA, indicando que o glutamato pode estar mediando a citotoxicidade induzida por 6-OHDA (Leaver et al., 2008). Sendo assim, a EGCG poderia estar atenuando a excitotoxicidade do glutamato por aumentar sua captação neste modelo de Parkinson, da mesma forma que nosso grupo comprovou anteriormente em células C6 (Abib et al., 2008).

A 6-OHDA pode reduzir a fosforilação da proteína cinase C (PKC) e diminuir a atividade da ERK1/2 em neuroblastoma (Levites et al., 2002a). A PKC tem um papel fundamental na regulação da sobrevivência celular (Berra et al., 1997) e intervenções farmacológicas destinadas a sua modulação ou em vias de sinalização por ela mediadas têm um grande potencial terapêutico para o envelhecimento e patologias relacionadas com a idade, incluindo a DP (Pascale et al., 2007). Acredita-se que a indução da atividade de PKC ϵ em neurônios seja pré-requisito para neuroproteção em muitos modelos de lesão (Mandel et al., 2008). De fato, já foi demonstrado que a EGCG, em baixas concentrações, pode ativar a PKC e diminuir os níveis de Bad em neuroblastomas (Kalfon et al., 2007), o que sugere que essa modulação da PKC possa ser um dos possíveis mecanismos neuroprotetores da EGCG.

Além disso, sabe-se que as catequinas podem modular proteínas cinases ativadas por mitógeno (MAPKs), que podem ativar a expressão gênica, fatores de transcrição e outras enzimas (ex: proteínas cinases e a óxido nítrico sintase) (Cuschieri and Maier, 2005; Kaminska, 2005), e assim afetar o processo inflamatório. A EGCG, especificamente, pode prevenir a diminuição de atividade de ERK1/2 causada pela 6-

OHDA em neuroblastomas (Levites et al., 2002a), inibir a translocação do NF- κ B para o núcleo em células PC12, e desta forma, inibir a resposta inflamatória (Levites et al., 2002b). Alguns estudos têm demonstrado alteração nos níveis plasmáticos de uma série de citocinas em paciente com DP, sugerindo o possível envolvimento de mecanismos de neuroinflamação na sua patogênese (Mogi *et al.*, 1994; Bessler *et al.*, 1999; Reale *et al.*, 2009; Scalzo *et al.*, 2009). Na DP, estão aumentadas citocinas pró-inflamatória como a interleucina-1 β (IL-1 β), interleucina-8 (IL-8), fator de necrose tumoral alfa (TNF- α), interferon gamma e interleucina-6 (IL-6) (Reale et al., 2009), que inclusive tem sido associada a déficits motores (Scalzo et al., 2010).

O TNF- α é uma das mais potentes citocinas pró-inflamatórias, e está envolvida no controle de vários processos biológicos como viabilidade celular, expressão gênica, homeostase de íons e integridade sináptica (Park and Bowers, 2010). No SNC, o TNF- α é produzido por astrócitos, microglia e neurônios em resposta a estímulos intrínsecos e extrínsecos, e tem se mostrado tóxico especialmente sobre a viabilidade de neurônios dopaminérgicos (De Lella Ezcurra et al., 2010). Como já observado na literatura em células epiteliais, a EGCG pode inibir a expressão de TNF- α , via redução da atividade do NF κ -B (Lee et al., 2009), sendo esta uma via importante a ser estudada para melhor esclarecer seu mecanismo de ação neuroprotetor.

A administração de L-DOPA, até o momento, é o tratamento mais efetivo para os pacientes com DP. Entretanto, a maioria destes pacientes sofre de complicações relacionadas a este tratamento, o que representa o maior problema no manejo clínico desta doença. Em um estudo que testou a administração de L-DOPA em ratos mostrou que ocorre um aumento no conteúdo de GSH oxidada assim como aumento na atividade da óxido nítrico sintase (NOS), tanto na substância negra quanto no estriado desses ratos (Calabrese et al., 2007). A neurodegeneração dopaminérgica seletiva

sugere que o estresse oxidativo e nitrosativo estejam associados ao metabolismo da DA (Tsang and Chung, 2009) e que existe uma interdependência entre funções mitocondriais, metabolismo de GSH e neurodegeneração na DP (Vali et al., 2007).

