UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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DEPARTAMENTO DE BIOQUÍMICA PROF. TUISKON DICK

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

CARACTERIZAÇÃO DA DIFERENCIAÇÃO NEURONAL INDUZIDA POR ÁCIDO RETINÓICO DA LINHAGEM DE NEUROBLASTOMA HUMANO SH-SY5Y E SEU USO COMO FERRAMENTA PARA PESQUISA EM NEUROCIÊNCIAS

FERNANDA MARTINS LOPES

PORTO ALEGRE, MARÇO DE 2012

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ALUNA: FERNANDA MARTINS LOPES

ORIENTADOR: PROF. DR. FÁBIO KLAMT

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PARTE I

RESUMO

Os mecanismos moleculares que levam ao dano da via nigroestriatal durante a progressão da Doença de Parkinson (DP) ainda não estão totalmente elucidados. Dessa forma, existe a necessidade de desenvolver modelos experimentais adequados para o estudo desse distúrbio neurodegenerativo. A linhagem de neuroblastoma humano SH-SY5Y tratada com neurotoxinas indutoras deste distúrbio (*ex.:* 6-hidroxidopamina - 6-OHDA) é amplamente utilizada como modelo *in vitro* da DP. Muitos estudos mostram que esta linhagem pode ser diferenciada em células dopaminérgicas através da combinação da diminuição do soro fetal bovino (SFB) em meio de cultura e da adição de neurotrofinas como o ácido retinóico (AR). No entanto, há poucos estudos mostrando as diferenças entre células proliferativas e diferenciadas da linhagem de neuroblastoma SH-SY5Y, além do efeito do tratamento com 6-OHDA. Ainda, não há um consenso nos protocolos de diferenciação. Dessa forma, o objetivo deste estudo foi estabelecer um protocolo de diferenciação dopaminérgica da linhagem de neuroblastoma humano SH-SY5Y, bem como avaliar a potencialidade do modelo como plataforma para o *screening* de neurotoxicidade/neuroproteção de compostos e a possibilidade de manipulação gênica.

As células proliferativas SH-SY5Y foram mantidas em meio de cultura DMEM/F12 (1:1) suplementado com 10% de SFB. A diferenciação foi induzida pela combinação de 10 µM de AR e meio de cultura com 1% de SFB durante 4, 7 e 10 dias. Foram avaliados parâmetros morfológicos (presença de neuritos) e neuroquímicos, através marcadores de diferenciação neuronal (DAT- transportador de dopamina; TH – tirosina hidroxilase; ENS – enolase neurônio específica; NeuN – proteína nuclear de neurônio; Nestina). Ainda, avaliamos parâmetros de estresse oxidativo através da atividade de enzimas antioxidantes e dos níveis de tióis reduzidos.

Nossos dados mostraram que as células SH-SY5Y diferenciadas por 7 dias apresentaram mudanças morfológicas e o aumento do imunoconteúdo de todos os marcadores neuronais testados, e a concomitantemente diminuição do imunoconteúdo de nestina (marcador de células indiferenciadas). Além disso, o fenótipo neuronal apresentou uma maior atividade de alguns sistemas antioxidantes. Também foi avaliada a citotoxicidade frente ao H_2O_2 e à 6-OHDA nos dois fenótipos. As células diferenciadas se mostraram mais resistentes ao dano causado pelo H_2O_2 e mais sensíveis à 6-OHDA. Dessa forma, a citotoxicidade da 6-OHDA pode estar relacionada com o aumento do imunoconteúdo do DAT, visto que a neurotoxina entra na célula dopaminérgica através deste transportador. Interessantemente, as células diferenciadas apresentaram aumento dos níveis da proteína neuroprotetora DJ-1, que está relacionada a uma forma prematura de Parkinsonismo em humanos.

Após a caracterização do modelo, nós utilizamos o fenótipo diferenciado como plataforma experimental para o *screening* de compostos neuroprotetores como os organocalcogênios. Nós determinamos a citotoxidade destes compostos em células diferenciadas da linhagem de neuroblastoma SH-SY5Y. A partir destes dados, foram selecionados compostos com baixa citotoxicidade e avaliamos a morfologia celular (densidade de neuritos). Nós verificamos que antes da perda de viabilidade, ocorre a perda de neuritos, sendo que este parâmetro é outra vantagem do modelo de célula diferenciada para avaliação da neurototoxicidade. Ainda, verificamos que estes compostos são capazes de prevenir o dano celular causado pela 6-OHDA.

Além disso, nós caracterizamos a capacidade do modelo de ser manipulado geneticamente através da transfecção e superexpressão de plasmídeo contendo a proteína verde fluorescente, onde verificamos que a expressão é mantida durante a diferenciação.

Dessa forma, nossos dados mostraram a eficácia da padronização da diferenciação induzida por AR da linhagem de neuroblastoma humano SH-SY5Y, pois estas células apresentam características morfológicas e neuroquímicas adequadas de neurônio dopaminérgico bem como pode ser aplicado não só para avaliação de neurototoxicidade/neuroproteção, mas também pode ser manipulado geneticamente.

Palavras-chave: Doença de Parkinson, SH-SY5Y, 6-hidroxidopamina, modelo experimental, diferenciação celular, estresse oxidativo.

ABSTRACT

The molecular mechanisms underlying the massive cellular loss found in the nigrostriatal pathway during the progression of Parkinson's disease (PD) are not completely understood. Therefore, it is important to develop more suitable experimental models to study the molecular mechanisms of this neurodegenerative disorder. Proliferative human neuroblastoma cell line SH-SY5Y challenged with neurotoxins (*e.g.*: 6-hydroxydopamine – 6-OHDA) has been widely used as an *in vitro* model for PD. Many lines of evidence showed that this cell line differentiates with the combination of lower fetal bovine serum (FBS) and retinoic acid (RA) to dopaminergic-like neural cell. However, there are few studies addressing the differences between proliferative and RA-differentiated SH-SY5Y cells as well as their responses to 6-OHDA cytotoxicity. Moreover, there is no consensus in differentiation protocols. Hence, the objective of this study was to establish a RA-induced dopaminergic differentiation protocol and also evaluate its capabilities for drug screening of neurotoxicity/neuroprotection and genetic manipulation.

Exponentially growing SH-SY5Y cells were maintained with DMEM/F12 (1:1) medium plus 10% FBS. Differentiation was triggered by the combination of 10 µM of RA plus medium with 1% of FBS during 4, 7 and 10 days. We evaluated the cell morphology (neurites) and the neuronal markers (Dopamine Transporter- DAT, Tyrosine Hydroxylase-TH, Neuron-Specific Enolase-NSE, Neuronal Nuclei Protein-NeuN, and Nestin immunocontent). Furthermore, we verify the activity of antioxidant enzymes and the reduced thiol levels.

Our data demonstrated that SH-SY5Y cells differentiated for 7 days expresses all neuronal markers tested with concomitant decrease in non-differentiated marker (nestin). Besides, they showed a higher activity of some antioxidant systems. We also evaluated the cytotoxicity of H_2O_2 and 6-OHDA in both phenotypes. Differentiated cells are more resistant to H_2O_2 and more sensitive to 6-OHDA. Hence, the damage caused by 6-OHDA could be related with the increase of DAT immuncontent, because this neurotoxin enters into the dopaminergic cell through this transporter. Interestingly, the differentiated cells have more levels of neuroprotective DJ-1 protein, which is related with a juvenile Parkinsonism.

After establish the conditions of differentiation, we used the neuronal phenotype to perform a drug screening with organoselenide compounds. We verify the cytotoxicity of these compounds in differentiated cells. From these data, we selected compounds with low toxicity and evaluated the cell morphology (neurites density). We verify that before the loss of viability, there is a loss of neurites. This parameter is another advantage of the differentiated cells model to neurotoxicity evaluation. Moreover, these compounds were able to prevent neuronal cell death caused by 6-OHDA.

We also characterized the ability of the model to be manipulated genetically through transfection and overexpression of a green fluorescent protein (GFP) plasmid. We verify that the expression of GFP is maintained during the differentiation.

Hence, our data showed the efficacy of the RA-induced differentiation protocol of the neuroblastoma cell line SH-SY5Y, because these cells have morphological and neurochemical characteristics of dopaminergic neurons. Furthermore, the neuronal phenotype can be applyed not only to evaluate neurocytotoxicity/neuroprotection but also can be manipulated genetically.

Key words: Parkinson Disease, SH-SY5Y, 6-hydroxydopamine, experimental model, cell differentiation, oxidative stress.

LISTA DE ABREVIATURAS

6-OHDA: 6-hidroxidopamina

AAPA: 2-acetilamino-3-[4-(2-acetilamino-2-carboxietilsulfaniltiocarnilamino) phenil tiolcarbamoilsulfanil] ácido propiônico

AR: ácido retinóico

Au: Auronofina

CAT: catalase

DAT: transportador de dopamina

DP: doença de Parkinson

ENS: enolase neurônio específica

GFP: proteína verde fluorescente

GPx: glutationa peroxidase

GR: glutationa redutase

GSH: glutationa reduzida

GST: glutationa-S-transferase

L-DOPA: 3,4-dihidroxi-*L*-fenilalaina

MPP^+: 1-metil-4-fenilpiridina

MPTP: 1-metil-4-fenil-1,2,3,6-tetrahidropiridina

NeuN: proteína nuclear de neurônio

SFB: soro fetal bovino

SNC: sistema nervoso central

SNPc: *substancia nigra pars compacta*

SOD: superoxide dismutase

TH: tirosina hidroxilase

TrxR: tioredoxina redutase

1. INTRODUÇÃO

1.1 A DOENÇA DE PARKINSON (DP)

A doença de Parkinson (DP) é uma desordem crônica, progressiva e neurodegenerativa que afeta de 0,1 a 0,3 % da população mundial (Wirdefeldt *et al.,* 2011; Baumann, 2012). O índice aumenta para 4% da população com idade superior a 85 anos (Rao *et al.,*2006; Meissner *et al.,* 2011). Esses dados podem variar de acordo com a região devido às diferenças na taxa de sobrevivência bem como a realização do diagnóstico correto (Muangpaisan *et al.,* 2011). A incidência desta doença, assim como outras doenças neurodegenerativas, está se elevando com o aumento da expectativa de vida (De Rijk *et al.,* 1995). A média de idade para o aparecimento da DP é de 60 anos, e a média de duração da doença do diagnóstico ao óbito é de 15 anos (Katzenschlager *et al.,* 2008).

Os sintomas primários da síndrome motora da DP incluem a bradicinesia, que é a dificuldade de iniciar movimentos voluntários, a rigidez muscular, a instabilidade postural e os tremores nas mãos e na mandíbula. Ainda, são observados alguns sintomas não-motores, como a disfunção cognitiva (Jankovic, 2008; Muangpaisan *et al.,* 2011). Estes sintomas só se desenvolvem quando há perda de 50-60% das células dopaminérgicas e quando há 80-85% menos liberação de dopamina no corpo estriado (Marsden, 1996; Morais & De Strooper, 2010).

Além dos sintomas clínicos, a DP só é confirmada através de estudos *post-mortem*, onde são verificadas alterações morfológicas, como os corpos de Lewy (Braak *et al.,* 2003). Esta estrutura é caracterizada por inclusões citoplasmáticas de vários tipos de forma e de tamanho, cujos componentes principais são acúmulos de proteínas filamentosas anormais, como a α-sinucleína (Gasser *et al.,* 2009).

A síndrome motora é conseqüência da degeneração de neurônios dopaminérgicos na *substancia nigra pars compacta* (SNpc), que está localizada no mesencéfalo. Estes neurônios são parte integrante da via nigroestriatal, onde seus corpos celulares estão localizados na SNpc e os axônios no corpo estriado (Dauer and Przedborski, 2003). O resultado disso é a perda de dopamina nas áreas de projeção estriatal, como ilustra a Figura 1 (Dawson &Dawson, 2003; Powe, 2009).

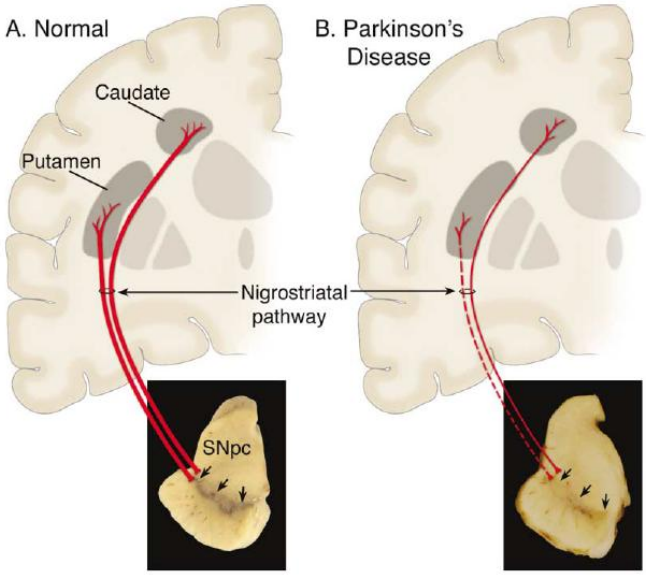

Figura 1: Corte transversal na altura cérebro médio, destacando os gânglios da base e uma representação esquemática da via nigroestriatal (em vermelho). À esquerda, uma representação de um indivíduo normal. À direita, a representação de um indivíduo com DP, onde se verifica uma significativa perda de neurônios dopaminérgicos na SNpc (retirada de Dauer e Przedborski, 2003).

Embora a degeneração da via nigroestriatal contribua para o aparecimento de muitos dos sintomas da DP, os mecanismos moleculares que provocam a morte neuronal ainda não estão esclarecidos. Dessa forma, determinar a causa da DP tem sido o foco de muitas pesquisas em neurociências (Shapira & Jenner, 2011). Os

avanços nesta área mostraram que a causa pode ser uma interação entre a suscetibilidade genética e os fatores ambientais (Schapira & Tolosa, 2010).

O estudo das formas genéticas da DP vem contribuindo para o entendimento da sua patogênese. Dessa forma, existem estudos mostrando o efeito genético nesse distúrbio bem como identificando os principais genes envolvidos (Marder *et al.,* 1996). Estes genes são denominados *PARK* e pelo menos 13 *locus* distintos são responsáveis por forma mendelianas da DP, sendo que uma das principais mutações é na α-sinucleína (Dawson & Dawson, 2003; Henchcliffe & Beal*,* 2008).

No entanto, a maioria dos casos da DP é esporádica. Mesmo antes dos estudos genéticos da DP, análises bioquímicas de cérebros *post-mortem* estabeleceram a disfunção mitocondrial, o estresse oxidativo e agregação de proteínas como os principais fatores da patogênese desta doença (Schapira*,* 2009; Schapira & Jenner, 2011).

Estudos de necropsias de pacientes com DP mostraram que a atividade do complexo I, componente da cadeia transportadora de elétrons, está diminuída na SNpc. Esta deficiência limita-se apenas a esta região cerebral (Schapira *et al.,* 1990; Parker *et al.,* 2008). A importância da mitocôndria na patogênese da DP aumentou devido à identificação de mutações nos genes que codificam proteínas mitocondriais (Henchcliffe & Beal*,* 2008).

Outra causa discutida para a patogênese da DP é o estresse oxidativo. Existem evidências mostrando o dano oxidativo ao DNA, a proteínas e a lipídios na SNpc, além da diminuição dos níveis de antioxidantes, como a glutationa (GSH) (Parker & Swerdlow, 1998; Yokota *et al*., 2003; Taira *et al*., 2004; Betarbet *et al*., 2006). Isso pode ser causado tanto pela disfunção mitocondrial como pelo metabolismo da dopamina (Dawson & Dawson, 2003). Ainda, a SNpc apresenta um

alto conteúdo de ferro. Isso somado ao metabolismo da dopamina que produz H_2O_2 propicia a Reação de Fenton, gerando mais espécies reativas, como o radical hidroxil ($^{\bullet}OH$). Por fim, a SNpc apresenta baixos níveis de antioxidantes enzimáticos e não-enzimáticos favorecendo o estresse oxidativo (Jenner, 2003).

Além disso, o dano ao sistema ubiquitina-proteossoma e o estresse proteolítico podem contribuir para esta doença. O déficit funcional e estrutural do proteossoma e o acúmulo de proteínas citotóxicas, como a α-sinucleína, são encontrados em neurônios dopaminérgicos de pacientes com a DP (Chung *et al.,*2001; Mcnaught & Olanow*,* 2003).

No entanto, estas hipóteses não são excludentes e podem atuar em conjunto, tendo como base a mitocôndria e o estresse oxidativo, como ilustra a Figura 2. A disfunção mitocondrial afeta um grande número de vias celulares, culminando em dano nos componentes citoplasmáticos e na morte celular. Algumas funções e morfologia anormais foram observadas na mitocôndria em algumas formas da DP (Henchcliffe & Beal*,* 2008).

A mitocôndria é a maior fonte de radicais livres da célula, resultando no estresse oxidativo. No entanto, esta organela também é integrante da resposta ao estresse oxidativo (Zhang *et al.,* 1999). Ainda, o aumento do dano oxidativo leva ao desequilíbrio do sistema ubiquitina-proteossoma. Os radicais livres induzem a agregação da α-sinucleína e impedem a degradação de proteínas (Jenner*,* 2003). Estudos mostraram uma diminuição na atividade das subunidades 26/20S do proteossoma na SNpc de pacientes com a DP (Mcnaught & Olanow, 2003).

Por fim, a mitocôndria possui um papel importante na apoptose. Os neurônios dopaminérgicos da SNpc apresentam canais de cálcio voltagem dependente (Schapira, 2008). Dessa forma, durante os processos de excitotoxicidade, esta

organela seqüestra o cálcio intracelular (Chan *et al.,* 2007), resultando na liberação de citocromo c e de fatores pró-apoptóticos, como o fator de iniciação da apoptose, que inicia uma cascata de eventos culminando na morte do neurônio dopaminérgico (Henchcliffe & Beal, 2008).

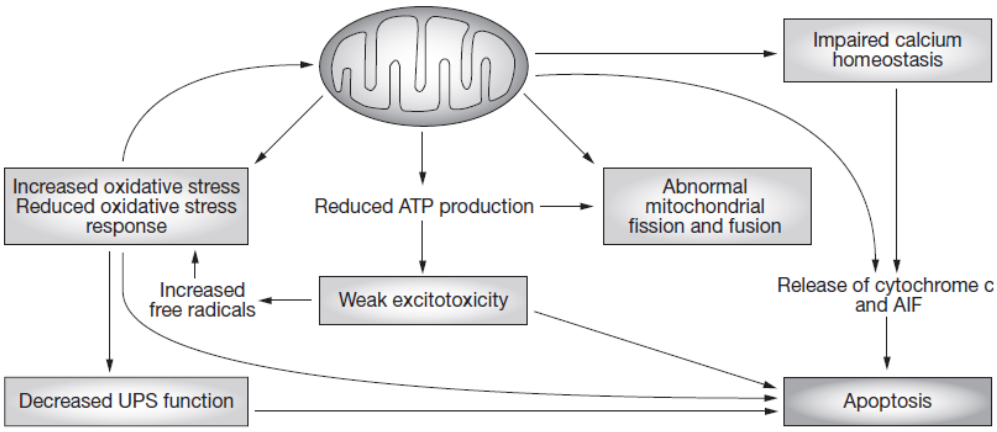

Figura 2: Patogênese da DP (retirada de Henchcliffe & Beal, 2008).

1.2 MODELOS EXPERIMENTAIS DA DP

Os modelos experimentais para DP são de grande importância para os avanços tanto na fisiopatologia bem como no desenvolvimento de novas terapias. Um modelo ideal *in vivo* para este distúrbio deve atender às seguintes características: os neurônios dopaminérgicos devem estar normais nas fases iniciais do desenvolvimento pré-natal, com degeneração progressiva na fase adulta; e esta degeneração deve implicar em sintomas motores observados na doença. Atualmente, nenhum dos modelos experimentais atende a essas características (Beal, 2010).

Os modelos experimentais da DP induzem a doença através do tratamento com neurotoxinas, as quais mimetizam os mecanismos fisiopatológicos do distúrbio em células dopaminérgicas (Beal, 2010). A seguir, discutiremos as principais

neurotoxinas indutoras da DP bem como as características de um neurônio dopaminérgico.

1.2.1 Indução da DP através de neurotoxinas

Vários estudos epidemiológicos sugerem que pesticidas (Bove *et al.,* 2005; Hatcher *et al.,* 2008) e outras toxinas ambientais (Uversky, 2004) que inibem o complexo I e causam estresse oxidativo estejam envolvidas na patogênese da DP. Os inibidores do complexo I causam morte de células dopaminérgicas e levam à formação de inclusões semelhantes a corpos de Lewy, sendo dessa forma utilizados para mimetizar a DP em modelos experimentais (Schapira, 2008). Exemplos de neurotoxinas são o 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), a rotenona, o paraquat e a 6-hidroxidopamina (6-OHDA). A ação das neurotoxinas descritas está ilustrada na Figura 3.

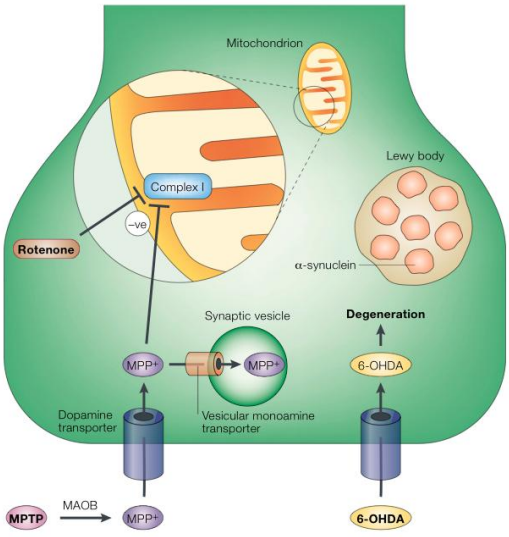

Figura 3: Ação das neurotoxinas indutoras da DP (Retirado de Beal, 2001)

O MPTP causa perda das células dopaminérgicas na SNpc e induz parkinsonismo em primatas e roedores (Fahn, 1996). A toxina é capaz de atravessar

a barreira hematoencefálica devido à sua alta lipofilicidade (Riachi *et al.,* 1989; Bove & Parier, 2011). O MPTP não é um composto tóxico, por outro lado, quando convertido pela monoamina oxidase B, seu metabólito MPP^+ (1-metil-4-fenilpiridina) concentra-se nos neurônios dopaminérgicos via transportadores de dopamina. Dessa forma, o MPTP é responsável pela morte celular através da inibição do complexo I (Schober, 2004; Lee *et al.,* 2006). Esta toxina também gera radicais livres, dentre eles o óxido nítrico ($^{\bullet}NO$), que provocam a morte dos neurônios por apoptose (Kitamura *et al.,* 2003).

O paraquat (dicloreto de 1,1'-dimetil-4,4'-bipiridínio), um dos herbicidas mais utilizados no mundo, também é inibidor do complexo I. Essa substância provoca a agregação da proteína α-sinucleína resultando na degeneração de neurônios dopaminérgicos (Uversky, 2004; Hatcher *et al.*, 2008).

A rotenona é um potente e específico inibidor do complexo I da cadeia respiratória, muito utilizado como pesticida nos EUA. Diferentemente do MPTP e do paraquat, a rotenona não se concentra nas células, mas também induz morte de neurônios dopaminérgicos, o que sugere uma possível sensibilidade dessas células a danos no complexo I (Uversky, 2004; Betarbet *et al.,* 2006).

No entanto, o modelo mais usado para degeneração catecolaminérgica é a 6-OHDA (Reichman *et al.,* 1994; Bove & Perier, 2011). Esta neurotoxina foi a primeira usada em modelos animais da DP (Beal, 2001). Desde então, a 6-OHDA é utilizada tanto para estudos *in vivo* como para *in vitro*. Ainda, os níveis desta neurotoxina estão elevados no soro de pacientes da DP, por isso, diferentemente das outras toxinas, ela pode ser encontrada fisiologicamente (Schober *et al.,* 2004; Bove *et al.,* 2005).

A 6-OHDA é metabólito da dopamina e a sua estrutura é muito semelhante a esse neurotransmissor. Dessa forma, a toxina possui uma alta afinidade pelo transportador de dopamina (DAT). Assim, a 6-OHDA é capaz de entrar no neurônio dopaminérgico causando dano às projeções catecolaminérgicas (Lehmensiek *et al.,* 2006). Uma vez dentro do neurônio, a 6-OHDA acumula no citosol e ocorre a sua auto-oxidação, promovendo a formação de espécies reativas de oxigênio bem como a diminuição dos níveis de antioxidantes (Bove & Perier, 2011). Entretanto, ainda não foram elucidadas as contribuições dos sistemas antioxidantes frente ao dano causado pela 6-OHDA. Além disso, a 6-OHDA pode acumular-se na mitocôndria, onde inibe o complexo I da cadeia respiratória (Schober, 2004).

Os neurônios dopaminérgicos da SNpc*,* além de conter níveis significantes de dopamina, também apresentam H_2O_2 e ferro. Uma reação não-enzimática destes três elementos pode levar à formação de 6-OHDA (Blum *et al.,* 2001). Entretanto, a seqüência de eventos moleculares que leva à morte seletiva desses neurônios pela ação da 6-OHDA ainda não está estabelecida.

Apesar da forte evidência do envolvimento das espécies reativas de oxigênio e danos à mitocôndria, os mecanismos moleculares *upstream* da citotoxicidade da 6-OHDA que levam a disfunção dessa organela e o estresse oxidativo ainda não foram elucidados (Gomez-Lazaro *et al.,* 2008). Estudos anteriores mostraram que a morte celular causada pela 6-OHDA é devida à apoptose. Ainda, acreditava-se no envolvimento do poro de transição de permeabilidade mitocondrial (Lee *et al.,* 2006). Entretanto, outros trabalhos mostraram o não envolvimento do poro e verificaram um importante papel das proteínas p53, p38 MAPK, PUMA e Bax (Gomez-Lazaro *et al.,* 2008; Berstein *et al.,* 2011).

Apesar de muitos estudos buscarem os mecanismos de morte neuronal causada pela 6-OHDA, os seus mecanismos de ação ainda devem ser elucidados.

1.2.2 Modelos experimentais de neurônio dopaminérgico

As células dopaminérgicas são a maior fonte de dopamina no sistema nervoso central (SNC). Estas células estão localizadas diencéfalo, no mesencéfalo e no bulbo olfatório. No entanto, a maior concentração encontra-se na SNPc, localizada no mesencéfalo (Björklund & Dunnett, 2007).

Para mimetizar uma célula dopaminérgica devemos saber suas características fisiológicas, bioquímicas e morfológicas. Os neurônios dopaminérgicos são um subtipo de neurônio catecolaminérgico, que secreta dopamina. Dessa forma, é importante conhecer a síntese deste neurotransmissor bem como os processos sinápticos, pois as proteínas envolvidas nestes processos podem servir como marcadores (Chinta & Andersen, 2005). Os neurônios dopaminérgicos maduros, além de possuírem marcadores neuronais comuns a outros neurônios, apresentam alguns marcadores específicos de células dopaminérgicas que serão discutidos a seguir (Liss & Roeper, 2008).

Exemplos de marcadores de neurônios maduros inespecíficos são enolase neurônio específica (ENS), proteína nuclear de neurônio (NeuN), sinaptobrevina e sinaptofisina. A ENS é uma enzima glicolítica de três subunidades *α, β* e *γ*. Sua subunidade *γ* é expressa primariamente em neurônios diferenciados (Kume *et al.,* 2008). Já a NeuN é uma proteína expressa na maioria dos neurônios. Sua imunorreatividade é observada em neurônios diferenciados (pós-mitóticos), não havendo sinal em zonas proliferativas (Weyer & Shiling, 2003). A sinaptobrevina e a sinaptofisina são proteínas integrais de membrana localizada nas vesículas

sinápticas de neurônios maduros e estão relacionadas com a liberação de neurotransmissores. Estas proteínas co-localizam junto aos neuritos (Sarkanen *et al.,*2007). Há também marcadores de célula indiferenciada da linhagem neuronal, como a nestina. Esta proteína é um filamento intermediário, responsável pela organização do citoesqueleto. A nestina sofre *down-regulation* quando os neuroblastos interrompem os processos de divisão celular e sofrem processos de diferenciação (Thomas *et al.,* 2004).

Entretanto, para verificarmos a origem dopaminérgica de um neurônio, devemos conhecer a síntese do neurotransmissor dopamina. Ele é sintetizado a partir do aminoácido aromático tirosina. No primeiro passo da reação, a tirosina hidroxilase (TH), uma enzima limitante da síntese de catecolaminas, catalisa a produção de 3,4-dihidroxi-*L*-fenilalaina (*L*-DOPA). As reações subseqüentes são catalisadas pela L-aminoácido descarboxilase e pela dopamina-β-hidroxilase, que formam a dopamina (Lawlor & During, 2004). Outro marcador dopaminérgico importante é o DAT, responsável pela recaptação do neurotransmissor na fenda sináptica (Presgraves *et al.,* 2007).

Ainda, quanto à fisiologia, os neurônios da SNpc, bem como em todo o SNC, apresentam baixos níveis de antioxidantes (Jenner, 2003). Há uma baixa atividade de catalase (CAT), superóxido dismutase (SOD) e a glutationa peroxidase (GPx) (Halliwell, 2006). O principal antioxidante do SNC é a GSH. Por isso, para mimetizar a fisiologia neuronal, os modelos experimentais de células dopaminérgicas devem apresentar baixos níveis de antioxidantes (Beal, 2010).

Os modelos experimentais *in vivo* são amplamente utilizados e são excelentes para avaliação da progressão da doença bem como patologia. Exemplos desse tipo de modelo são *Drosophila*, ratos e macacos. Os animais são tratados

com toxinas para induzir o distúrbio ou utilizam-se modelos genéticos (*knockout* para proteínas, como a α-sinucleína) (Beal, 2010). Entretanto, é difícil transpor os resultados dos estudos em modelos *in vivo* para mecanismos moleculares e celulares da DP. Além disso, há uma grande variabilidade intra-espécie e muitos fatores podem influenciar no estudo, por exemplo, o estresse (Bove *et al.,* 2005). Por isso, são utilizados modelos *in vitro*, como o cultivo celular. Este tipo de modelo pode ser dividido em três áreas: culturas primária, organotípica e de linhagens (Scülle *et al.,* 2009).

A cultura primária deriva de animais. Para preparar este tipo de modelo, utilizam-se embriões. A parte ventral do mesencéfalo é retirada e, após o isolamento, as células dopaminérgicas são colocadas em meio de cultura específico (Han *et al.,* 2003). Os neurônios rapidamente diferenciam e formam neuritos e sinapses (Falkenburger & Schulz, 2006). Apesar de apresentarem vantagens quanto à morfologia e à bioquímica, este tipo de cultura apresenta algumas limitações. Primeiramente, a praticidade do modelo é muito baixa, devido ao longo tempo de processamento. Este tipo de cultura tem uma grande variabilidade e por isso há uma baixa reprodutibilidade dos dados. Ainda, o isolamento de neurônios dopaminérgico é pouco rentável visto que somente 5-10% das células são TH-positivas (Liu *et al.* 2008). Por fim, como o isolamento não é muito eficaz, há uma grande contaminação de células gliais (Falkenburger & Schulz, 2006).

Outro exemplo são as culturas organotípicas. Elas são definidas como um modelo *in vitro* que mantém as interações celulares *in vivo* e a estrutura tridimensional (Studer, 2001). Uma vantagem deste tipo de modelo é a manutenção da via nigroestriatal (Cavaliere *et al.*, 2010). Para esta cultura também são utilizados os animais neonatos, de onde o cérebro é retirado. Este é cortado de forma

transversal e colocado em meio de cultura específico. Os cortes são mantidos durante de 10-12 dias (Stahl *et al.,* 2009). No entanto, é difícil a realização de ensaios de viabilidade celular bem como verificar produção de dopamina nesta cultura (Falkenburger & Schulz, 2006)

Devido às desvantagens destes modelos *in vitro*, uma alternativa é o uso de linhagens celulares. Elas são células imortalizadas e podem ser mantidas em cultura em um grande período de tempo devido à capacidade de proliferação. As principais vantagens quando comparados com os demais modelos é a praticidade e a reprodutibilidade, pois a população celular é homogênea (Schülle *et al.,* 2009). PC12 (feocromocitoma de rato) e MN9D (neuroblastoma de camundongo) são exemplos de linhagens celulares usadas como modelo de células dopaminérgicas, por secretarem dopamina (Choi *et al.,* 1992; Lee *et al.,* 2006). Entretanto, a linhagem de neuroblastoma humano SH-SY5Y é a linhagem mais utilizada na literatura para o estudo da DP.

Os neuroblastomas são tumores malignos neuroendócrinos, onde os sítios primários são localizados nas glândulas adrenais. Estas células secretam catecolaminas, que são usadas como marcadores tumorais na clínica para o diagnóstico e monitoramento deste câncer. Por isso, estas células podem ser utilizadas como modelos *in vitro* para DP (Howman-Giles *et al.,* 2007).

A linhagem de neuroblastoma humano SH-SY5Y é um subclone derivado do neuroblastoma SK-N-SH e são derivadas de sítio metastático de medula óssea. Estas células, quando em cultura, se assemelham com neuroblastos do sistema simpático, apresentando morfologia epitelial com citoplasma escasso (Biedler *et al.,* 1978). Células desta linhagem estão estacionadas nos estágios iniciais da diferenciação neuronal e são caracterizadas bioquimicamente pela escassez de

marcadores neuronais (Conroy & Berg, 1995; Gilany *et al.,* 2008). Além disso, estas células expressam baixos níveis de enzimas da via de síntese das catecolaminas, como a TH e a dopamina β-hidroxilase. (Xie *et al.,* 2010).

As células SH-SY5Y podem ser definidas como neuroblastos imaturos que proliferam durante um grande período de tempo. Isso é uma grande desvantagem do modelo, pois os neurônios apresentam baixas taxas de proliferação (Luchtman & Song, 2010). Apesar da origem tumoral, muitos estudos mostraram que as células de neuroblastoma SH-SY5Y podem ser diferenciadas em neurônios dopaminérgicos através do tratamento com ácido retinóico (AR) (Pahlman *et al.*, 1984).

O AR é a forma biologicamente ativa da vitamina A necessária para o desenvolvimento normal, incluindo apoptose, diferenciação e morfogênese de diversos órgãos e sistemas, como o sistema nervoso (Bain *et al.,* 1995). O papel desta neurotrofina na diferenciação neuronal é mediado através de receptores ao AR e receptores retinóides X (Mark *et al.*, 2006). *In vitro*, o AR promove a diferenciação e inibe a divisão celular, bloqueando a fase G1/S do ciclo celular (Malik *et al.,* 2000). Desta forma, o AR regula a transição das células precursora para a célula diferenciada pós-mitótica (Ross *et al.,* 2000), como ilustra a Figura 4.

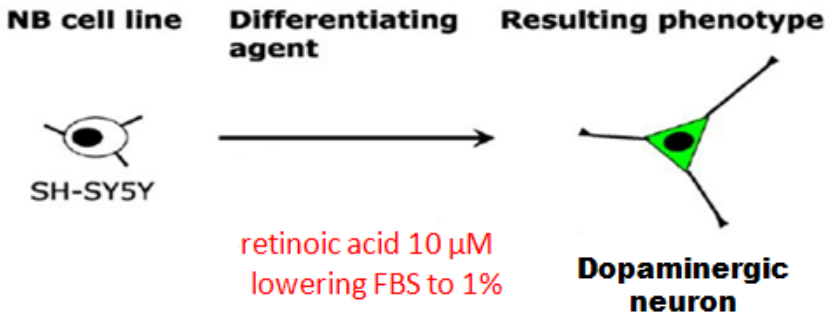

Figura 4: Diferenciação *in vitro* do neuroblastoma humano SH-SY5Y induzida por AR (Adaptada de Edsjö *et al.*, 2007).

A indução do processo de diferenciação ocorre pelo aumento da produção de catecolaminas, pelo aumento da expressão da enzima enolase e pela expressão de fatores de crescimento. Isso leva à formação de projeções citoplasmáticas (neuritos), induzindo a células a modificar a morfologia epitelial para a estrelada. Ainda, o processo de diferenciação acarreta em aumento do imunoconteúdo de marcadores dopaminérgicos, como TH e DAT (Edsjö *et al.*, 2007).

Apesar da diferenciação induzida por AR estar estabelecida, há poucos estudos mostrando as diferenças entre células proliferativas e diferenciadas desta linhagem. Além disso, embora a diferenciação produza um fenótipo dopaminérgico, não há aplicação deste modelo para estudo da DP. Por fim, como não há um consenso dos protocolos de diferenciação apresentados na literatura, existe a necessidade de estabelecer as melhores condições da diferenciação dopaminégica do neuroblastoma humano SH-SY5Y (Xie *et al.,* 2010).

2. OBJETIVOS

2.1 Objetivo Geral

O objetivo deste estudo foi estabelecer um protocolo de diferenciação dopaminérgica da linhagem de neuroblastoma humano SH-SY5Y e demonstrar a aplicação potencial do fenótipo diferenciado como modelo *in vitro* para o *screening* neurotoxicidade/neuroproteção e a possibilidade de manipulação genética.

2.2 Objetivos Específicos

- estabelecimento das melhores condições experimentais para a diferenciação dopaminérgica da linhagem de neuroblastoma humano SH-SY5Y;
- caracterização das diferenças morfológicas (presença de neuritos) e neuroquímicas (marcadores de diferenciação neuronal, como DAT, TH, NeuN, ENS e nestina) em células proliferativas e em diferenciadas da linhagem de neuroblastoma humano SH-SY5Y;
- determinação de parâmetros redox em células proliferativas e diferenciadas da linhagem de neuroblastoma humano SH-SY5Y;
- determinação da citotoxicidade (DL_{50}) da 6-OHDA e do H_2O_2 em células proliferativas e diferenciadas da linhagem de neuroblastoma humano SH-SY5Y e a contribuição do sistema Glutationa Redutase (GR) e Tioredoxina Redutase (TrxR) na defesa celular frente a ambos insultos;
- demonstrar o uso potencial do fenótipo diferenciado da linhagem de neuroblastoma humano SH-SY5Y como modelo *in vitro* para o *screening* de compostos neurotóxicos/neuroprotetores;
- estabelecer das condições experimentais para a transfecção e superexpressão de genes de interesse na linhagem diferenciada de neuroblastoma humano SH-SY5Y.

PARTE II

3. RESULTADOS

Os principais resultados dessa dissertação estão apresentados na forma de artigos científicos e dados suplementares (Capítulos I, II e Resultados Suplementares I e II).

CAPÍTULO I: Manuscrito publicado no periódico *Brain Research* em 06/2010

"Comparison Between Proliferative and Neuron-like SH-SY5Y Cells as an In Vitro Model for Parkinson Disease Studies"

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BRAIN RESEARCH

Research Report

Comparison between proliferative and neuron-like SH-SY5Y cells as an *in vitro* model for Parkinson disease studies

Fernanda Martins Lopes[a,b], *Rafael Schröder*[a], *Mário Luiz Conte da Frota Júnior*[a], *Alfeu Zanotto-Filho*[a], *Carolina Beatriz Müller*[a,b], *André Simões Pires*[a], *Rosalva Thereza Meurer*[c], *Gabriela Delevati Colpo*[b,d], *Daniel Pens Gelain*[a], *Flávio Kapczinski*[b,d], *José Cláudio Fonseca Moreira*[a], *Marilda da Cruz Fernandes*[c], *Fabio Klamt*[a,b,*]

[a]*Department of Biochemistry, ICBS/UFRGS, Porto Alegre/RS, Brazil*
[b]*National Institute for Translational Medicine (INCT-TM), Porto Alegre/RS, Brazil*
[c]*Laboratory of Pathology Research, UFCSPA, Porto Alegre/RS, Brazil*
[d]*Molecular Psychiatry Laboratory, HCPA/UFRGS, Porto Alegre/RS, Brazil*

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ABSTRACT

The molecular mechanisms underlying the cellular lost found in the nigrostriatal pathway during the progression of Parkinson's disease (PD) are not completely understood. Human neuroblastoma cell line SH-SY5Y challenged with 6-hydroxydopamine (6-OHDA) has been widely used as an *in vitro* model for PD. Although this cell line differentiates to dopaminergic *neuron-like* cells in response to low serum and retinoic acid (RA) treatment, there are few studies investigating the differences between proliferative and RA-differentiated SH-SY5Y cells. Here we evaluate morphological and biochemical changes which occurs during the differentiation of SH-SY5Y cells, and their responsiveness to 6-OHDA toxicity. Exponentially growing SH-SY5Y cells were maintained with DMEM/F12 medium plus 10% of fetal bovine serum (FBS). Differentiation was triggered by the combination of 10 μM RA plus 1% of FBS during 4, 7 and 10 days in culture. We found that SH-SY5Y cells differentiated for 7 days show an increase immunocontent of several relevant neuronal markers with the concomitant decrease in non-differentiated cell marker. Moreover, cells became two-fold more sensitive to 6-OHDA toxicity during the differentiation process. Time course experiments showed loss of mitochondrial membrane potential triggered by 6-OHDA (mitochondrial dysfunction parameter), which firstly occurs in proliferative than *neuron-like* differentiated cells. This finding could be related to the increase in the immunocontent of the neuroprotective protein DJ-1 during differentiation. Our data suggest that SH-SY5Y cells differentiated by 7 days with the protocol described here represent a more suitable experimental model for studying the molecular and cellular mechanisms underlying the pathophysiology of PD.