As citocinas pró-inflamatórias, bem como a produção de prostaglandinas, óxido nítrico (NO), ERO e ativação imunológica exacerbada têm impacto significativo na resposta imunológica de doenças crônicas. O NO, por exemplo, que é produzido pela NOS através da conversão de L-arginina a L-citrulina utilizando NADPH e O₂ como cofatores, desempenha diversas funções no organismo dependendo da sua concentração e do tipo celular em que atua. O NO pode ser considerado uma molécula de sinalização envolvida em diversos eventos fisiológicos, incluindo relaxamento da musculatura lisa, inflamação, defesa, regulação da pressão sanguínea, inibição da agregação plaquetária, aterosclerose e choque séptico (Duval *et al.*, 1996; Day *et al.*, 1999; Guo *et al.*, 2001; Connelly *et al.*, 2005). Entretanto, a super expressão da NOS tem sido associada a várias condições patológicas em humanos, como na DP (Hunot *et al.*, 1996; Zhang *et al.*, 2000). O NO pode reagir com diferentes moléculas de forma direta ou indireta, e formar peroxinitrito (ONOO⁻), envolvido com morte celular e neurodegeneração (Floyd, 1999; Oh-Hashi *et al.*, 2001; Pan *et al.*, 2004).

Contudo, estudos com camundongos demonstraram que a utilização de inibidores da NOS diminui o estresse oxidativo e nitrosativo, reduz a liberação de citocromo C, além de restabelecer a atividade do complexo I, que também é afetado na DP (Tapias *et al.*, 2009) e no modelo de Parkinson induzido por 6-OHDA (Singh *et al.*, 2010). A diminuição dos sintomas motores também foi observada em tratamento com inibidores da NOS (Padovan-Neto et al., 2009), indicando que o NO contribui significativamente para apoptose neuronal em ratos. O tratamento com EGCG pode estar aumentando expressão do RNAm da gama-glutamilcisteína ligase, enzima

limitante da biossíntese de GSH (Kim et al., 2009), auxiliando na manutenção do estado redox e, como já descrito com os polifenóis do chá verde, pode inibir a expressão do NOS tanto *in vitro* em neuroblastomas expostos a 6-OHDA (Guo et al., 2005; Guo et al., 2007), quanto *in vivo* (Kumar and Kumar, 2009), evitando assim formação de NO e suas consequências. Portanto, a inibição da NOS poderia ser outro possível mecanismo de ação desta catequina e alvo de estudos posteriores.

Neste sentido, já foi demonstrado na literatura que a administração de EGCG 20 mg/Kg durante 6 dias, no modelo MPTP, diminuiu NOS neuronal (nNOS), e por consequência, a formação de NO na SN dos camundongos, cujos autores sugeriram que esta seja uma das principais rotas envolvidas na neuroproteção pela EGCG (Choi et al., 2002). Corroborando com estes dados, outro grupo examinou a mistura de quatro compostos polifenólicos do chá verde 150 e 450 mg/Kg via oral por sete dias em modelo animal de Parkinson induzido por 6-OHDA e demonstrou efeito neuroprotetor destes compostos, via inibição da nNOS, lipoperoxidação e apoptose (Guo et al., 2007).

Em relação à prevenção da expressão de genes envolvidos com a morte celular por apoptose, pode-se dizer que o aumento na expressão de genes pró-apoptóticos e a diminuição de RNAm de Bcl-2 e Bcl-xL causado por 6-OHDA estão implicados na sua toxicidade (Jordan et al., 2004; Manakova et al., 2004). A indução de genes pró-apoptóticos como Bax e Bad por 6-OHDA pode ocasionar a liberação de citocromo C da mitocôndria, culminando na ativação de caspases (Blum et al., 1997; Cutillas et al., 1999; Yuan and Yankner, 2000). Em contrapartida, a EGCG pode prevenir a expressão de Bax e Bad, além de modular a expressão de Bcl-2 e Bcl-xL (Levites et al., 2002a; Mandel et al., 2004). Esses efeitos da EGCG podem também estar contribuindo com seu papel protetor contra toxicidade da 6-OHDA.