* *Corresponding author*. Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul (UFRGS). Rua Ramiro Barcelos, 2600. Porto Alegre (RS), Brazil. 90035-003.
E-mail address: 00025267@ufrgs.br (F. Klamt).

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1. Introduction

Parkinson disease (PD) is one of the most common neurodegenerative disorders, affecting about 2% of the population over the age of 60 (Lo Bianco et al., 2004). This chronic disturb causes severe motor dysfunction, such as bradykinesia, resting tremor, rigidity, postural instability, and also affects autonomic function and cognition (Poewe, 2008; Lesage and Brice, 2009). Pathologically, it is associated with the profound loss of dopamine-producing neurons in the *substantia nigra pars compacta* and the presence of Lewy bodies in affected regions of the central nervous system (Schapira, 2008). Although several factors, including mitochondrial dysfunction, oxidative stress and apoptosis have been suggested to contribute to cell death in PD, its etiology remains unknown (Prabhakara et al., 2008).

6-hydroxydopamine (6-OHDA) is the most used toxin in experimental models of PD (Gomez-Lazaro et al., 2008; Ikeda et al., 2008; Mu et al., 2009). Because this neurotoxin has similar structure to dopamine, it shows high affinity for the dopamine transporter and for this reason selectively destroys dopaminergic/catecholaminergic neurons (Lehmensiek et al., 2006). Once inside the neuron, 6-OHDA accumulates and undergoes non-enzymatic auto-oxidation, promoting free radical formation (Bladini et al., 2008). The inhibitory effect over complex I activity in mitochondria also accounts for the described mechanism of reactive oxygen species (ROS) generation by this neurotoxin (Lehmensiek et al., 2006; Inden et al., 2006; Chin et al., 2008). Moreover, 6-OHDA induces cell death of human neuroblastoma SH-SY5Y (Jordan et al., 2004) and mouse pheochromocytoma PC12 cell lines (Nie et al., 2002) and selectively kills tyrosine hydroxylase (TH)-immunoreactive neurons in *substantia nigra* and *striatum* in animal models of intranigral-administration (Inden et al., 2006).

Exponentially growing SH-SY5Y cells treated with 6-OHDA are often used as an *in vitro* model for PD (Hwang and Jeong, 2008; Lev et al., 2008). This cell line is a human catecholaminergic neuroblastoma derived from SK-N-SH, which resembles immature sympathetic neuroblasts in culture (Biedler et al., 1978). These cells are typically locked in an early neuronal differentiation stage, characterized biochemically by the low presence of neuronal markers (Biedler et al., 1978; Gilany et al., 2008). In this regard, the proliferative SH-SY5Y cells do not represent a suitable experimental model for studying the molecular and cellular mechanisms underlying the pathophysiology of PD, a disease that affects primarily differentiated dopaminergic neurons (Schapira, 2008).

In spite of that, many lines of evidence have indicated that human neuroblastoma SH-SY5Y cells are able to acquire neuron-like phenotypes with neurite outgrowth and branches by all-*trans*-retinoic acid (RA) treatment (Pahlman et al., 1984; Miloso et al., 2004). RA is essential in embryonic development and maintenance of growth and differentiation of epithelial, fibroblastic and myelomonocytic cells (Bastien and Rochette-Egly, 2004). Hence, RA controls cellular differentiation processes by modulating the expression of several RA-responsive genes by the activation of retinoic acid/retinoid nuclear receptors (Mark et al., 2006). *In vitro*, RA also plays a role in regulating transition from the proliferating precursor cell to post-mitotic differentiated cell (López-Carballo et al., 2002). Although some previous reports have addressed the RA-differentiation process in SH-SY5Y cells (López-Carballo et al., 2002; Savickiene et al., 2009), there are few studies that compare the changes in neuronal markers in SH-SY5Y cells undergoing the differentiation process, and their cellular response to 6-OHDA (Cheung et al., 2009).

Here we evaluate morphological, biochemical and cytotoxic parameters related to human neuroblastoma SH-SY5Y cells differentiated by RA treatment and challenged with 6-OHDA. Our results suggest that SH-SY5Y cells differentiated by 7 days with 1% FBS and RA represent a more suitable experimental model for studying the molecular and cellular mechanisms underlying the pathophysiology of PD.

2. Results

2.1. *Cell morphology analysis*

To demonstrate that SH-SY5Y cells can be differentiated to a *neuronal-like* phenotype with the combination of lowering fetal bovine serum (FBS) and RA treatment, we first analyzed changes in cell morphology. We observed a complete decrease in cellular proliferation rate (data not shown) and the induction of extensive neurites outgrowth by the

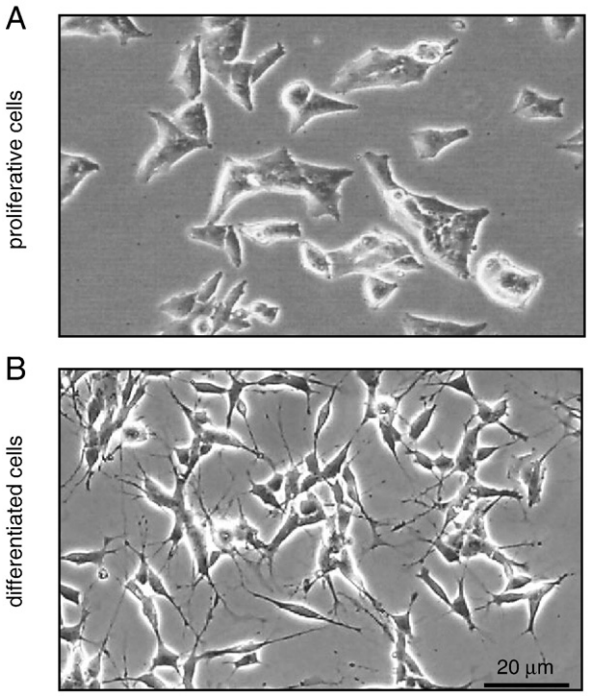

Fig. 1 – Morphological parameter of human neuroblastoma SH-SY5Y cells differentiated with retinoic acid. Representative phase contrast images of proliferative SH-SY5Y cells in culture medium with 10% of FBS (A). Representative phase contrast images of RA-differentiated human neuroblastoma cell line SH-SY5Y cultured for 7 days with 10 µM RA in culture medium with 1% of FBS (B). Note the stellate morphology and the abundance of neurites in differentiated cells (400× magnification).

differentiation protocol of SH-SY5Y cells (Fig. 1). This change in morphology was observed as early as 24 h of treatment. No further differences were observed during the differentiation process (4, 7 and 10 days). Exponentially growing cells maintained a typical epithelial morphology (Fig. 1A). Fig. 1B shows a representative image of SH-SY5Y cells over 7 days of differentiation.

2.2. *Western blot and immunocytochemistry*

Changes in specific neuronal and non-differentiated cell markers during the differentiation process were evaluated by Western Blot immunoassay. A significant increase in the immunocontent of the neuronal markers TH, neuron specific enolase (NSE) and neuronal nuclei protein (NeuN) in differentiated cells (4, 7 and 10 days of differentiation) as compared to proliferative cells (representative western blots in Fig. 2A, and respective densitometry analysis in Fig. 2B) were found. On the other hand, a significant decrease in the non-differentiated cell marker nestin was observed only in 7 and 10 days of differentiation (Fig. 2). Based on these data, we re-evaluate the differentiation cells markers through immunocytochemistry and western blot analysis in proliferative and 7- day RA-differentiated SH-SY5Y cells (Fig. 3). These data confirmed that only 7-day RA-differentiated cells expressed TH, NSE and NeuN, with the concomitant decrease in nestin immunocontent (Fig. 3). Insert boxes show the intense cytosolic immunoreactivities of NSE and TH in differentiated cells and nestin in proliferative cells, and nuclear immunoreactivity of NeuN in differentiated cells (Fig. 3A).

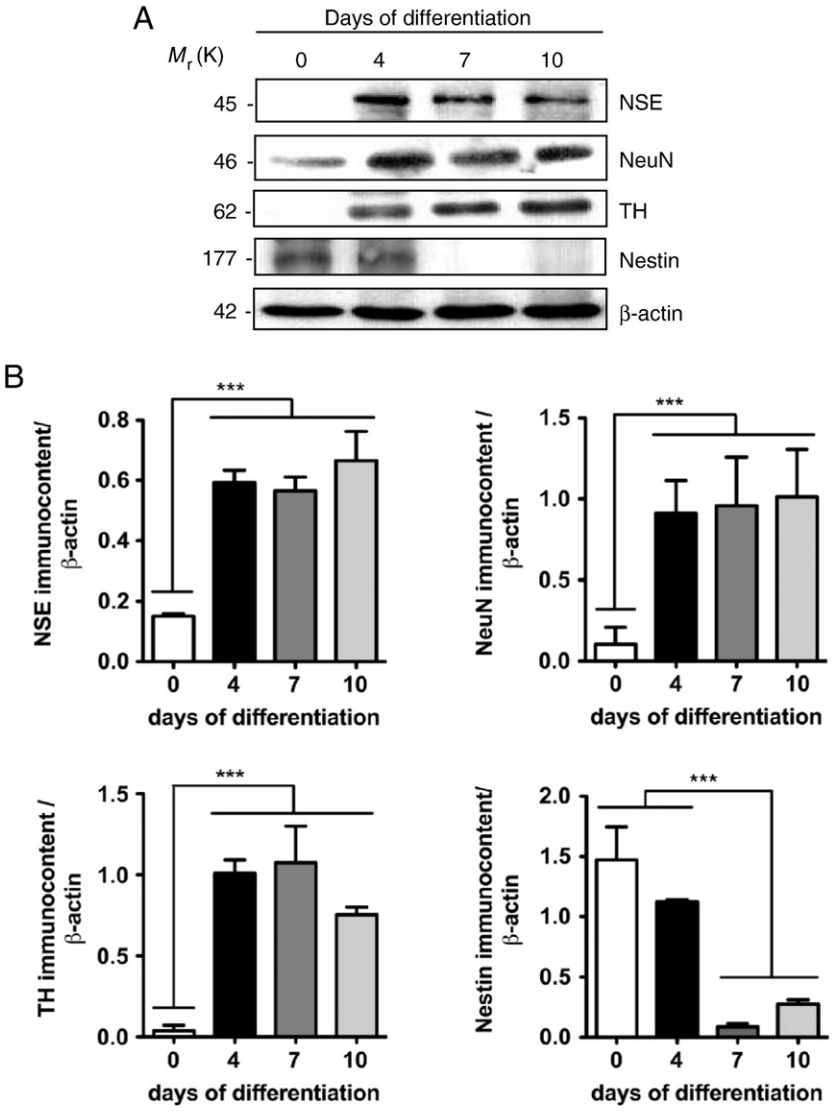

Fig. 2 – Changes in the immunocontent of selected neuronal and non-differentiated cell markers in human neuroblastoma cell line SH-SY5Y during 4, 7 and 10 days of differentiation. (A) Representative immunoblots of the neuronal markers neuron-specific enolase (NSE), neuronal nuclei protein (NeuN) and tyrosine hydroxylase (TH) and the non-differentiated marker nestin. (B) Densitometric analysis of bands representing means ± SD of three independent experiments. β-actin was used as loading control, as described in experimental procedure section. ***Statistically different from the corresponding control values, $p < 0.001$ (one-way analysis of variance).

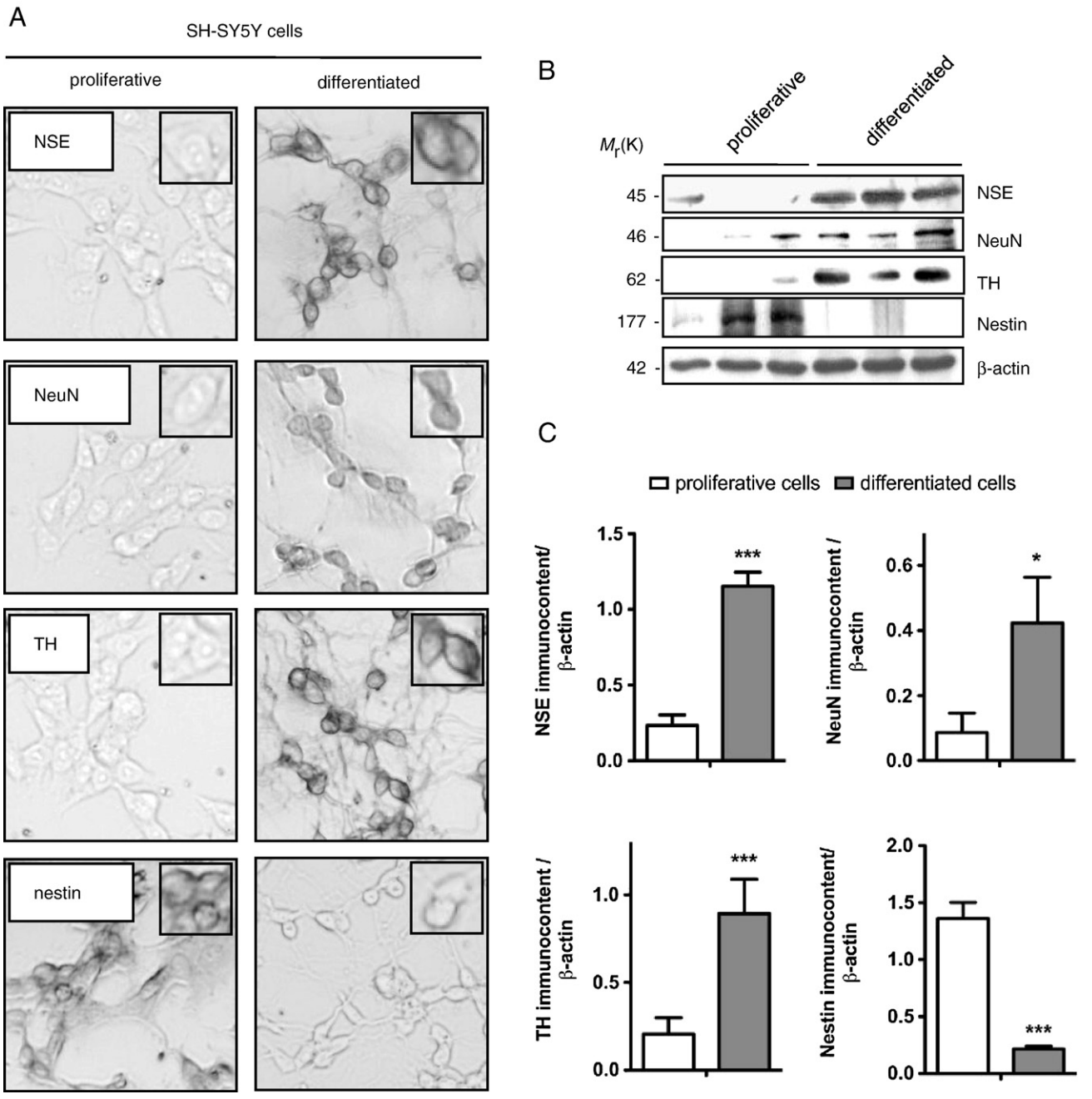

Fig. 3 – Immunocytochemistry and Western blot analysis of selected neuronal and non-differentiated cell markers in proliferative and 7-days-differentiated human neuroblastoma cell line SH-SY5Y. (A) Representative immunocytochemistry images (Insert boxes: representative images with higher magnification) and (B) western blots showing the immunocontent in triplicates of neuronal and non-differentiated markers. (C) Densitometric analysis of bands representing means ± SD of three independent experiments. β-actin was used as loading control, as described in experimental procedure section. *Statistically different from the corresponding control values, $p<0.05$; ***$p<0.001$ (Student t test).

2.3. *6-OHDA cytotoxicity*

After exploring the differences between proliferative and RA-differentiated SH-SY5Y cells, we examined their susceptibility to 6-OHDA. Proliferative and differentiated SH-SY5Y cells were exposed to different 6-OHDA concentrations for 24 h and drug neurotoxicity was assessed. To establish the optimal experimental dosage, we first plotted a dose-response curve (Fig. 4A). We found that RA-differentiated cells are more susceptible to this neurotoxin. However, in 4 days of differentiation, the drug GI_{50} value is not distinct from proliferative cells. Only in 7 and 10 days of RA-differentiation, the drug GI_{50} value had a significant decrease (i.e., increased sensitivity) compared to proliferative cells. Based on these data, we next decided to run the ensuing experiments using 35 or 15 μM 6-OHDA in proliferative and in cells with 7 days of differentiation,

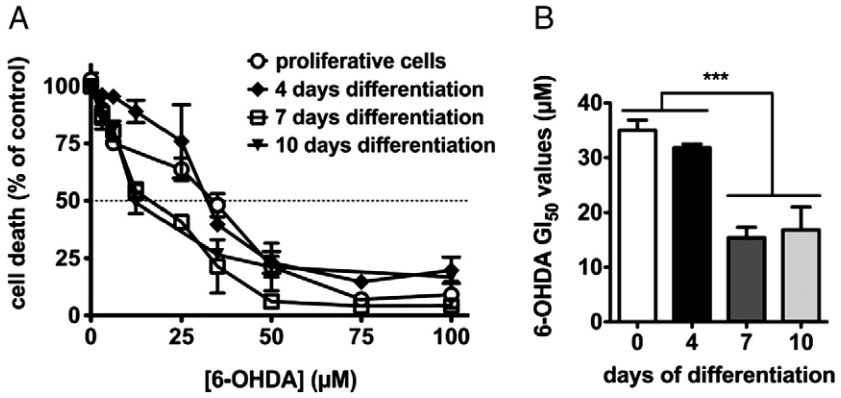

Fig. 4 – Differences in sensitivity of proliferative and differentiated human neuroblastoma cell line SH-SY5Y to the 6-OHDA neurotoxin. Cells were treated with different concentrations of 6-OHDA for 24 h, and the drug cytotoxicity was evaluated using the MTT assay, as described in experimental procedure section. (A) Cytotoxicity curves for 6-OHDA. (B) GI_{50} value for 6-OHDA obtained during the different days of differentiation. The data represent means ± SD for three experiments carried out in quadruplicates. ***Statistically different from the corresponding control values, $p < 0.001$ (one-way analysis of variance).

respectively, which were the drug concentration that reduced cell viability about 50% (drug GI_{50} value).

2.4. *Mitochondrial membrane potential and DJ-1 immunocontent*

6-OHDA treatments induce loss of mitochondrial membrane potential in both proliferative and differentiated SH-SY5Y cells (Fig. 5A). Interestingly, even though the proliferative cells are more resistant to this neurotoxin (higher GI_{50} value), time course experiments showed that mitochondrial depolarization firstly occur in proliferative than *neuron-like* differentiated cells challenged with 6-OHDA (3 and 6 h of treatment, respectively) (Fig. 5A). In order to further investigate the molecular mechanism involved in the delay in mitochondria depolarization found in differentiated cells, we examined the levels of the neuroprotective protein DJ-1 in both experimental groups. We found an increase in DJ-1 levels during the differentiation process. To confirm these data, we performed another western blot in proliferative and 7-day RA-differentiated SH-SY5Y cells. Differentiated cells expressed significantly higher amount of this neuroprotective protein (Fig. 5B).

3. Discussion

The etiology of Parkinson disease is still not fully understood, but genetic analyses, epidemiologic studies and development of new *in vitro* experimental models of PD will potentially provide important new insights into the pathogenesis of PD (Dawson and Dawson, 2003). While primary rat midbrain cell cultures have been widely used to study the biochemical cascades involved in PD, they are not derived from human source and they present only 6 - 15% TH-positive neuronal cells (Liu et al., 2008), which limits the translation of potential findings into the pathophysiology of PD. Due to the difficulty to find a suitable and accessible model for PD studies, exponentially growing human neuroblastoma or rat pheochromocytoma cell lines (e.g. SH-SY5Y and PC12) are often used to study the idiopathic PD with respect to molecular and neurochemical parameters (Lee et al., 2002; Gomez-Lazaro et al., 2008). However, these cell lines are tumor cells, so that they have oncogenic and mitogenic properties (Biedler et al., 1978).

The novel findings presented here are that the human neuroblastoma SH-SY5Y cell line differentiates into a dopaminergic *neuron-like* by the combination of lowering the FBS to 1% and 10 μM RA treatment for 7 days, leading to the acquisition of all desired morphological and biochemical characteristics of an *in vitro* cellular model to study PD. Moreover, during the differentiation process this cell line presents an increase in DJ-1 protein immunocontent, a neuroprotective protein that has been extensively related to an early onset of PD.

Even though the differentiation properties of retinoic acid over human neuroblastoma cell line SH-SY5Y have been widely investigated (Miloso et al., 2004; Guarnieri et al., 2009), few works explored these features to be used as an *in vitro* model for PD research (Tieu et al., 1999; Cheung et al. 2009). To address whether RA-differentiated SH-SY5Y cells are functionally mature, our study showed the detailed characteristics of proliferative and RA-differentiated SH-SY5Y through the immunoreactivity of several neuronal markers, cellular morphology and response to 6-OHDA treatment.

We observed an increase of the neuronal markers TH, NSE and NeuN, starting at 4 days of RA-dependent differentiation process. TH, a classical dopaminergic cell marker, has low immunocontent and is not active in proliferative SH-SY5Y cells. TH is the rate-limiting enzyme in catecholamine synthesis, hence, these cells do not secrete dopamine (Biedler et al., 1978; Seitz et al., 2000). RA treatment induces TH expression and activates phosphatase 2A, which dephosphorylates and activates TH, turning them into dopamine-producing cells (Pahlman et al., 1984; Dunkley et al., 2004). Therefore, our findings are in agreement to these data. However, this subject is still controversial since there are few studies showing the expression of dopaminergic cells markers in proliferative cells (Cheung et al., 2009; Guarnieri et al., 2009). Therefore, another neuronal marker studied here was NSE. The differentiation protocol causes an increase of NSE immunocontent and its activity, reinforcing that RA-differentiated SH-SY5Y

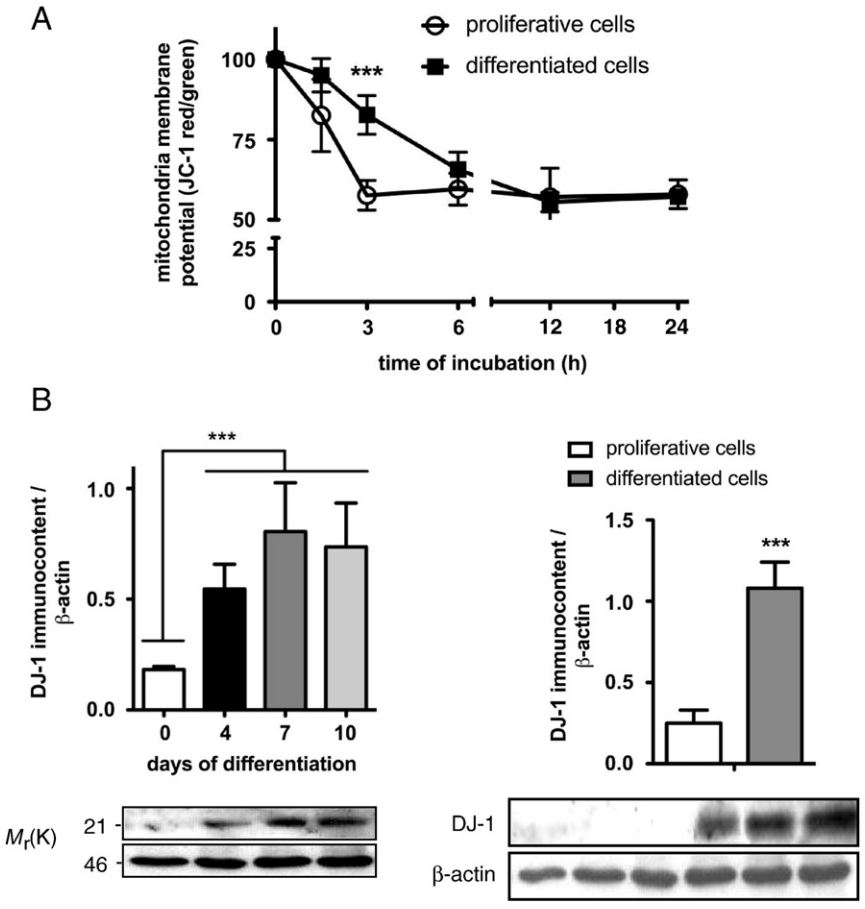

Fig. 5 – Loss of membrane potential in proliferative and differentiated cells of the neuroblastoma cell line SH-SY5Y challenged with 6-OHDA and the DJ-1 protein immunocontent. (A) Cell were treated with respective 6-OHDA GI_{50} value, harvested and incubated with JC1 (10 μg/mL) at 37 °C for 15 min. The data represent means ± SD for three experiments carried out in quadruplicate. ***Statistically different from the corresponding control values, $p < 0.001$ (one-way analysis of variance). (B) DJ-1 immunocontent of proliferative and 7 days differentiated SH-SY5Y cells. The figure represents immunoblots showing representative high DJ-1 expression in differentiation cells. The graph shows the quantification of DJ-1 immunocontent. ***Statistically different from the corresponding control values, $p < .001$ (one-way analysis of variance). (B) Densitometric analysis of DJ-1 immunocontent bands representing means ± SD of three independent experiments. β-actin was used as loading control, as described in experimental procedure section. ***Statistically different from the corresponding control values, $p < 0.001$ (one-way analysis of variance/Student *t* test).

cells acquired a more neuronal phenotype (Pahlman et al., 1984; Cheung et al., 2009). Moreover, we also observed an increase in NeuN, which is only expressed in neurons upon maturation, and therefore served as a neuronal marker (Weyer and Schilling, 2003; Cheung et al., 2009). Although we found an increase in the immunocontent of all these neuronal markers as early as 4 days of differentiation, there is an equal amount of nestin protein in proliferative and in 4 days differentiated SH-SY5Y cells. After 7 days of RA differentiation, the expression of nestin decreases significantly, keeping this cellular profile through 10 days of treatment. Nestin, an intermediate filament, is expressed in a cell-cycle-dependent manner. Nestin is down-regulated as neuroepithelial stem cells cease division and differentiate along their respective neuronal lineages (Hockfield and McKay, 1985; Fuchs and Weber, 1994; Thomas et al., 2004).

Immunocytochemistry assay confirmed the changes in the immunocontent of the cells markers. We detected immunoreactivity of NSE and TH in the cytoplasm of RA-differentiated cells and the NeuN immunoreactivity was verified in the nuclear compartment. On the other hand, nestin immunoreactivity was found in the cytoplasm of proliferative cells. Our data are not in agreement with a previous study that showed immunoreactivity of NSE and TH in both cytoplasm and nucleus (Cheung et al., 2009). In spite of that, there are few studies showing that these are cytosolic proteins (Odelstad et al., 1981; Dunkley et al., 2004).

As previously shown, SH-SY5Y cells must be differentiated *in vitro* at least 7 days to show mature excitability, action potential propagation and formation of synaptic vesicles (Sarkanen et al., 2007, Cheung et al., 2009). These data are in agreement with our findings. Therefore, our study confronts previous data that suggested there are limited changes in dopaminergic markers immunocontent after RA differentiation (Cheung et al., 2009).

After characterizing the differences between these experimental models, we analyzed their responsiveness to 6-OHDA. Proliferative and 4-day-RA-differentiated SH-SY5Y cells responded equally to this neurotoxin. In 7 and 10 days of differentiation, we found an increased sensitivity to this neurotoxin. The only dramatic difference between 4-, 7- and 10-day RA-differentiated cells found here was in nestin immunocontent. Previous studies showed that the reduction of nestin expression by siRNA resulted in clearly vulnerability to oxidant-induced (H_2O_2) cell death in neuronal progenitors (Sahlgren et al., 2006). Furthermore, this protein has been associated with tumor aggressiveness (Weggen et al., 1997), and downregulation of nestin in neurons activates Cdk5/p35-dependent apoptosis, suggesting that nestin serves as a survival factor, protecting neuronal progenitors from stress-induced cell death (Thomas et al., 2004; Reimer et al., 2009). So that, the role of nestin in the resistance to 6-OHDA remains to be described.

In contrast to our data, there are studies showing that RA-differentiated SH-SY5Y cells are more resistant to neurotoxins like 6-OHDA (Tieu et al., 1999; Cheung et al., 2009). Tieu et al. showed that RA-differentiated SH-SY5Y cells decreased p53 protein (proapoptotic), thus resulting in vulnerability to 6-OHDA. They discuss this data based on both proliferative and RA-differentiated SH-SY5Y cells have an inactive p53, which is sequestered in the cytoplasm (Moll et al., 1995). Nevertheless, another study verified that transcriptional active p53 is found in the nucleus of RA-differentiated cells (Chen et al., 2007). Hence, the association between p53 and neurotoxin resistance suggested by this study is not known. Moreover, Cheung et al. also showed that RA-differentiated SH-SY5Y cells are less susceptible to the 6-OHDA cytotoxicity, probably because of the up-regulation of the survival signaling pathways, including the increase of Akt and Erk1/2 expression. Based on these, they suggest that the best experimental model for study PD is proliferative cell. The data present here support the opposite conclusion.

Even though proliferative cells present a two-fold higher GI_{50} value for 6-OHDA, time course experiments showed that mitochondrial depolarization, a marker of organelle dysfunction, firstly occurs in proliferative (3 h) than in *neuron-like* differentiated cells (6 h) once challenged with 6-OHDA. These data are in agreement with another study that showed a collapse of the mitochondrial membrane potential in proliferative SH-SY5Y cells rapidly after 6-OHDA treatment (Ikeda et al., 2008). This study also showed that 6-OHDA induces the formation of intracellular reactive oxygen species (ROS). The resultant oxidative stress triggers the activation of the p38 MAPK cascade, leading to the lost of $\Delta\Psi_m$ and cytochrome *c* release, which results in caspase 9- and 3-dependent apoptosis. On the other hand, there is no data showing the effect of 6-OHDA in mitochondrial dysfunction in RA-differentiated cells.

Differences in mitochondria depolarization kinetics trigged by 6-OHDA suggest that differentiated cells present an extra mitoprotective feature against this neurotoxin. This finding could be related to the significant increase in the levels of the protein DJ-1 during differentiation treatment found here. DJ-1 protein belongs to the DJ-1/ThiJ/PfpI superfamily of proteins, which are conserved in many different organisms (Mizote et al. 1999). Mutations in this protein have been strongly related to an early onset of PD (Bonifati et al., 2003). It was demonstrated that the expression of DJ-1 can be induced by ROS (Sekito et al., 2006), and their mutant forms make the neuron more susceptible to the cell death induced by oxidative stress (Xu et al., 2005; Miyazaki et al., 2008). DJ-1 is exclusive cytosolic, however, upon oxidative damage this protein translocates to the mitochondria (Zhang et al., 2005). So, DJ-1 plays a role in mitochondrial maintenance (Zhong and Xu, 2008). Hence, the expression of this protein protects the mitochondria of differentiated cells from the 6-OHDA toxicity and delays mitochondria depolarization (REF), but the neuroprotective mechanism of DJ-1 over the mitochondria remains to be described. Furthermore, previous studies showed the increase of Akt in RA-differentiated cells (López-Carballo et al., 2002; Cheung et al., 2009). Cheung et al. associated the increase of Akt immunocontent with the resistance to 6-OHDA in differentiated cells. Since it is known that DJ-1 can activate Akt (Clements et al., 2006), this mechanism could be related to the delay in mitochondria depolarization found here in differentiated cells challenged with 6-OHDA. More studies are necessary to explore the role of DJ-1/Akt in 6-OHDA-induced neuronal dysfunction.

Hence, our data suggest that 7-day-RA-differentiated form of SH-SY5Y cells represents a more suitable experimental model for studying the molecular and cellular mechanisms underlying the pathophysiology of PD.

4. Experimental procedures

4.1. *Reagents*

Materials used in cell culture were acquired from Gibco®/Invitrogen (São Paulo, SP Brazil). Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

4.2. *Cell culture and differentiation*

Exponential growing human neuroblastoma cell line SH-SY5Y, obtained from ATCC (Manassas, VA, USA), were maintained in a mixture 1:1 of Ham's F12 and Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM of glutamine, 0.28 μg/μL of gentamicin and 250 μg of amphotericin B, in a humidified atmosphere of 5% of CO_2 in air at 37 °C. Cell medium were replaced each 3 days and the cells were sub-cultured once they reached 90% confluence. All treatments were performed when cells were ~75% confluence. For the immunocytochemistry, MTT assay and JC-1 fluorescence, the cells were plated in a 24-well plated at density of 6×10^4 cells per well. For western blot analysis, the cells were seeded into bottles of 75 cm^3 at density of 5×10^6 cells. After 24 h of cell plating, differentiation was induced by lowering the FBS in culture medium to 1% plus RA at 10 μM during 4, 7 and 10 days. This treatment was replaced each 3 days to replenish RA in culture media. Hence we choose these specific days of differentiation because these are the days of retinoic acid replacement in the culture medium. To evaluate the differences in cell morphology in proliferative and cells differentiated for 4, 7 and 10 days, we analyzed them under phase contrast light microscopy. Images were captured with NIS-elements software.

4.3. Western blot

After 24 h plating (proliferative cell) or after the differentiation treatment, cells were washed with PBS and resuspended in a Tris-buffer (pH 7.0) with protease inhibitor (Roche®), sonicated, and its total protein extracts (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane. Thereafter, nonspecific binding was blocked with 5% of BSA in TTBS for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with rabbit anti-TH (1:5000) from Sigma Chemical Co (St. Louis, MO, USA), rabbit anti-NSE (1:20000), rabbit anti-nestin (1:5000), mouse anti- NeuN (1:1000) or mouse anti-DJ-1 (1:10000) from Chemicon/Millipore® (Billerica, MO, USA). After washing, the membrane was incubated with peroxidase-conjugated secondary antibodies (1:5000 or 1:10000) from Dako® (Glostrup, DK) for 2 h at room temperature. Bands were visualized with Super Signal West Pico Chemiluminescence Substrate from PIERCE® (Rockford, Il, USA). Membranes were then striped and reprobed with rabbit anti-β-actin antibody (1:5000) from Sigma Chemical Co (St. Louis, MO, USA) followed by goat anti-rabbit peroxidase-conjugated secondary antibody (1:5000) from Dako® (Glostrup, DK).

4.4. Immunocytochemistry

Cells were washed twice with PBS to remove the excess of culture medium followed by the fixation in acetone:methanol (1:1 v/v) for 20 min. The endogenous peroxidase was inhibited by methanol:hydrogen peroxide (95:5% v/v). Non-specific binding were blocked with 1% BSA in PBS-Triton-X100 for 1 h. Cells were incubated with the rabbit anti-TH (1:500), rabbit anti-NSE (1:2000), rabbit anti-nestin (1:500) and mouse anti-NeuN antibodies overnight at 4 °C. After washing the cells with PBS/ 0.02% Triton-X100, they were incubated with biotinylated secondary antibody for 1 h at room temperature followed by the incubation of tertiary antibody linked with horseradish peroxidase–streptavidin complex. The color reaction is constituted by 0.06% of diaminobenzidine (DAB) and 1% of hydrogen peroxide in PBS. The cells were covered in this solution for 5 min at room temperature. Images were visualized under light microscopy and captured NIS-elements software.

4.5. 6-OHDA cytotoxicity

6-OHDA was freshly prepared in 0.1% ascorbic acid to avoid oxidation. Cytotoxicity of 6-OHDA were evaluated by exposing cells to different concentrations of this neurotoxin for 24 h at 37 °C, and cell viability was estimated by the quantification of the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to a blue formazan product by cellular dehydrogenases (Fang et al., 2005). At the end of the treatments, the medium was discarded and a new medium containing 0.5 mg/mL MTT was added. These cells were incubated for 1 h at 37 °C. This medium was discarded after the incubation and the cells were washed three times with PBS. DMSO was added to solubilize the formazan crystals for 30 min. Absorbance was determined at 560 nm and 630 nm in a SoftMax Pro Microplate Reader (Molecular Devices®, USA).

4.6. Mitochondrial membrane potential ($\Delta\Psi_m$)

Proliferative and 7-day RA-differentiated SH-SY5Y cells were treated by different time with their correspondent GI_{50} value of 6-OHDA (35 and 15 μM, respectively) to evaluate the mitochondrial depolarization, as previously described (Klamt and Schacter, 2005). Cells (0.5×10^6 cells/mL) were incubated for 20 min at 37 °C with 10 μg/μL of a lipophilic cationic probe 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), centrifuged, washed once with PBS, transferred to a 96-well plate (10^5 cells/well), and assayed using SoftMax Pro fluorescence plate reader (Molecular Devices®, USA) with the following settings: excitation at 485 nm, emission at 540 and 590 nm, and cut-off at 530 nm. $\Delta\Psi_m$ was estimated using the ratio of 590 nm (J-aggregates)/ 540 nm (monomeric form).

4.7. Protein quantification

The proteins contents were measured by the Bradford assay (Bradford, 1976).

4.8. Statistical analysis

Band intensities of western blots were quantified by a densitometer and expressed as relative values to the controls. Data are expressed as means ± SD from at least three independent experiments. For statistical analysis, quantitative data were analyzed by Student *t* test and the one-way analysis of variance (ANOVA) followed by Neuman–Keuls test. Differences were considered significant at $p<0.05$.

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REFERENCES

Bastien, J., Rochette-Egly, C., 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 328, 1–16.

Biedler, J.L., Roffler-Tarlov, S., Schachner, M., Freedman, L.S., 1978. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res. 38, 3751–3757.

Bladini, F., Armentero, M.T., Martignoni, E., 2008. The 6-hydroxydopamine model: news form the past. Parkinsonism. Relat Disord. 14, 124–129.

Bonifati, V., Rizzu, P., Squitieri, F., Krieger, E., Vanacore, N., van Swieten, J.C., Brice, A., van Duijn, C.M., Oostra, B., Meco, G., Heutink, P., 2003. Mutations in the DJ-1 gene associated with autosomal recessive early-onset Parkinsonism. Science 299, 256–259.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chen, L., Malcolm, A.J., Wood, K.M., Cole, M., Variendm, S., Cullinane, C., Pearson, A.D.J., Lunec, J., Tweddle, D.A., 2007. p53 is nuclear and functional in both undifferentiated and differentiated neuroblastoma. Cell cycle 6, 2685–2696.