A 6-OHDA, além de atuar na modulação de vias de transdução de sinal, pode agir diretamente inibindo o complexo I e IV da cadeia respiratória mitocondrial (Glinka and Youdim, 1995), gerando H_2O_2 e outras espécies reativas (Thakar and Hassan, 1988), embora este não seja o principal mecanismo desta neurotoxina (Wu et al., 1996).

Muitos estudos confirmam que o estresse oxidativo está envolvido na toxicidade induzida pela 6-OHDA tanto *in vivo* (Lopez-Real et al., 2005; Smith and Cass, 2007b, a), quanto *in vitro* (Hara et al., 2003; Mazziro et al., 2004). O estresse oxidativo oriundo da neurotoxina 6-OHDA pode diminuir os níveis de GSH (Tirmenstein et al., 2005), alterar permeabilidade de membrana mitocondrial (Lee et al., 2006) e pode ser um forte desacoplador da fosforilação oxidativa (Wagner and Trendelenburg, 1971). Além disso, a diminuição da morte de neurônios dopaminérgicos em camundongos transgênicos que super expressam SOD e GPX (Asanuma et al., 1998; Bensadoun et al., 1998), atestam o efeito pró-oxidante da 6-OHDA. Isto poderia explicar o efeito protetor da utilização de moléculas antioxidantes neste modelo, como a EGCG.

Esse estresse oxidativo pode ser gerado por outros dois mecanismos distintos: a desaminação da 6-OHDA pela monoamino oxidase (MAO) ou pela sua auto-oxidação (Blum et al., 2001), entretanto os polifenóis do chá verde são capazes de inibir esta oxidação e remover as ERO geradas (Guo et al., 2005). Inclusive um trabalho realizado com cultura de células PC12, que comparou o efeito de cinco catequinas contra os danos induzidos por 6-OHDA, demonstrou que apenas a EGCG e a ECG possuem o efeito protetor, sugerindo que o grupamento galato possa estar contribuindo efetivamente neste mecanismo (Nie et al., 2002).

Ainda em relação ao caráter antioxidante das catequinas, recentemente foi mostrado em células neuronais, que a EGCG é capaz de aumentar as defesas

antioxidantes e a expressão da enzima heme oxigenase-1, possivelmente por ativar o fator de transcrição Nrf2 (Romeo et al., 2009) via ERK1/2, como visto também em células endoteliais (Wu et al., 2006). A ativação de Nrf2 tem sido apontada como uma importante rota de defesa contra danos induzidos por 6-OHDA em cultura de neurônios dopaminérgicos (Jakel *et al.*, 2007; Siebert *et al.*, 2009), sugerindo que a EGCG possa estar agindo também por este mecanismo.

Além disso, os níveis de ferro encontram-se aumentados no estriado após injeção de 6-OHDA (He *et al.*, 1996; Wang *et al.*, 2004), o que pode acarretar no aumento do estresse oxidativo através da reação de Fenton (Blum et al., 2001). Assim, parte dos efeitos deletérios da 6-OHDA poderia estar sendo prevenida pela EGCG, também por ser quelante de ferro (Borisenko *et al.*, 2000; Ryan and Hynes, 2007). A Figura 10 ilustra resumidamente os possíveis mecanismos neuroprotetores da EGCG sobre o modelo de Parkinson induzido por 6-OHDA.