Cheung, Y.T., Lau, W.K., Yu, M.S., Lai, C.S., Yeung, S.C., So, K.F.,

Chang, R.C., 2009. Effect of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology 30, 127–135.
Chin, M.H., Qian, W.J., Wang, H., Petryuk, V.A., Bloom, J.S., Sforza, D.M., Lacán, G., Liu, D., Khan, A.H., Cantor, R.M., Bigelow, D.J., Melega, W.P., Camp 2nd, D.G., Smith, R.D., Smith, D.J., 2008. Mitochondrial dysfunction, oxidative stress, and apoptosis revealed by proteomic and transcriptomic analyses of the striata in two mouse models of Parkinson's disease. J. Proteome Res. 7, 666–677.
Clements, C.M., McNally, R.S., Conti, B.J., Mak, T.W., Ting, J.P., 2006. DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. Proc. Natl Acad. Sci. USA 103, 15091–15096.
Dawson, T.M., Dawson, V.L., 2003. Molecular pathways of neurodegeneration in Parkinson's disease. Science 302, 819–821.
Dunkley, P.R., Bobrovskaya, L., Graham, M.E., von Nagy-Felsobuki, E.I., Dickson, P.W., 2004. Tyrosine hydroxylase phosphorylation: regulation and consequences. J. Neurochem. 91, 1025–1043.
Fang, F., Wang, A.P., Yang, S.F., 2005. Antitumor activity of a novel recombinant mutant human tumor necrosis factor-related apoptosis-inducing ligand. Acta pharmacol. Sin. 26, 1373–1381.
Fuchs, E., Weber, K., 1994. Intermediate filaments: structure, dynamics, function, and disease. Annu. Rev. Biochem. 63, 345–382.
Gilany, K., Elzen, R.V., Mous, K., Coen, E., Dongen, W.V., Vandamme, S., Gevaert, K., Timmerman, E., Vandekerckhove, J., Dewilde, S., Ostade, X.V., Moens, L., 2008. The proteome of the human neuroblastoma cell line SH-SY5Y: an enlarged proteome. Biochim. Biophys. Acta 1784, 983–985.
Gomez-Lazaro, M., Bonekamp, N.A., Galindo, M.F., Jordan, J., Schrader, M., 2008. 6-Hydroxydopamine (6-OHDA) induces Drp1-dependent mitochondrial fragmentation in SH-SY5Y cells. Free Radic. Biol. Med. 11, 1690–1699.
Guarnieri, S., Pilla, R., Morabito, C., Sacchetti, S., Mancinelli, R., Fano, G., Mariggiò, M.A., 2009. Extracellular guanosine and GTP promote expression of differentiation markers and induce S-phase cell-cycle arrest in human SH-SY5Y neuroblastoma cells. Int. J. Dev. Neurosci. 27, 135–147.
Hockfield, S., McKay, R.D., 1985. Identification of major cell classes in the developing mammalian nervous system. J. Neurosci. 5, 3310–3328.
Hwang, Y.P., Jeong, H.G., 2008. The coffee diterpene kahweol induces heme oxygenase-1 via the PI3K and p38/Nrf2 pathway to protect human dopaminergic neurons from 6-hydroxydopamine-derived oxidative stress. FEBS Lett. 17, 2655–2662.
Inden, M., Taira, T., Kitamura, Y., Yanagida, T., Tsuchiya, D., Takata, K., Yanagisawa, D., Nishimura, K., Taniguchi, T., Kiso, Y., Yoshimoto, K., Agatsuma, T., Koide-Yoshida, S., Iguchi-Ariga, S.M., Shimohama, S., Ariga, H., 2006. PARK7 DJ-1 protects against degeneration of nigral dopaminergic neurons in Parkinson's disease rat model. Neurobiol. Dis. 24, 144–158.
Ikeda, Y., Tsuji, S., Satoh, A., Ishikura, M., Shirasawa, T., Shimizu, T., 2008. Protective effects of astaxanthin on 6-hydroxydopamine-induced apoptosis in human neuroblastoma SH-SY5Y cells. J. Neurochem. 107, 1730–1740.
Klamt, F., Schacter, E., 2005. Taurine chloramine, an oxidant derived from neutrophilis, induces apoptosis in human B lymphoma cells through mitochondrial damage. J. Biol. Chem. 280, 21346–21352.
Jordan, J., Galindo, M.F., Tornero, D., Gonzalez-Garcia, C., Cena, V., 2004. Bcl-xL blocks mitochondrial multiple conductance channel activation and inhibits 6-OHDA-induced death in SH-SY5Y cells. J. Neurochem. 89, 124–133.
Lee, C.S., Han, J.H., Jang, Y.Y., Song, J.H., Han, E.S., 2002. Differential effect of catecholamines and MPP on membrane permeability in brain mitochondria and cell viability in PC12 cells. Neurochem. Int. 40, 361–369.
Lehmensiek, V., Tan, E.M., Liebau, S., Lenk, T., Zettlmeisl, H., Schwarz, J., Storch, A., 2006. Dopamine transporter-mediated cytotoxicity of 6-hydroxydopamine in vitro depends on expression of mutant a-synucleins related to Parkinson's disease. Neurochem. Int. 48, 329–340.
Lesage, S., Brice, A., 2009. Parkinson's disease: from monogenic forms to genetic susceptibility factors. Hum. Mol. Genet. 18, 48–50.
Lev, N., Ickowicz, D., Melamed, E., Offen, D., 2008. Oxidative insults induce DJ-1 upregulation and redistribution: implications for neuroprotection. Neurotoxicology 29, 397–405.
Liu, F., Nguyen, J.L., Hulleman, J.D., Li, L., Rochet, J.C., 2008. Mechanisms of DJ-1 neuroprotection in a cellular model of Parkinson's disease. J Neurochem.
Lo Bianco, C., Schneider, B.L., Bauer, M., Sajadi, A., Brice, A., Iwatsubo, T., Aebischer, P., 2004. Lentiviral vector delivery of parkin prevents dopaminergic degeneration in an a-synuclein rat model of Parkinson's disease. Proc. Natl Acad. Sci. USA 101, 17510–17515.
López-Carballo, G., Moreno, L., Masiá, S., Pérez, P., Barettino, D., 2002. Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells. J. Biol. Chem. 28, 25297–25304.
Mark, M., Ghyselinck, N.B., Chambon, P., 2006. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. Annu. Rev. Pharmacol. Toxicol. 46, 451–480.
Miloso, M., Villa, D., Crimi, M., Galbiati, S., Donzelli, E., Nicolini, G., Tredici, G., 2004. Retinoic acid-induced neuritogenesis of human neuroblastoma SH-SY5Y cells is ERK independent and PKC dependent. J. Neurosci. Res. 75, 241–252.
Miyazaki, S., Yanagida, T., Nunome, K., Ishikawa, S., Inden, M., Kitamura, Y., Nakagawa, S., Taira, T., Hirota, K., Niwa, M., Iguchi-Ariga, S.M., Ariga, H., 2008. DJ-1-Binding Compounds Prevent Oxidative Stress-Induced Cell Death and Movement Defect in Parkinson's Disease Model Rats. J Neurochem.
Mizote, T., Tsuda, M., Smith, D.D.S., Nakayama, H., Nakazawa, T., 1999. Cloning and characterization of the thiD/J gene of *Escherichia coli* encoding a thiamin-synthesizing bifunctional enzyme, hydroxymethylpyrimidine kinase/phosphomethylpyrimidine kinase. Microbiology 145, 495–501.
Moll, U.M., LaQuaglia, M., Benard, J., Riou, G., 1995. Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proc. Natl Acad. Sci. USA 92, 4407–4411.
Mu, X., He, G., Cheng, Y., Li, X., Xu, B., Du, G., 2009. Baicalein enxerts neuroprotective effects in 6-hydroxydopamine-induced experimental parkinsonism in vivo and in vitro. Pharmacol. Biochem. Behav. 92, 642–648.
Nie, G., Cao, Y., Zhao, B., 2002. Protective effects of green tea polyphenols and their major component, (–)-epigallocatechin-3-gallate (EGCG), on 6-hydroxydopamine-induced apoptosis in PC12 cells. Redox Rep. 7, 171–177.
Odelstad, L., Pahlman, S., Nilsson, K., Larsson, E., Lackgren, G., Johansson, K.E., Hjerten, S., Grotte, G., 1981. Neuron specific enolase in relation to differentiation in human neuroblastoma. Brain Res. 224, 69–82.
Pahlman, S., Ruusala, A.I., Abrahamsson, L., Mattsson, M.E., Esscher, T., 1984. Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. Cell Differ. 14, 135–144.
Prabhakara, J.P.R., Feist, G., Thomasson, S., Thompson, A., Schommer, E., Ghribi, O., 2008. Differential effects of 24-hydroxycholesterol and 27-hydroxycholesterol on tyrosine hydroxylase and α-synuclein in human neuroblastoma SH-SY5Y cells. J. Neurochem. 107, 1722–1729.
Poewe, W., 2008. Non-motor symptoms in Parkinson's disease. Eur. J. Neurol. 15, 14–20.
Reimer, R., Helmbold, H., Szalay, B., Hagel, C., Hohenberg, H., Deppert,

W., Bohn, W., 2009. Nestin modulates glucocorticoid receptor function by cytoplasmic anchoring. Plos One 29, e6084.
Sahlgren, C.M., Pallari, H.M., He, T., Chou, Y.H., Goldman, R.D., Eriksson, J.E., 2006. A nestin scaffold links Cdk5/p35 signaling to oxidant-induced cell death. Embo J. 25, 4808–4819.
Sarkanen, J.R., Nykky, J., Siikanen, J., Selinummi, J., Ylokomi, T., Janolen, T.O., 2007. Cholesterol supports the retinoic acid-induced synaptic vesicle formation in differentiating human SH-SY5Y neuroblastoma cells. J. Neurochem. 102, 1941–1952.
Savickiene, J., Treigyte, G., Magnusson, K.E., Navakauskiene, R., 2009. Response of retinoic acid-resistant KG1 cells to combination of retinoic acid with diverse histone deacetylase inhibitors. Ann. N. Y. Acad. Sci. 1171, 321–333.
Schapira, A.H., 2008. Mitochondrial dysfunction in neurodegenerative disease. Neurochem. Res. 12, 2502–2509.
Seitz, G., Stegmann, H.B., Jager, H.H., Schlude, H.M., Wolburg, H., Roginsky, V.A., Niethammer, D., Bruchelt, G., 2000. Neuroblastoma cells expressing the noradrenaline transporter are destroyed more selectively by 6-fluorodopamine than by 6-hydroxydopamine. J. Neurochem. 75, 511–520.
Sekito, A., Koide-Yoshida, S., Niki, T., Taira, T., Iguchi-Ariga, S.M., Ariga, H., 2006. DJ-1 interacts with HIPK1 and affects H_2O_2-induced cell death. Free Radic. Res. 40, 155–165.
Thomas, S.K., Messam, C.A., Spengler, B.A., Biedler, J.L., 2004. Nestin is a potential mediator of malignancy in human neuroblastoma cells. J. Biol. Chem. 279, 27994–27999.
Tieu, K., Zuo, D.M., Yu, P.H., 1999. Differential effects of staurosporine and retinoic acid on the vulnerability of the SH-SY5Y neuroblastoma cells: involvement of Bcl-2 and p53 proteins. J. Neurosci. Res. 58, 426–435.
Weggen, S., Bayer, T.A., Koch, A., Salewski, H., Scheidtmann, K.H., Pietsch, T., Wiestler, O.D., 1997. Characterization of neural cell lines derived from SV40 large T-induced primitive neuroectodermal tumors. Brain Pathol. 7, 731–739.
Weyer, A., Schilling, K., 2003. Developmental and cell type-specific expression of the neuronal marker NeuN in the murine cerebellum. J. Neurosci. Res. 73, 400–409.
Xu, J., Zhong, N., Wang, H., Elias, J.E., Kim, C.Y., Woldman, I., Pifl, C., Gygi, S.P., Geula, C., Yankner, B.A., 2005. The Parkinson's disease-associated DJ-1 protein is a transcriptional co-activator that protects against neuronal apoptosis. Hum. Mol. Genet. 14, 231–1241.
Zhang, L., Shimoji, M., Thomas, B., Moore, D.J., Yu, S.H., Marupudi, N.I., Torp, R., Torgner, I.A., Ottersen, O.P., Dawson, T.M., Dawson, V.L., 2005. Mitochondrial localization of the Parkinson's disease related protein DJ-1: implications for pathogenesis. Hum. Mol. Genet. 14, 203–2073.
Zhong, N., Xu, J., 2008. Synergistic activation of the human MnSOD promoter by DJ-1 and PGC-1alpha: regulation by SUMOylation and oxidation. Hum. Mol. Genet. 17, 3357–3367.

RESULTADOS SUPLEMENTARES I: Caracterização redox entre células proliferativas e diferenciadas da linhagem de neuroblastoma humano SH-SY5Y.

No capítulo I, nós estabelecemos as condições da diferenciação induzida pelo AR através da morfologia, da neuroquímica e da citotoxicidade à 6-OHDA. Nesta sessão, nós iremos apresentar dados que não compõem um manuscrito, mas contribuirão para uma melhor compreensão da discussão deste trabalho quanto à caracterização dos fenótipos tumoral e neuronal da linhagem de neuroblastoma humano SH-SY5Y.

Nós avaliamos as diferenças entre os dois modelos quanto ao perfil redox (parâmetros enzimáticos: CAT, SOD, GPx, Glutationa-S-Transferase – GST, GR e TrxR; e não enzimáticos: níveis de tióis reduzidos e níveis de GSH). Foi observado que as células diferenciadas apresentam uma maior atividade de enzimas antioxidantes (CAT e GST) bem como maiores níveis de tióis reduzidos. Entretanto, dois parâmetros avaliados (atividade da TrxR e níveis de GSH) tiveram uma maior atividade nas células proliferativas (Tabela 1)

Ainda, nós verificamos a citotoxicidade de outra espécie reativa nos dois modelos, o H_2O_2 (Figura 1A), sendo que as células diferenciadas foram mais resistentes a este oxidante. Este dado pode estar relacionado com o aumento da atividade das enzimas antioxidantes encontradas no fenotótipo neuronal.

Além disso, avaliamos o imunoconteúdo do marcador dopaminérgico DAT, visto que este dado não foi discutido no capítulo I. O aumento dos níveis desse transportador podem estar relacionados com a suscetibilidade à 6-OHDA encontradas nas células diferenciadas (Figura 1C)

Também foi avaliada a contribuição dos sistemas antioxidantes de forma mais acurada através da inibição das enzimas TrxR (inibidor: Auronofina - Au) e da GR (inibidor: 2-acetilamino-3-[4-(2-acetilamino-2-carboxietilsulfaniltiocarnilamino) phenyltiolcarbamoilsulfanl] ácido propiônico – AAPA (Figura 2A). Após isso as células foram co-tratadas com inibidor e doses subletais das toxinas: 6-OHDA (6.25 µM) ou H_2O_2 (400 µM). Os dados mostraram que a TrxR tem um papel importante na defesa celular frente à 6-OHDA, devido a potencialização da morte quando as células diferenciadas são co-tratadas com Au. Já na citotoxicidade do H_2O_2, foi observado que ambas as enzimas estão relacionadas com a morte causada por este oxidante (Figura 2C).

Estes resultados complementam a primeira etapa desta dissertação, cujo objetivo era a caracterização de diferenças relevantes entre os fenótipos proliferativos e diferenciados da linhagem de neuroblastoma humano SH-SY5Y.

Table 1: Differences in redox parameters between human neuroblastoma SH-SY5Y cell line phenotypes.

	Proliferative cells	Differentiated cells	*P*
Antioxidant Enzyme Defenses			
CAT (U/mg protein)	0.36 ± 0.01	1.43 ± 0.27*	0.014
GPx (U/mg protein)	2.83 ± 0.87	3.85 ± 0.91	0.233
GR (U/mg protein)	18.45 ± 4.82	22.82 ± 4.71	0.203
TrxR (U/mg protein)	9.92 ± 2.52	5.17 ± 1.48*	0.019
GST (U/mg protein)	9.96 ± 4.46	25.31 ± 3.23**	0.003
SOD (U/mg protein)	10.18 ± 4.42	19.52 ± 5.35	0.080
Non-Enzymatic Defenses			
-SH levels (nmol/mg protein)	17.57 ± 9.69	39.16 ± 9.08**	0.002
GSH levels (nmol/mg protein)	16.44 ± 2.46	7.84 ± 1.16	

Exponentially growing human neuroblastoma SH-SY5Y or RA-differentiated cells were harvested, washed with 10 mM saline phosphate buffer (pH 7.4) and frozen at -80^{o}C and thawed twice. Cell extracts were centrifuged and the supernatant was collected for analysis. Data are expressed as mean ± SD from at least three independent experiments performed in triplicates (n = 3) and analyzed by Student *t* test. Differences were considered significant at $P \leq 0.05$ (*) or $P \leq 0.01$ (**).

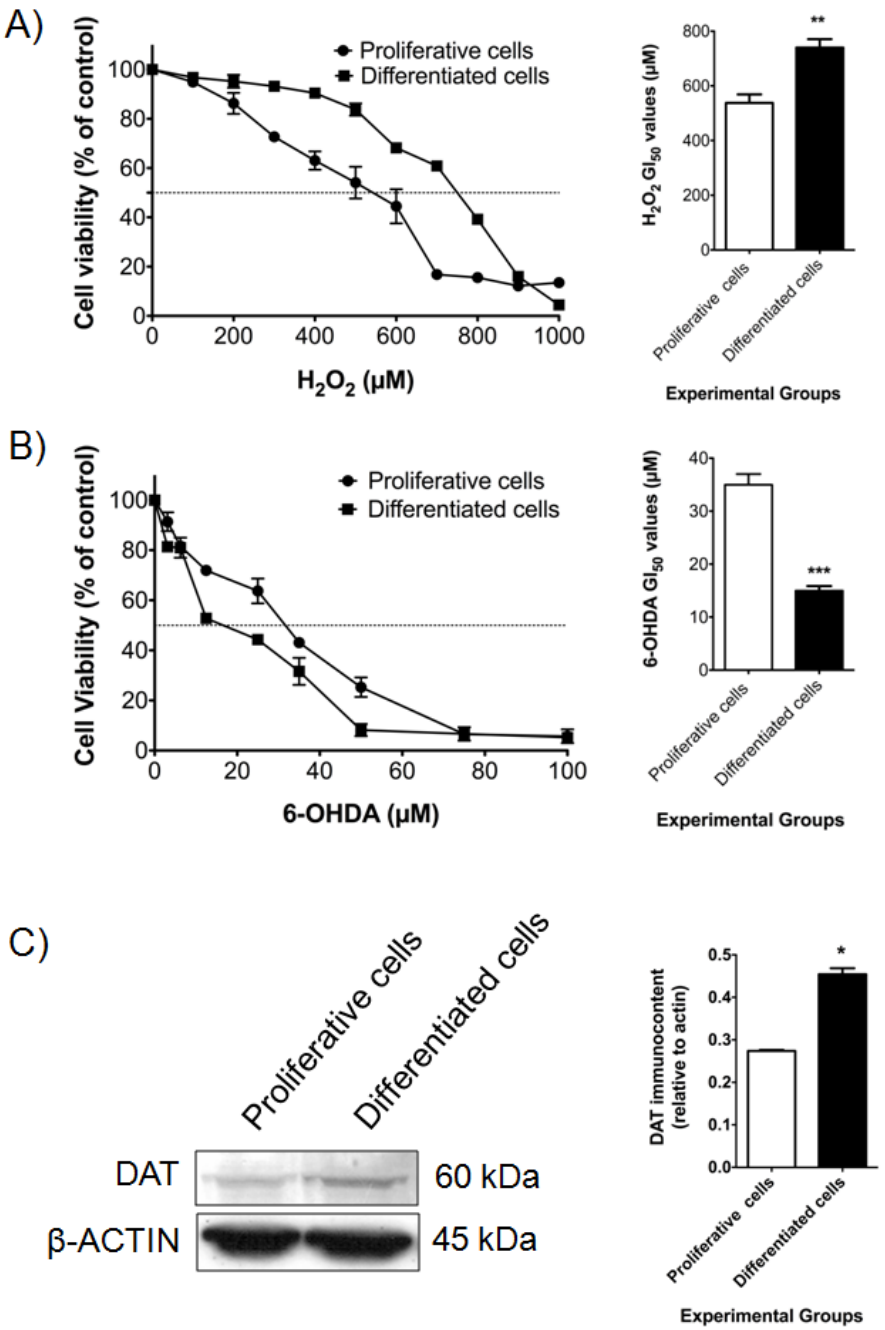

Figure 1: Differences in H_2O_2 and 6-OHDA cytotoxicities and changes in the immunocontent of DAT dopaminergic cell marker in proliferative and differentiated human neuroblastoma cell line SH-SY5Y. (A) Cytotoxicity curves and LD_{50} value for (A) H_2O_2 and for (B) 6-OHDA. (C) Representative immunoblot of DAT and densitometric analysis of bands representing means ± SD of three independent experiments. β-actin was used as loading control. Data are expressed as mean ± SD from three experiments performed in triplicates (n = 3) and analyzed by Student *t* test.

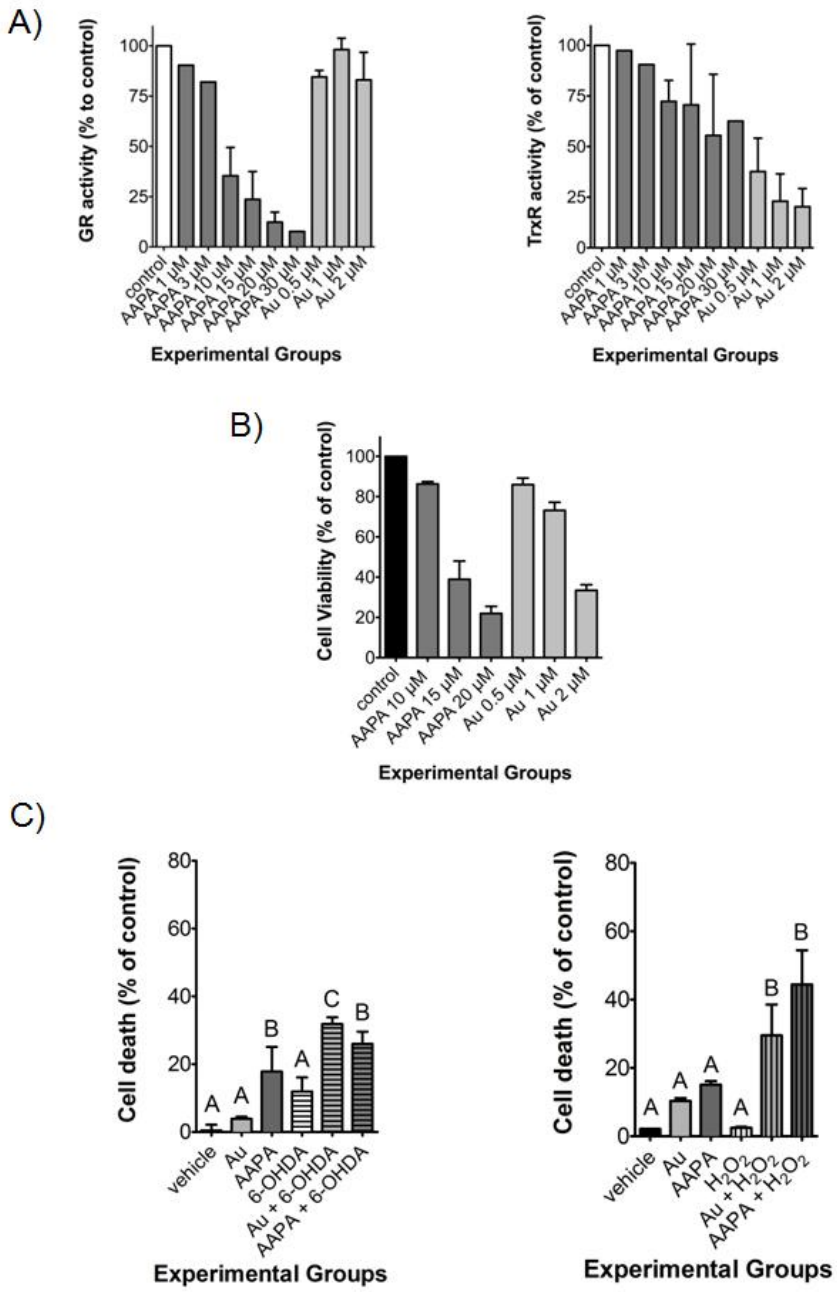

Figure 2: Activity of the antioxidant enzymes and cell viability in differentiated cells treated with Au and AAPA. Differentiated cells were pretreated for 30 min with the inhibitors and the (A) specific enzymatic activity and (B) cell viability was measured. (C) Cell viability in differentiated cells exposed after a 30-min pre-incubation with Au or AAPA. Cells were exposed to 6-OHDA and H_2O_2 for 24 hours. Cell death caused by inhibitor was discounted for the calculation in the groups “inhibitor + toxin”. Data are expressed as mean ± SD from three independent experiments (n = 3) and analyzed by one-way analysis of variance. Statistical differences are expresses by letters. $p<.05$ (one-way analysis of variance).