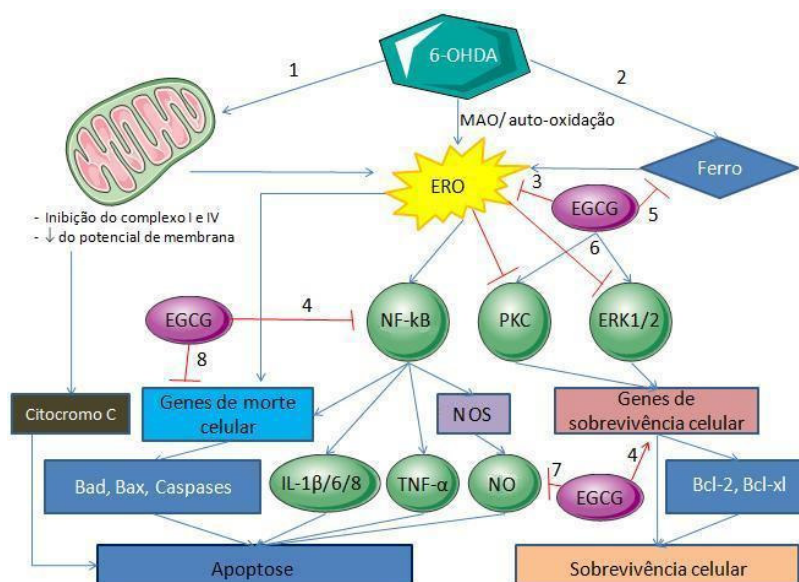


Figura 10: Possíveis mecanismos de neuroproteção da EGCG sobre o modelo de Parkinson induzido por 6-OHDA, baseados na literatura: 1 - (Glinka and Youdim, 1995); 2 - (Blum et al., 2001); 3 - (Kim et al., 2009); 4 - (Levites et al.,

2002b); 5 - (Borisenko et al., 2000); 6 - (Kalfon et al., 2007); 7 - (Kumar and Kumar, 2009); 8 - (Levites et al., 2002a).

A Figura 11 sintetiza os principais efeitos protetores das catequinas EGCG e ECG comprovados nesta tese. Contudo, ainda não está bem esclarecida a interação entre as catequinas e seus efeitos em conjunto, ressaltando a importância de mais estudos sobre estes polifenóis, principalmente *in vivo*, em que se possa analisar também seu metabolismo, para se ter uma maior segurança quanto a sua recomendação.

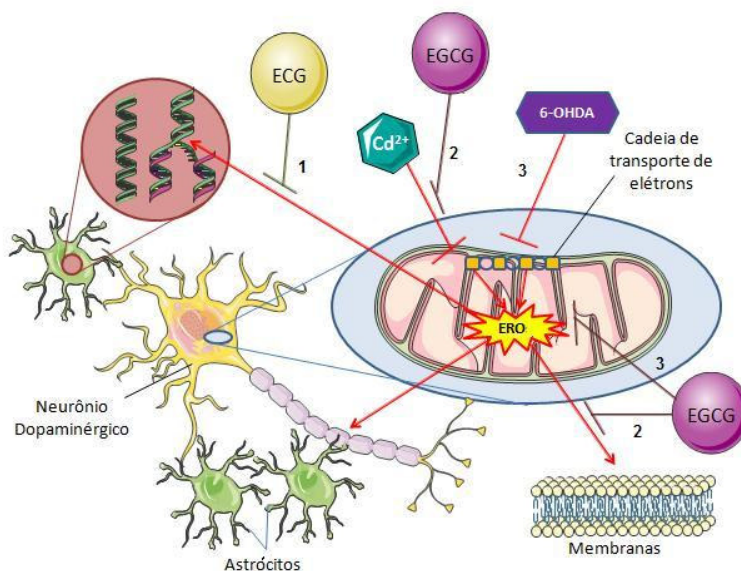


Figura 11: Efeitos protetores da EGCG e da ECG elucidados nesta tese. 1- Capítulo I: genoproteção da ECG frente a dano por ERO; 2- Capítulo II: proteção mitocondrial da EGCG frente ao dano induzido por cádmio; 3- Capítulo 3: possível efeito neuroprotetor da EGCG frente ao modelo de Parkinson induzido por 6-OHDA.

5 CONCLUSÕES

Capítulo I

- ECG (0,1 e 1 μ M) protegeu as células astrogliais contra dano em DNA induzido por H_2O_2 .
- ECG apresentou um caráter genotóxico dose-tempo-dependente *per se*.

Estes resultados demonstram que a ECG pode ser genoprotetora e antioxidante em baixas concentrações, como as fisiológicas, sugerindo que esta catequina possa estar atuando na remoção das EROs geradas pelo H_2O_2 , causadoras do dano ao DNA. Já em altas concentrações e em tempos prolongados, pode ter um efeito genotóxico.

Capítulo II

- EGCG foi capaz de proteger as mitocôndrias contra o dano induzido pelo cádmio no que se refere à viabilidade e à lipoperoxidação.
- EGCG não foi capaz de evitar a oxidação da GSH induzida pelo cádmio.
- EGCG apresentou efeito quelante de cádmio de forma equimolar.