CAPÍTULO II: Manuscrito aceito para publicação no periódico *Neurotoxicity Research* em 01/2012

"Evaluation of the Neurotoxic/Neuroprotective Role of Organoselenides Using Differentiated Human Neuroblastoma SH-SY5Y Cell Line Challenged with 6-hydroxydopamine"

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Evaluation of the Neurotoxic/Neuroprotective Role of Organoselenides Using Differentiated Human Neuroblastoma SH-SY5Y Cell Line Challenged with 6-Hydroxydopamine

Fernanda Martins Lopes · Giovana Ferreira Londero · Liana Marengo de Medeiros · Leonardo Lisbôa da Motta · Guilherme Antônio Behr · Valeska Aguiar de Oliveira · Mohammad Ibrahim · José Cláudio Fonseca Moreira · Lisiane de Oliveira Porciúncula · João Batista Teixeira da Rocha · Fábio Klamt

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Abstract It is well established that oxidative stress plays a major role in several neurodegenerative conditions, like Parkinson disease (PD). Hence, there is an enormous effort for the development of new antioxidants compounds with therapeutic potential for the management of PD, such as synthetic organoselenides molecules. In this study, we selected between nine different synthetic organoselenides the most eligible ones for further neuroprotection assays, using the differentiated human neuroblastoma SH-SY5Y cell line as in vitro model. Neuronal differentiation of exponentially growing human neuroblastoma SH-SY5Y cells was triggered by cultivating cells with DMEM/F12 medium with 1% of fetal bovine serum (FBS) with the combination of 10 μM retinoic acid for 7 days. Differentiated cells were further incubated with different concentrations of nine organoselenides (0.1, 0.3, 3, 10, and 30 μM) for 24 h and cell viability, neurites densities and the immunocontent of neuronal markers were evaluated. Peroxyl radical scavenging potential of each compound was determined with TRAP assay. Three organoselenides tested presented low cytotoxicity and high antioxidant properties. Pre-treatment of cells with those compounds for 24 h lead to a significantly neuroprotection against 6-hydroxydopamine (6-OHDA) toxicity, which were directly related to their antioxidant properties. Neuroprotective activity of all three organoselenides was compared to diphenyl diselenide $(PhSe)_2$, the simplest of the diaryl diselenides tested. Our results demonstrate that differentiated human SH-SY5Y cells are suitable cellular model to evaluate neuroprotective/neurotoxic role of compounds, and support further evaluation of selected organoselenium molecules as potential pharmacological and therapeutic drugs in the treatment of PD.

Keywords SH-SY5Y cells · Organoselenides · Oxidative stress · 6-Hydroxydopamine · Neuroprotection

F. M. Lopes · G. F. Londero · L. M. de Medeiros · L. L. da Motta · G. A. Behr · V. A. de Oliveira · J. C. F. Moreira · L. de Oliveira Porciúncula · F. Klamt (✉)
Departamento de Bioquímica, Laboratório 24, ICBS/ Universidade Federal do Rio Grande do Sul (UFRGS), Rua Ramiro Barcelos, 2600, Porto Alegre, RS 90035-003, Brazil
e-mail: 00025267@ufrgs.br

F. M. Lopes · G. F. Londero · L. M. de Medeiros · L. L. da Motta · G. A. Behr · V. A. de Oliveira · J. C. F. Moreira · L. de Oliveira Porciúncula · J. B. T. da Rocha · F. Klamt
Rede Gaúcha de Estresse Oxidativo e Sinalização Celular (FAPERGS), Porto Alegre, RS, Brazil

F. M. Lopes · G. F. Londero · L. M. de Medeiros · L. L. da Motta · V. A. de Oliveira · F. Klamt
Institutos Nacionais de Ciência e Tecnologia—Translacional em Medicina (MCT/CNPq INCT-TM), Porto Alegre, RS, Brazil

M. Ibrahim · J. B. T. da Rocha
Departamento de Química, CCNE/UFSM, Santa Maria, RS, Brazil

M. Ibrahim
Department of Chemistry, Abdul Wali Khan University Mardan (AWKUM) KPK, Mardan, Pakistan

Introduction

Many lines of evidence indicate that the central nervous system (CNS) is prone to oxidative damage (Halliwell 2006). Some attributes of this tissue can contribute to this situation: modest antioxidant defenses (Halliwell 2001), high amount of polyunsaturated fatty acids (Floyd and

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Hensley 2002), auto-oxidization of several neurotransmitters (Spencer et al. 1998), and high oxygen consumption (Halliwell 1992). The process of neurodegeneration also involves oxidative damage (Halliwell 2006). It is well known that many neurodegenerative disease share common pathophysiologic mechanisms: oxidative stress (Halliwell 2001); protein aggregation (Stefanis and Keller 2006); and mitochondrial dysfunction (Zeevalk et al. 2005).

One example of these disorders is Parkinson disease (PD). The motor symptoms of Parkinson's disease result from the death of dopamine (DA)-generating neurons of *substancia nigra pars compacta*, which are exposed to chronic oxidative stress generated by DA metabolism. High amount of iron present in this region also contributes to hydroxyl radical ($^{\bullet}$OH) generation by Fenton Chemistry (Barnham et al. 2004; Drechsel and Patel 2008). Moreover, the neurotoxin 6-hydroxydopamine (6-OHDA), an analog of DA, is widely used to mimic PD through the generation of a massive oxidative damage in cells (Lehmensiek et al. 2006).

Since the overproduction of reactive species (RS) is a milestone in PD, investigations on antioxidant molecules with neuroprotective potential are at the forefront of PD therapeutic management (Posser et al. 2008). Agents currently under investigation as potential treatments include anti-apoptotics (omigapil, CEP-1347), compound that improve mitochondrial activity (coenzyme Q10), antiglutamatergics, monoamine oxidase inhibitors (selegiline, rasagiline), calcium channel blockers (isradipine), and growth factors (GDNF) (Pedersen and Schmidt 2000; Mandel et al. 2003; McCarthy et al. 2004; Naoi and Maruyama 2010; Ilijic et al. 2011; Safi et al. 2011). One example of these candidates are organochalcogenides, especially Se-containing organic compounds (Bhabak and Mugesh 2007). Since the discovery that these compounds (in the form of selenocysteine) are a critical component of glutathione peroxidase (GPx) (Flohe et al. 1973; Rotruck et al. 1973) and thioredoxin reductase (Arnér and Holmgre 2000; Santos et al. 2009), the chemistry at the active site of GPx has been extensively investigated to make synthetic Se-containing compounds (Rossato et al. 2002; Luchese et al. 2007; Posser et al. 2006, 2008; Ibrahim et al. 2011).

One example of this compound is ebselen. Ebselen is a synthetic organoselenide that mimics the activity of GPx both in vitro and in vivo (Müller et al. 1984; Bhabak and Mugesh 2007). Due to its antioxidant function, it can neutralize the free radical damage and also has neuroprotective effects against brain injuries involving glutamatergic system (Porciúncula et al. 2003), ischemia (Xu et al. 2006), and spinal cord injury (Kalayci et al. 2005). Hence, the success obtained with ebselen inspired several research groups to synthesize other low-molecular-weight compounds with high availability, both qualities that improve its therapeutic potential (Sies 1993; Schewe 1995; Mugesh et al. 2001; Geoghegan et al. 2006; Talas et al. 2008; Ozdemir et al. 2010). Even though other ebselen analog, diphenyl diselenide $(PhSe)_2$, can inhibit glutamate uptake in rat hippocampus (Ardais et al. 2010) and also confers neuroprotection in hippocampus slices through antioxidant mechanisms (Posser et al. 2008), the organoselenides can be very toxic to many tissues, such as liver (Meotti et al. 2003), blood (Santos et al. 2009), and brain (Souza et al. 2010). Paradoxically, the abovementioned toxicity can be mediate due to pro-oxidative conditions, such as GSH depletion (Farina et al. 2004). Hence, due to its contradictory data, more studies are necessary to elucidate its mechanisms.

There are many studies evaluating the cytoprotective/cytotoxic features of organochalcogenides, however, there is little information regarding their effects over the CNS and neuronal cells models, such as differentiated human neuroblastoma SH-SY5Y cells (Posser et al. 2011). This cell line has been widely used as an in vitro experimental model and present several advantages for neuroscience studies, such as: (i) is derived from humans; (ii) as a cell line, is homogenous (which increases data reproducibility); (iii) has mitogenic potential, which decreases the time of the culture process compared to primary and organotypic culture; (iv) it can be differentiated into dopaminergic neurons through neurotrophins, for instance, retinoic acid (RA); and (v) once differentiated, it acquires important neuronal attributes (e.g., high expression of tyrosine hydroxylase—TH, dopamine transporter—DAT, neuron specific enolase—NSE, neuronal nuclear protein—NeuN, Synaptobrevin and others, and a typical neuronal morphology with abundant neurites outgrow). Hence, the RA-differentiated SH-SY5Y cells are considered a more suitable in vitro model to study not only the pathophysiological mechanism of CNS diseases, but also to evaluate neuroprotective/neurotoxicity of compounds (Lopes et al. 2010).

The aim of this study was to screen between different organoselenides, investigating their antioxidant potential and neurototoxicity, for subsequent selection the most promising compounds for neuroprotection assays. For this purpose, we used differentiated human neuroblastoma SH-SY5Y cells challenged with 6-OHDA. Our data demonstrate that at least two different organoselenides tested—compound IV (1,1′-(2,2′-diselanediylbis(2,1-phenylene)) diethanamine) and V (2,2′-(1*Z*,1′*E*)-(1,1′-(2,2′-diselanediylbis(2,1-phenylene))bis(ethane-1,1-diyl))bis(azan-1-yl-1-ylidene)bis(methan-1-yl-1-ylidene)diphenol) (Fig. 1) present neuroprotective potential and are promising candidates for further investigations for PD therapeutic management.

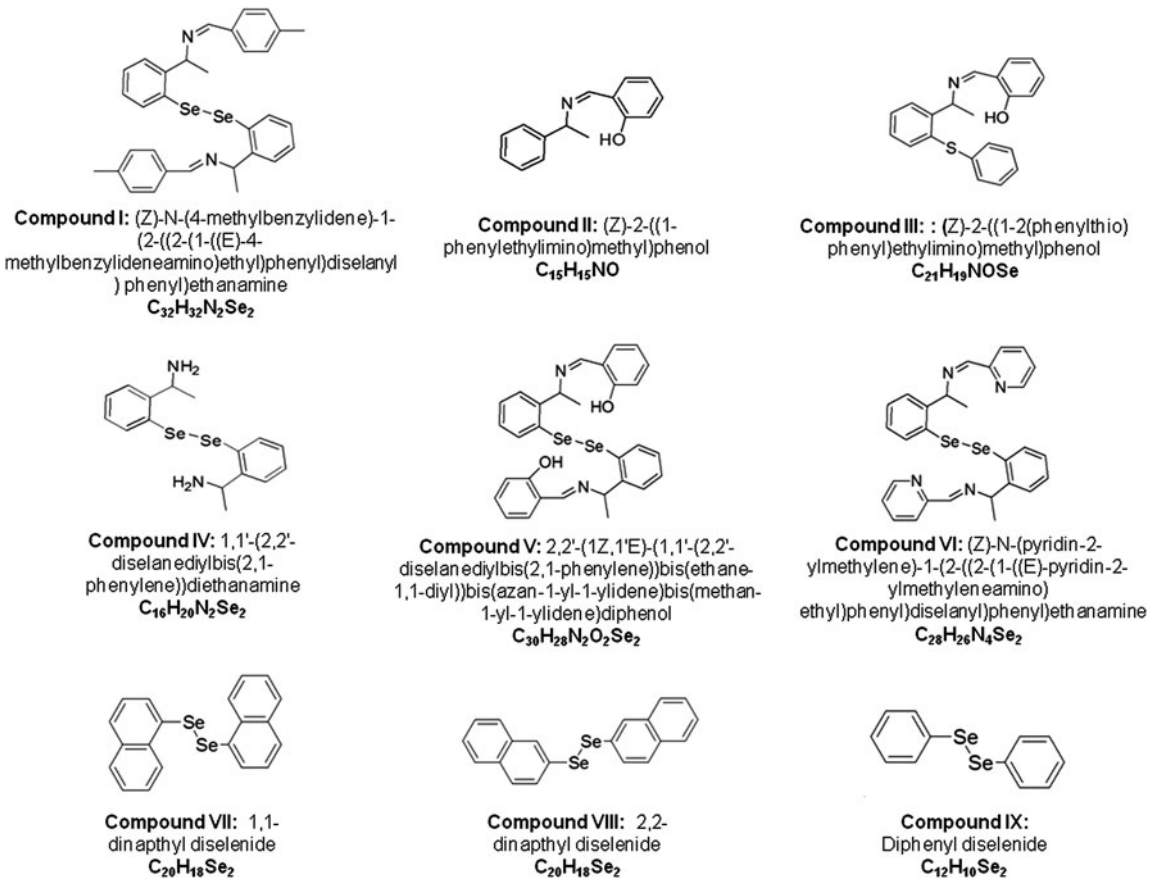

Fig. 1 Molecular structures of the organoselenides tested. Basic structures of compounds were derived from ebselen (not shown) and diphenyl diselenide $(PhSe)_2$, the simplest diaryl diselenides tested. All the compounds names were substitute for roman numerals (I–IX)

Experimental Procedures

Chemicals

Materials used in cell culture were acquired from Gibco®/Invitrogen (São Paulo, SP, Brazil). Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The structures of the nine organoselenides tested in this study are shown in Fig. 1. Analysis of the ^{1}H-NMR and ^{13}C-NMR spectra showed that all the compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structures. The purity of the compounds were assessed by high resonance mass spectroscopy (HRMS) and was higher than 99.9%. To simplify the understanding of this work, the compound name was replaced by roman numerals. All of them were diluted in ultrapure dimethyl sulfoxide (DMSO).

Cell Culture, Differentiation, and Treatments

Exponentially growing human neuroblastoma SH-SY5Y cell line, obtained from ATCC (Manassas, VA, USA), was maintained at 37°C in a humidified atmosphere of 5% CO_2. The cells were grown in a mixture of 1:1 of Ham's F12 and Dulbecco's Modified Eagle Minimum (DMEM) supplemented with 10% of fetal bovine serum (FBS), 2 mM of glutamine, 0.28 μg/μL of gentamicin, and 250 μg of amphotericin B. Medium was changed each 3 days and cells were sub-cultured once they reached ~80% confluence. After 24 h of plating, cell differentiation was triggered by lowering the FBS in medium to 1% with the addition of 10 μM of RA during 7 days (Lopes et al. 2010). In the seventh day of RA-induced differentiation, the SH-SY5Y cells were treated with different concentrations of the nine compounds (0.1; 0.3; 3; 10; 30 μM) for 24 h at 37°C. For viability assay, cells were seeded in 96-wells plate at density of 2×10^4 cells/well. For the evaluation of the redox parameters, cells were seeded into flasks of 75 cm^3 at a density of 3×10^6 cells/well.

Total Radical-Trapping Antioxidant Potential

To evaluate the non-enzymatic antioxidant capacity of samples, we used the total radical-trapping antioxidant potential (TRAP) assay, which is based on the measurement of luminescence generated by luminol oxidation by

AAPH (2, 20-azobis 2-amidinoprepane) decomposition, in glycine buffer (pH 8.6). After system stabilization (buffer plus luminol and AAPH), sample was added (organoselenides or extract of cells pre-incubated with selected concentration of compounds) and the luminescence decreases proportionately to its antioxidant potential. The luminescence was monitored in a Wallace 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer (Perkin Elmer). For data analysis, a time per chemiluminescence curve was obtained and the relative "area under the curve" (AUC) in the recovery phase was used, as previously established (Lissi et al. 1995; Dresch et al. 2009).

Cellular Viability

Cell viability was evaluated by the quantification of 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to a blue formazan product by cellular dehydrogenases. At the end of the treatment, cells were incubated with 0.5 mg/mL of MTT during 1 h at 37°C. Then, medium was discarded and DMSO was added to solubilize the formazan crystals. The absorbance was determined at 560 and 630 nm in a SoftMax Pro Microplate Reader (Molecular Devices®, USA).

Neurites Density and Immunocontent of Neuronal Markers

To further evaluate the implication of the organoselenides in neuronal parameters, we evaluated the stellate morphology and neurites densities in treated cells. Cellular treatments were performed in six-well plates (density: 2×10^5 cell/well) for 24 h. First, 10 microscopic fields (200× magnification) were randomly selected from three independent experiments ($n = 3$), photographed using an Olympus IX70 inverted microscope and analyzed with NIS-elements software. Neurite density was assessed by counting the number of "nodes" per cell. Primary nodes were considered branches from the cell body. The results were expressed in percentage of untreated cells (mean ± SD value).

For western blot analysis of neuronal markers, after 24 h treatment, cells were washed with PBS and resuspended in a Tris-buffer (pH 7.0) with protease inhibitor (Roche®), lysed, and total protein extracts (30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane. Thereafter, nonspecific binding was blocked with 5% of BSA in TTBS for 1 h at room temperature. Membranes were then incubated overnight at 4°C with rabbit anti-tyrosine hydroxylase—TH (1:3,000) from Abcam®, rat anti-dopamine transporter—DAT (1:1,000) from Santa Cruz®, and mouse anti-synaptobrevin (1:1,000) from Sigma® (Billerica, MO, USA). After washing, the membrane was incubated with peroxidase-conjugated secondary antibodies (1:5,000) from Dako® (Glostrup, DK) for 2 h at room temperature. Bands were visualized with Super Signal West Pico Chemiluminescence Substrate from PIERCE® (Rockford, IL, USA). Membranes were then striped and reprobed with rabbit anti-β-actin antibody (1:2,000) from Sigma Chemical Co. (St. Louis, MO, USA) followed by goat anti-rabbit peroxidase-conjugated secondary antibody (1:5,000) from Dako® (Glostrup, DK). Quantification of band was done with ImageJ 1.36b software (National Institutes of Health).

Antioxidant Enzymes Activities and –SH levels

Differentiated SH-SY5Y cells were treated with selected compounds during 24 h. The medium was removed and the cells were washed with PBS to avoid the contamination of non-incorporated compound. After that, cells were frozen at −80°C and thawed twice in 10 mM PBS. Cells extracts were centrifuged and the supernatant was collected and Elman's sulfhydryl group (–SH) levels were determined. Briefly, samples were dilute in 10 mM of boric acid with 0.2 mM EDTA (pH 8.5). 10 mM of DTNB was added and –SH levels were determined by reacting samples with 5-thio-2-nitrobenzoic acid (Nbs) and measuring absorbance at 412 nm ($\varepsilon_{412\ nm} = 27{,}200\ M^{-1}\ cm^{-1}$). Results are expressed an nmol –SH/mg protein (Ellman 1959). GPx activity (E.C. 1.11.1.9) was assayed measuring NADPH oxidation at 340 nm in the presence of GSH, *tert*-butyl hydroperoxide and glutathione reductase. GPx unit was defined as nmol NADPH oxidized/min (Wendel1981). Catalase (CAT) (E.C. 1.11.1.6) activity was determined by monitoring the rate of H_2O_2 consumption at 240 nm (Aebi 1984). Superoxide dismutase (SOD) (E.C. 1.15.1.1) activity was assayed by measuring the inhibition of adrenaline auto-oxidation at 480 nm. SOD unit was defined as the sample amount that inhibits 50% of adrenaline auto-oxidation (Misra and Fridovich 1972). Glutathione *S*-transferase (GST) (E.C. 2.5.1.13) activity is determined measuring the formation of the conjugate of GSH and chloro-dinitro benzene (CDNB) (Pabst et al. 1974).