O fato de que a EGCG foi capaz de proteger totalmente a diminuição da viabilidade e o aumento na lipoperoxidação causada por cádmio, nos leva a concluir que esta catequina esteja promovendo mecanismos de defesa antioxidante, independente da recuperação da forma reduzida de GSH. Além disso, o efeito quelante indica outro mecanismo provável de ação protetora da EGCG.

Capítulo III

Dados preliminares indicam que:

- EGCG 10 mg/kg não é capaz de diminuir o número de rotações induzidas por metilfenidato.
- EGCG protege da morte neuronal dopaminérgica, evidenciada pela prevenção da perda do imunoconteúdo de tirosina hidroxilase.

Pode-se sugerir que a EGCG apresenta efeito neuroprotetor para neurônios dopaminérgicos expostos à 6-OHDA. Entretanto, é necessário aumentar o tamanho amostral para confirmar estes efeitos.

Os resultados do atual projeto indicam um possível papel neuroprotetor decorrente do uso de catequinas na dieta e abrem muitas perspectivas a serem seguidas para ampliar a compreensão dos mecanismos envolvidos na neuroproteção, particularmente na DP.

6 PERSPECTIVAS

- Aumentar tamanho amostral para confirmação do efeito neuroprotetor da EGCG no modelo de DP induzido por 6-OHDA;
- Avaliar sinalização celular mediada por EGCG no modelo de Parkinson, avaliando fosforilação das MAPKs e PKC;
- Avaliar efeito anti-inflamatório mediado por EGCG no modelo de Parkinson, medindo citocinas pró-inflamatórias, NO, NFkB e TNF- α ;
- Avaliar efeito antioxidante mediado por EGCG no modelo de Parkinson, medindo atividade e expressão de enzimas antioxidantes (SOD, CAT e GPX, heme oxigenase-1) e Nrf-2;
- Avaliar interação entre diferentes catequinas, por exemplo, na proporção encontrada no chá e seus efeitos;
- Avaliar interação da EGCG com outros metais, como o manganês e o metilmercúrio *in vitro* e *in silico*;
- Avaliar se o efeito encontrado da EGCG é preventivo ou curativo sobre o modelo de Parkinson;
- Avaliar a expressão gênica de de mediadores inflamatórios e apoptóticos.

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LISTA DE FIGURAS

Figura 1: Cadeia de transporte de elétrons (http://www.bioq.unb.br , compilado em 21 de julho de 2011)	2
Figura 2: Reação de Fenton.....	3
Figura 3: Estrutura química das catequinas.....	6
Figura 4: Efeitos da ECG e da EGCG sobre enzimas antioxidantes indicados com as setas vermelhas. GR: glutathione redutase; GPX: glutathione peroxidase; SOD: superóxido dismutase; CAT: catalase.....	9
Figura 5: Interação dos astrócitos. Adaptado da literatura (Seifert et al., 2006).....	13
Figura 6: Estrutura química da 6-OHDA.	15
Figura 7: Neurotoxicidade da 6-OHDA. Adaptado da literatura (Simola et al., 2007).....	17
Figura 8: Regiões afetadas na doença de Parkinson. Adaptado da literatura (Brodsky and Nutt, 2010)	18
Figura 9: Formação da dopamina.....	19
Figura 10: Possíveis mecanismos de neuroproteção da EGCG sobre o modelo de Parkinson induzido por 6-OHDA, baseados na literatura: 1 - (Glinka and Youdim, 1995); 2 - (Blum et al., 2001); 3 - (Kim et al., 2009); 4 - (Levites et al., 2002b); 5 - (Borisenko et al., 2000); 6 - (Kalfon et al., 2007); 7 - (Kumar and Kumar, 2009); 8 - (Levites et al., 2002a).....	79
Figura 11: Efeitos protetores da EGCG e da ECG elucidados nesta tese. 1- Capítulo I: genoproteção da ECG frente a dano por ERO; 2- Capítulo II: proteção mitocondrial da EGCG frente ao dano induzido por cádmio; 3- Capítulo 3: possível efeito neuroprotetor da EGCG frente ao modelo de Parkinson induzido por 6-OHDA.	80