Neuroprotection and RS Generation

Cells were incubated with selected compounds for 24 h, washed with PBS and challenged with 6-OHDA ($LD_{50} = 15\ \mu M$) to evaluate the neuroprotection features of the organoselenides through MTT assay. Moreover, to evaluate if the compounds were able to decrease the RS generated by 6-OHDA, we use the probe DCF-DA (2′, 7′-dichlorodihydrofluorescein diacetate) (Wang and Joseph 1999; Halliwell and Whiteman 2004). After treatment, the

medium was removed and 10 μM DCF-DA was added. Cells were treated with 6-OHDA (LD_{50} = 15 μM) and the fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm.

Protein Quantification

The proteins contents were measured by the Lowry assay (Lowry et al. 1951).

Statistical Analysis

Data are expressed as percentage of untreated cells (control) (mean ± SD) from at least three independent experiments. For statistical analysis, data were analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls test. Differences were considered significant at $P < 0.05$.

Results

Screening of Organoselenides Compounds for Neuroprotective Evaluations

This study was designed to screen between different synthetic organoselenides compounds to select the most eligible ones for further neuroprotection evaluation. The organoselenides evaluated here were synthesized using the basic structure of $(PhSe)_2$, the simplest diaryl diselenides tested (Fig. 1, compound IX). Since it is well known that oxidative stress is related to pathophysiologic mechanisms of many neurodegenerative diseases, including PD, our screening was based on two distinct parameters: find the concentration of compounds that presents high in vitro antioxidant potential with concomitant low cytotoxicity against the differentiated human neuroblastoma SH-SY5Y cells. TRAP assay showed that the majority of the organoselenides tested were not able to scavenge the peroxyl radical generated by AAPH decomposition (Fig. 2). In the concentrations evaluated in this study (0.1, 0.3, 3, 10, and 30 μM) only compound III (concentration ranging from 10 to 30 μM), IV, V, and VI (concentration ranging from 3 to 30 μM) presented antioxidant activity against the peroxyl radical.

MTT assay was used to evaluate the basal cytotoxicity of the nine compounds over the differentiated human neuroblastoma SH-SY5Y cells. Figure 3 shows that compound III, IV, V, and VI, considered as antioxidant based on the in vitro TRAP assay, presented also high cytotoxicity in treated cells. In all concentrations tested, compound I, II, VII, and VIII did not show any significant signs of cytotoxicity. Hence, based on TRAP and MTT assays, we selected the concentrations of three compounds that presented high antioxidant potential and low cytotoxicity: compound III at 10 μM, IV at 3 μM, and V at 3 μM. Even though the compound IX $(PhSe)_2$ presented no antioxidant potential in the concentrations tested, we select this compound based on previous findings about its neuroprotective features (Porciúncula et al. 2003; Posser et al. 2008; Ardais et al. 2010).

Effect of Selected Organoselenides Compounds Over Cellular Morphology and Neuronal Markers

The RA-induced differentiated SH-SY5Y cells have several morphological and biochemical parameters of a dopaminergic cell (Lopes et al. 2010). One important feature of this cell model is the stellate (neuron-like) morphology and the abundance of neurites, which are important parameters to be evaluated in neuroscience studies (Radio and Mundy 2008). Here, we treated the SH-SY5Y cells with the previously selected concentration of compounds and further evaluated their effect over neurites density and the immunocontent of several neuronal markers (Fig. 4). Even though the concentration selected of all compounds was considered sub-lethal by MTT assay, our data showed a significant change in cell morphology with concomitant decrease in neurites density caused by compound III and IX (Fig. 4a, b). Densitometric analysis showed no change in the immunocontent of the neuronal markers tested (Synaptobrevin, DAT, and TH) (Fig. 4c).

Effect of Selected Organoselenides Compounds Over Cellular Redox Parameters

Based on the widely documented peroxidase-like activity of organoselenides, we first determined the TRAP capacity and Elman's reduced thiol levels of cells incubated with compound III at 10 μM, IV at 3 μM, and V at 3 μM for 24 h, in order to evaluate the effect of these three compounds in cellular redox status. All these experiments were compared to DMSO to determine the solvent effect. The results shown that control and vehicle groups were not different from each other and all the data were expressed in percentage to control cells.

TRAP assay showed that all the selected compounds were able to increase radical scavenging capacity when compared to vehicle ($P < 0.05$) (Fig. 5a). These increases in TRAP capacity were followed by an increase in reduced thiol levels found in treated cells (Fig. 5b). Incubation with compounds III, IV, and V increased the GPx activity (Fig. 5e), and SOD activity was increased in SH-SY5Y cells treated with compounds IV and V (Fig. 5d). No

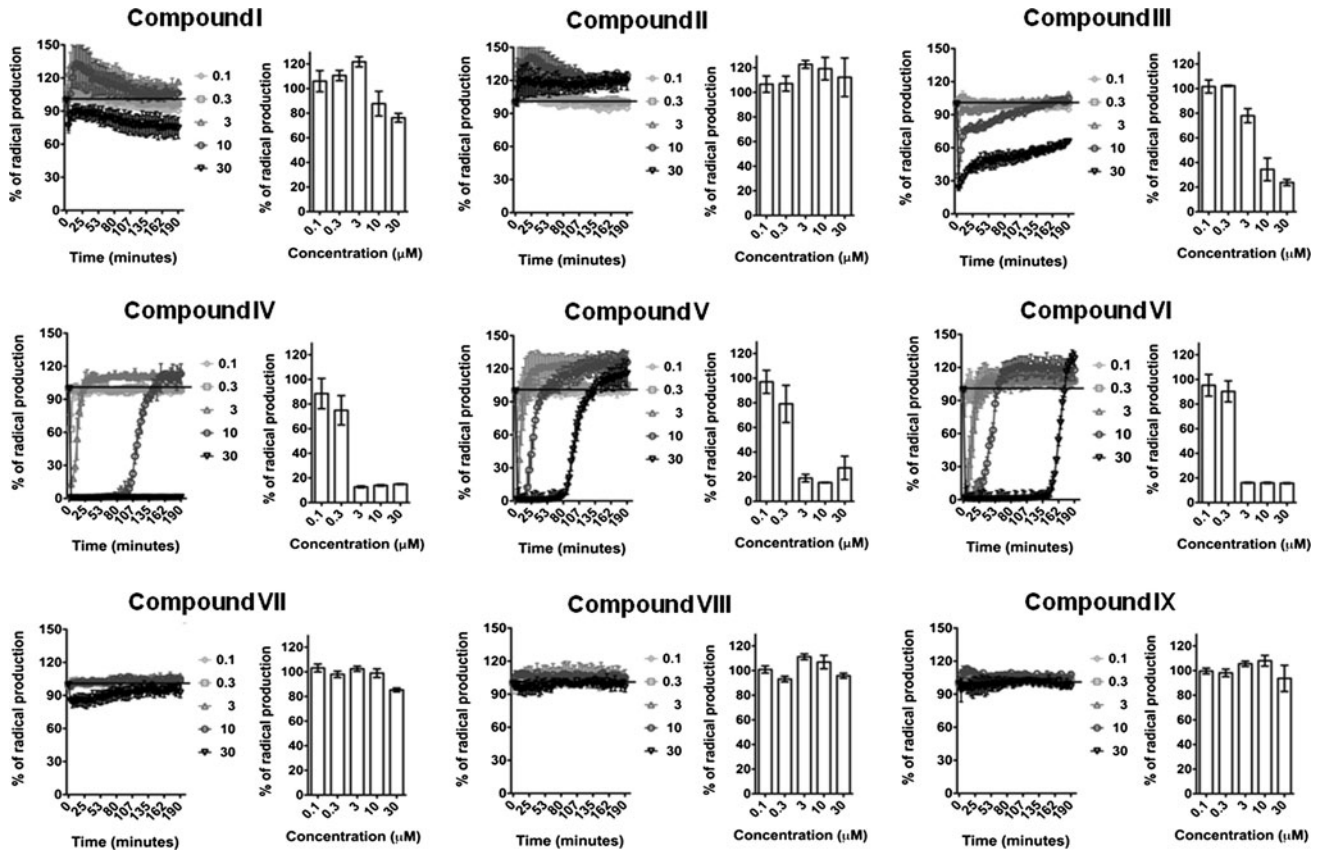

Fig. 2 In vitro total radical-trapping antioxidant potential (TRAP) of the organoselenides. *Left figures* represents TRAP traces, evidencing the system measurements (AAPH + buffer + luminol) (*black line*), which represent 100% of radical production, and the effect of the addition of compounds at the concentrations of 0.1, 0.3, 3, 10, and 30 μM. *Right figures* represent the "AUC" values and expressed as % of radical production, as described in "Experimental Procedures" section. Data are presented as mean ± SD of four independent experiments carried out in triplicates ($n = 4$)

differences were observed in CAT and GST activities (Fig. 5c, f).

Evaluation of Neuroprotection by Selected Organoselenides Against 6-Hydroxydopamine Toxicity

To evaluate neuroprotection, we pre-incubate the differentiated SH-SY5Y cells with compounds III, IV, V, and IX during 24 h. After cells were washed and challenged with 6-OHDA ($LD_{50} = 15$ μM). All the compounds were able to inhibit the cell death (Fig. 6a) and to decrease the generation of RS (Fig. 6b) caused by 6-OHDA treatment.

Discussion

The ongoing management of neurodegenerative diseases is only palliative; basically aimed to decrease the symptoms of these debilitating illnesses. Finding a drug that can reduce disease progression and thus, delay the onset of disability is a critical goal (Shobha et al. 2006; Meissner et al. 2011). However, to develop targeted therapeutics, it is fundamental to understand the molecular mechanisms of these diseases. Even though the pathophysiology of neurodegenerative diseases is unknown, for instance, PD, there are many lines of evidence supporting the major involvement of oxidative stress (Schapira 2008). In this context, the search for antioxidant molecules as therapeutic adjuvant can be an alternative for PD (Jaisin et al. 2011).

Organoselenides have been documented as promising pharmacological agents against a number of diseases associated with oxidative stress, mainly related to hepatotoxicity (Ibrahim et al. 2010). However, it is not well elucidated their role in neuroprotection. These molecules present several features that support their potential role in the management of neurodegenerative disease, such as their low-molecular weight in combination with high antioxidant potential, high availability and hydrophobicity, all qualities that improve its therapeutic potential. For instance, one clinical trial showed that early treatment with ebselen improved the patient outcome in acute ischemic stroke (Yamaguchi et al. 1998). Further studies demonstrated a

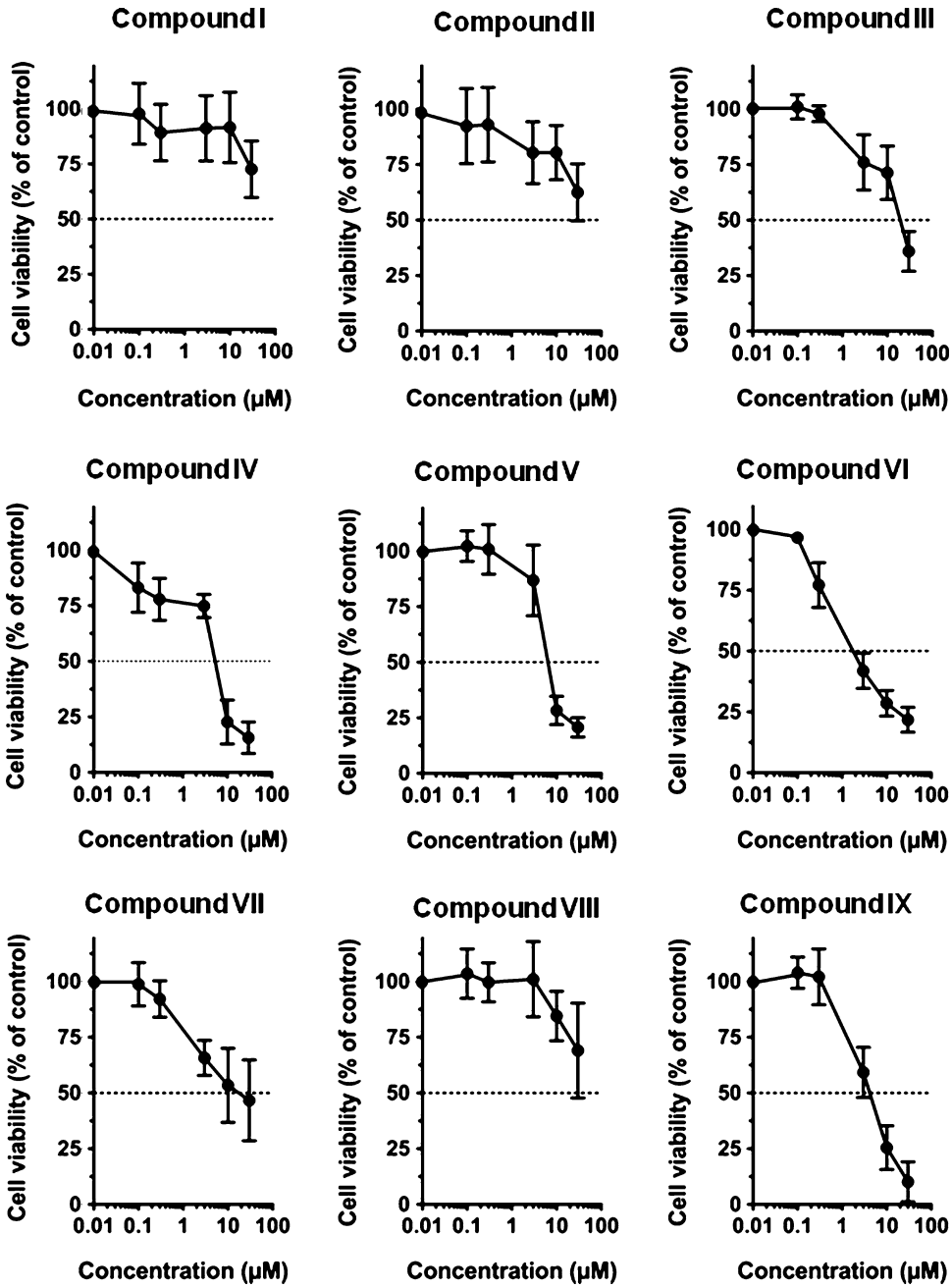

Fig. 3 Cytotoxicity curves of the organoselenides compounds. Cells were treated with each compound at the concentrations of 0.1, 0.3, 3, 10, and 30 μM during 24 h and cell viability was evaluated by MTT assay. *Dashed line* represents LD_{50} value of compound. Data are presented as mean ± SD for four independent experiments carried out in quadruplicates ($n = 4$)

neuroprotective effect of organoselenides in hippocampal slices submitted to oxygen-glucose deprivation (Porciúncula et al. 2003) and hydrogen peroxide administration (Posser et al. 2008). However, there is no data exploring the neurotoxic or neuroprotective role of organoselenides in cell systems, such as differentiated human neuroblastoma SH-SY5Y cells.

We aimed here to screen between different synthetic organoselenides compounds to select the most eligible ones for neuroprotection evaluation. Taking into account the results obtained in this study, we found a significant correlation between the antioxidant potential of synthetic organoselenides compounds and cellular toxicity in RA-differentiated human neuroblastoma SH-SY5Y cells. The concept of antioxidant supplementations being beneficial to human health should be taking with caution. Antioxidant is any substance that, when present in low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substances so that, they keep the RS in low levels (Halliwell 2006). However, oxidants are not only villains in biological systems and they can act in normal physiology of the cells, such as in the activity of cytochrome P450, the immune response and in normal signal transduction pathways

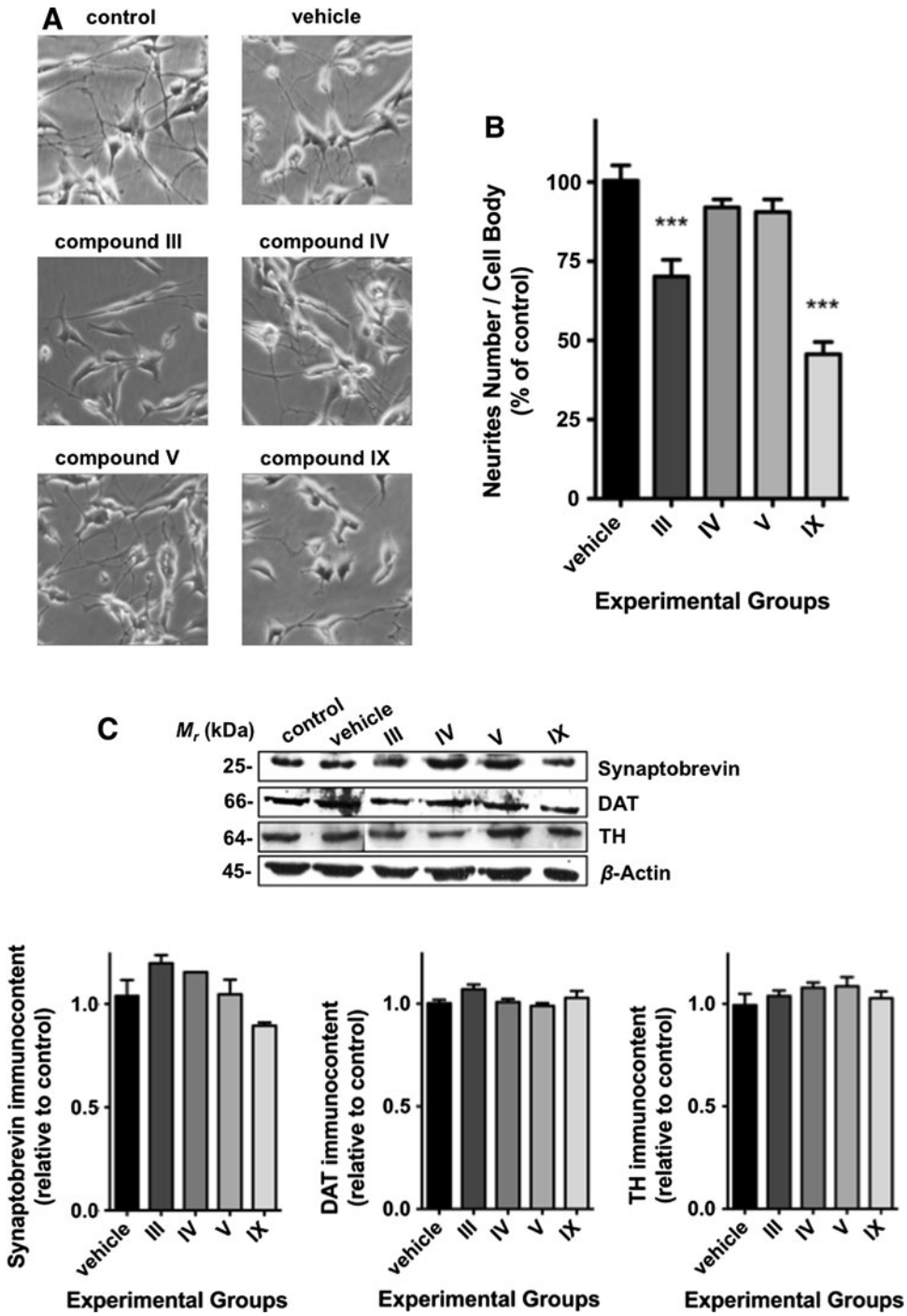

Fig. 4 The effect of sub-lethal doses of selected organoselenides over the cellular morphology and neuronal markers of differentiated human neuroblastoma SH-SY5Y cells. **a** Representative phase contrast images of cells treated by 24 h with 10 μM of compound III, 3 μM of compound IV, 3 μM of compound V, 0.3 μM of compound IX or vehicle, evidencing the stellate morphology with abundant neurites outgrow of cells (200× magnification). Note the change in cell morphology and the lost of neurites caused by compound III and IX. **b** Representative quantification of the neurites density per cell body in treated cells. Data are presented as mean ± SD for three independent experiments carried out in quadruplicates ($n = 3$). ***$P < 0.001$ (one-way analysis of variance). **c** Western blot analysis of neuronal markers (synaptobrevin, tyrosine hydroxylase—TH, dopamine transporter—DAT) of differentiated SH-SY5Y cells treated with selected sub-lethal doses of compounds. β-Actin was used as loading control. Representative blots of three independent experiments ($n = 3$)

(Stone and Yang 2006). Thus, as found with the organoselenides studied here, any antioxidant compound may alter the intracellular redox balance toward a more reduced state, causing as much cytotoxicity response as oxidative stress (Gutteridge and Halliwell 2010).

Our study also established several advantages of the use of differentiated human neuroblastoma SH-SY5Y cells as an in vitro experimental model for neurotoxicity/neuroprotection drug screening evaluations. Even though the differentiated SH-SY5Y cells posses many desired features of an in vitro cell model for neuroscience research (e.g., neuronal morphology with abundant of neurites outgrow and the expression of several neuronal markers) (Pahlman et al. 1984; Lopes et al. 2010), there is still some controversy about the necessity of cellular differentiation (Xie et al. 2010). As an example, in a recent report Cheung et al. (2009) found no change in the expression of dopaminergic markers during SH-SY5Y cellular differentiation by RA.

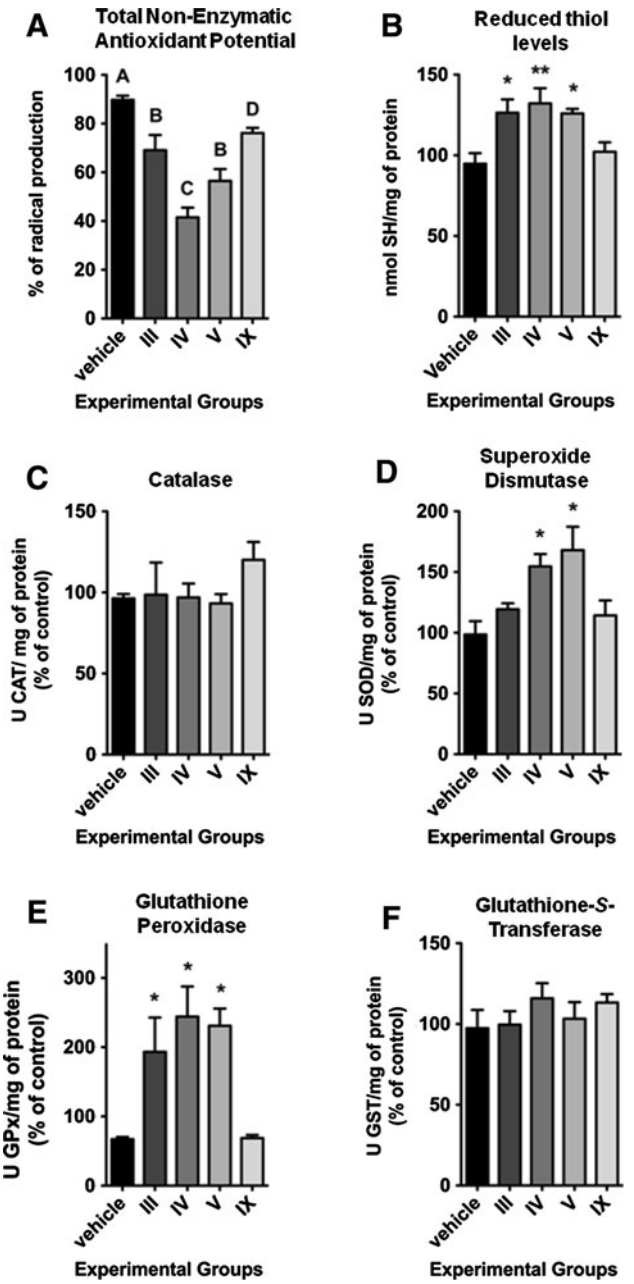

Fig. 5 The effect of sub-lethal doses of selected organoselenides over the redox parameters in differentiated human neuroblastoma SH-SY5Y cells. SH-SY5Y cells were treated by 24 h with 10 μM of compound III, 3 μM of compound IV, 3 μM of compound V, 0.3 μM of compound IX or vehicle, washed and the redox parameters were determined as described in "Experimental Procedures" section. All the results were compared to control cells and expressed as % of control. **a** Total radical-trapping antioxidant potential (TRAP) of treated cells. Data represent the "AUC" values and expressed as % of radical production, as described in "Experimental Procedures" section. Statistical differences are expressed by *letters*. $P < 0.05$ (one-way analysis of variance). **b** Elmann's reduced thiol levels (basal levels ranging from 24 to 48 nmol –SH/mg of protein). **c** Catalase activities in treated cells (basal levels ranging from 1.18 to 1.73 U CAT/mg of protein). **d** SOD activities in treated cells (basal levels ranging from 9.50 to 16.23 U SOD/mg of protein). **e** GPx activities in treated cells (basal levels ranging from 2.84 to 4 U GPx/mg of protein). **f** Glutathione-*S*-transferase activities in treated cells (basal levels ranging from 21.11 to 28.35 U GST/mg of protein). Data are presented as mean ± SD for four independent experiments carried out in quadruplicates ($n = 4$). $^{*}P < 0.05$; $^{**}P < 0.01$ (one-way analysis of variance)

Furthermore, they found a reduced sensitivity to the neurotoxins MPP^{+} and 6-OHDA in RA-differentiated cells, which was attributed to an increased activity of survival signals such as Akt pathway. Thus, with the lack of change in dopaminergic properties and decreased susceptibility to neurotoxins during RA differentiation, they claimed that undifferentiated SH-SY5Y cell might be a more appropriate model in experimental PD research (Cheung et al. 2009). These conclusions have been criticized (Luchtman and Song 2010), and contrast with our previous report, in which we found an increase in the expression of several neuronal markers and an increased sensitivity to 6-OHDA in RA-differentiated SH-SY5Y cells (Lopes et al. 2010). These contradictories results could be explained by the differences in differentiation protocols used, for instance, serum origin and amount (Dr. Raymond Chang, personal communication). The study of neurotoxicity induced by chemicals represents a major challenge due to the physiological and morphological complexity of nervous system (Radio and Mundy 2008; Bal-Price et al. 2010). Most of in vitro models uses cell lines derived from CNS tumors and do not exhibit the same phenotype as the progenitor cells (Banker and Goslin 1998). Moreover, most of the screening strategies use simple cell viability assays (such as MTT, sulforhodamine B, trypan blue exclusion, and others) as outcome of the studies. Using the differentiated SH-SY5Y cells, we found that sub-lethal doses of two compounds (III and IX) significantly decrease the neurites densities of treated cells. The term neurite refers to axons and dendrites extended by neuronal cell lines, and neurites densities are important morphological parameters in neuroscience studies (Carmeliet 2003). These results should be taken into account in futures neurotoxic/neuroprotective evaluations of organoselenides or other potential molecules.

The synthesis of different organoselenides, such as ebselen and $(PhSe)_2$, was based on the structure of the selenocysteine in the active site of GPx enzyme (Rotruck et al. 1973; Brigelius-Flohé 1999; Sarma and Mugesh 2005). Since they all present a peroxidase-like activity in vitro, is not surprising that incubation of cells with selected compounds leads to an increased in TRAP and reduced thiol levels, in combination with increased GPx activity. The increase in the cellular antioxidant defense in response to organoselenides treatment is the basis of the neuroprotection against 6-OHDA toxicity. 6-OHDA is an analog of DA with similar structural characteristics and affinity with DA transporter. Once inside the neuron, it accumulates in the

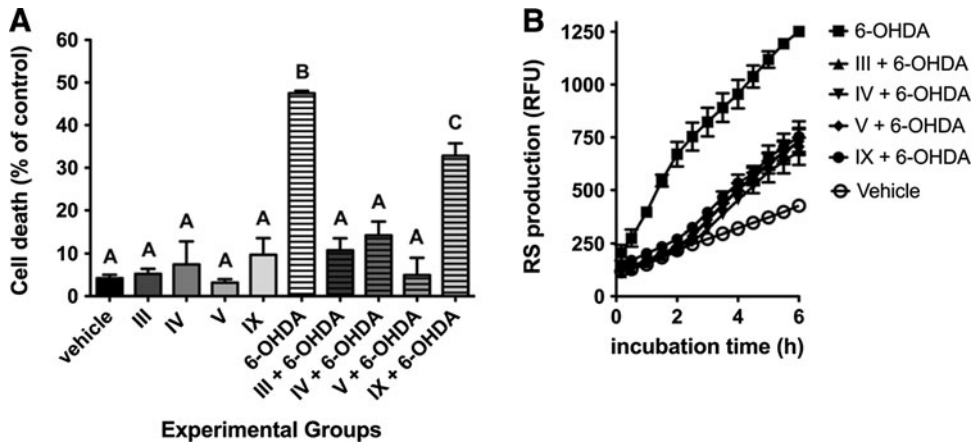

Fig. 6 Evaluation of neuroprotection of sub-lethal doses of compounds III, IV, V, and IX against 6-OHDA toxicity. SH-SY5Y cells were pre-incubated by 24 h with 10 μM of compound III, 3 μM of compound IV, 3 μM of compound V, 0.3 μM of compound IX or vehicle, washed with PBS, and further treated with 6-OHDA (LD_{50} = 15 μM) for 24 h. **a** Cell viability was determined by MTT assay, as described in "Experimental Procedures" section. **b** RS production was evaluated by DCF assay. Data are presented as mean ± SD of four independent experiments carried out in quadruplicates ($n = 4$). Statistical differences are expressed by *letters*. $P < 0.05$ (one-way analysis of variance)

cell causing cellular alterations, such as oxidative damage (Lehmensiek et al. 2006). This neurotoxin is a potent complex I inhibitor and may lead to the production of superoxide radicals and H_2O_2 (Glinka et al. 1997). 6-OHDA can suffer auto-oxidation and consequently can generate a massive RS production, such as quinones, hydrogen peroxide, superoxide, and hydroxyl radicals (Blandini et al. 2010; Bove and Perier 2011). Moreover, 6-OHDA leads to a reduction in endogenous cellular antioxidants activities such as SOD and GPx (Ahmad et al. 2011). The neuroprotection against 6-OHDA in differentiated SH-SY5Y cells should be related to a high radical scavenge capacity of the incorporated organoselenides. High peroxidase-like activity and thiol levels found in treated cells are important (and desired) features of organoselenides, since many lines of evidence indicate that the CNS posses modest antioxidant defenses and is highly susceptibility to oxidative damage (Halliwell 2006; Jaisin et al. 2011). These features of organoselenides suggest their use as therapeutic adjuvant for PD and demand futures investigations.

In conclusion, our data demonstrated that RA-differentiated human neuroblastoma SH-SY5Y cells treated with sub-lethal doses of both compounds IV and V present an increase in cellular antioxidant status without alteration of neurites densities and neuronal markers. Moreover, these treatments also confer a complete protection against 6-OHDA neurotoxicity and we can infer that these organoselenides are the most promising ones as potential drugs in the treatment of PD.

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References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126

Ahmad S, Khan MB, Hoda MN, Bhatia K, Haque R, Fazili IS, Jamal A, Khan JS, Katare DP (2011) Neuroprotective effect of sesame seed oil in 6-hydroxydopamine induced neurotoxicity in mice model: cellular, biochemical and neurochemical evidence. Neurochem Res. doi:10.1007/s11064-011-0638-4

Ardais AP, Viola GG, Costa MS, Nunes F, Behr GA, Klamt F, Moreira JC, Souza DO, Rocha JB, Porciúncula LO (2010) Acute treatment with diphenyl diselenide inhibits glutamate uptake into rat hippocampal slices and modifies glutamate transporters, SNAP-25, and GFAP immunocontent. Toxicol Sci 113:434–443

Arnér ES, Holmgre A (2000) Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem 267:6102–6109

Bal-Price AK, Hogberg HT, Buzanska L, Coecke S (2010) Relevance of in vitro neurotoxicity testing for regulatory requirements: challenges to be considered. Neurotoxicol Teratol 32:36–41

Banker G, Goslin K (1998) Type of neurvous cells, their advantages and limitations. The MIT Press, Cambridge

Barnham KJ, Masters CL, Bush AI (2004) Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov 3:205–214

Bhabak KP, Mugesh G (2007) Synthesis, characterization, and antioxidant activity of some ebselen analogues. Chemistry 13:4594–4601

Blandini F, Cova L, Armentero MT, Zennaro E, Levandis G, Bossolasco P, Calzarossa C, Mellone M, Giuseppe B, Deliliers GL, Polli E, Nappi G, Silani V (2010) Transplantation of undifferentiated human mesenchymal stem cells protects against 6-hydroxydopamine neurotoxicity in the rat. Cell Transplant 19:203–217

Bové J, Perier C (2011) Neurotoxin-based models of Parkinson's disease. Neuroscience. doi:10.1016/j.neuroscience.2011.10.057

Brigelius-Flohé R (1999) Tissue-specific functions of individual glutathione peroxidases. Free Radic Biol Med 27:951–965

Carmeliet P (2003) Blood vessels and nerves: common signals, pathways and diseases. Nat Rev Genet 4:710–720

Cheung YT, Lau WK, Yu MS, Lai CS, Yeung SC, So KF, Chang RC (2009) Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology 30:127–135

Drechsel DA, Patel M (2008) Role of reactive oxygen species in the neurotoxicity of environmental agents implicated in Parkinson's disease. Free Radic Biol Med 44:1873–1886

Dresch MT, Rossato SB, Kappel VD, Biegelmeyer R, Hoff ML, Mayorga P, Zuanazzi JA, Henriques AT, Moreira JC (2009) Optimization and validation of an alternative method to evaluate total reactive antioxidant potential. Anal Biochem 385:107–114

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77

Farina M, Soares FA, Zeni G, Souza DO, Rocha JB (2004) Additive pro-oxidative effects of methylmercury and ebselen in liver from suckling rat pups. Toxicol Lett 146:227–235

Flohe L, Günzler WA, Schock HH (1973) Glutathione peroxidase: a selenoenzyme. FEBS Lett 32:132–134

Floyd RA, Hensley K (2002) Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases. Neurobiol Aging 23:795–807

Geoghegan M, McAuley D, Eaton S, Powell-Tuck J (2006) Selenium in critical illness. Curr Opin Crit Care 12:136–141

Glinka Y, Gassen M, Youdim MBH (1997) Mechanism of 6-hydroxydopamine neurotoxicity. J Neural Transm 104:55–66

Gutteridge JM, Halliwell B (2010) Antioxidants: molecules, medicines, and myths. Biochem Biophys Res Commun 393:561–564

Halliwell B (1992) Reactive oxygen species and the central nervous system. J Neurochem 59:1609–1623

Halliwell B (2001) Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 18:685–716

Halliwell B (2006) Oxidative stress and neurodegeneration: where are we now? J Neurochem 97:1634–1658

Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 142:231–255

Ibrahim M, Prigol M, Hassan W, Nogueira CW, Rocha JB (2010) Protective effect of binaphthyl diselenide, a synthetic organoselenium compound, on 2-nitropropane-induced hepatotoxicity in rats. Cell Biochem Funct 28:258–265

Ibrahim M, Luchese C, Pinton S, Roman SS, Hassan W, Nogueira CW, Rocha JB (2011) Involvement of catalase in the protective effect of binaphthyl diselenide against renal damage induced by glycerol. Exp Toxicol Pathol 63:331–335

Ilijic E, Guzman JN, Surmeier DJ (2011) The L-type channel antagonist isradipine is neuroprotective in a mouse model of Parkinson's disease. Neurobiol Dis 43:364–371

Jaisin Y, Thampithak A, Meesarapee B, Ratanachamnong P, Suksamrarn A, Phivthong-Ngam L, Phumala-Morales N, Chongthammakun S, Govitrapong P, Sanvarinda Y (2011) Curcumin I protects the dopaminergic cell line SH-SY5Y from 6-hydroxydopamine-induced neurotoxicity through attenuation of p53-mediated apoptosis. Neurosci Lett 489:192–196

Kalayci M, Coskun O, Cagavi F, Kanter M, Armutcu F, Gul S, Acikgoz B (2005) Neuroprotective effects of ebselen on experimental spinal cord injury in rats. Neurochem Res 30:403–410

Lehmensiek V, Tan EM, Liebau S, Lenk T, Zettlmeisl H, Schwarz J, Storch A (2006) Dopamine transporter-mediated cytotoxicity of 6-hydroxydopamine in vitro depends on expression of mutant a-synucleins related to Parkinson's disease. Neurochem Int 48:329–340

Lissi E, Salim-Hanna M, Pascual C, del Castillo MD (1995) Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. Free Radic Biol Med 18:153–158

Lopes FM, Schröder R, da Frota ML Jr, Zanotto-Filho A, Müller CB, Pires AS, Meurer RT, Colpo GD, Gelain DP, Kapczinski F, Moreira JC, Fernandes MdaC, Klamt F (2010) Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain Res 1337:85–94

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Luchese C, Zeni G, Rocha JB, Nogueira CW, Santos FW (2007) Cadmium inhibits delta-aminolevulinate dehydratase from rat lung in vitro: interaction with chelating and antioxidant agents. Chem Biol Interact 165:127–137

Luchtman DW, Song C (2010) Why SH-SY5Y cells should be differentiated. Neurotoxicology 31:164–165

Mandel S, Grunblatt E, Riederer P, Gerlach M, Levites Y, Youdim MB (2003) Neuroprotective strategies in Parkinson's disease: an update on progress. CNS Drugs 17:729–762

McCarthy S, Somayajulu M, Sikorska M, Borowy-Borowski H, Pandey S (2004) Paraquat induces oxidative stress and neuronal cell death; neuroprotection by water-solubleCoenzyme Q10. Toxicol Appl Phamacol 201:21–31

Meissner WG, Frasier M, Gasser T, Goetz CG, Lozano A, Piccini P, Obeso JA, Rascol O, Schapira A, Voon V, Weiner DM, Tison F, Bezard E (2011) Priorities in Parkinson's disease research. Nat Rev Drug Discov 10:377–393

Meotti FC, Borges VC, Zeni G, Rocha JB, Nogueira CW (2003) Potential renal and hepatic toxicity of diphenyl diselenide, diphenyl ditelluride and ebselen for rats and mice. Toxicol Lett 143:9–16

Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247:3170–3175

Mugesh G, Panda A, Singh HB, Punekar NS, Butcher RJ (2001) Glutathione peroxidase-like antioxidant activity of diaryl diselenides: a mechanistic study. J Am Chem Soc 123:839–850

Müller A, Cadenas E, Graf P, Sies H (1984) A novel biologically active seleno-organic compound—I. Glutathione peroxidase-like activity in vitro and antioxidant capacity of PZ 51 (ebselen). Biochem Pharmacol 33:3235–3239

Naoi M, Maruyama W (2010) Monoamine oxidase inhibitors as neuroprotective agents in age-dependent neurodegenerative disorders. Curr Pharm Des 16:2799–2817

Ozdemir I, Talas ZS, Gok Y, Ates B, Yilmaz I (2010) Changes in tyrosine hydroxylase activity, adrenomedullin and total RNA levels by treatment of organoselenium compounds in rat hypothalamus exposed to DMBA. Regul Pept 159:137–141

Pabst MJ, Habig WH, Jakoby WB (1974) Glutathione S-transferase. A novel kinetic mechanism in which the major reaction pathway depends on substrate concentration. J Biol Chem 249:140–147

Pahlman S, Rusala AI, Abrahamsson L, Mattsson ME, Esscher T (1984) Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. Cell Differ 14:135–144

Pedersen V, Schmidt WJ (2000) The neuroprotectant properties of glutamate antagonists and antiglutamatergic drugs. Neurotoxic Res 2:179–204

Porciúncula LO, Rocha JBT, Cimarosti H, Vinadé L, Ghisleni G, Salbego CG, Souza DO (2003) Neuroprotective effect of ebselen on rat hippocampal slices submitted to oxygen-glucose deprivation: correlation with immunocontent of inducible nitric oxide synthase. Neurosci Lett 346:101–104

Posser T, Moretto MB, Dafre AL, Farina M, da Rocha JB, Nogueira CW, Zeni G, Ferreira J, Dos S, Leal RB, Franco JL (2006) Antioxidant effect of diphenyl diselenide against sodium nitroprusside (SNP) induced lipid peroxidation in human platelets and erythrocyte membranes: an in vitro evaluation. Chem Biol Interact 164:126–135

Posser T, Franco JL, dos Santos DA, Rigon AP, Farina M, Dafré AL, Teixeira Rocha JB, Leal RB (2008) Diphenyl diselenide confers neuroprotection against hydrogen peroxide toxicity in hippocampal slices. Brain Res 1199:138–147

Posser T, de Paula MT, Franco JL, Leal RB, da Rocha JB (2011) Diphenyl diselenide induces apoptotic cell death and modulates ERK1/2 phosphorylation in human neuroblastoma SH-SY5Y-cells. Arch Toxicol 85:645–651

Radio NM, Mundy WR (2008) Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. Neurotoxicology 29:106–118

Rossato JI, Ketzer LA, Centurião FB, Silva SJ, Lüdtke DS, Zeni G, Braga AL, Rubin MA, Rocha JB (2002) Antioxidant properties of new chalcogenides against lipid peroxidation in rat brain. Neurochem Res 27:297–303

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science 179:588–590

Safi R, Gardaneh M, Panahi Y, Maghsoudi N, Zaefizadeh M, Gharib E (2011) Optimized quantities of GDNF overexpressed by engineered astrocytes are critical for protection of neuroblastoma cells against 6-OHDA toxicity. J Mol Neurosci. doi: 10.1007/s12031-011-9654-8

Santos DB, Schiar VP, Paixão MW, Meinerz DF, Nogueira CW, Aschner M, Rocha JB, Barbosa NB (2009) Hemolytic and genotoxic evaluation of organochalcogens in human blood cells in vitro. Toxicol In Vitro 23:1195–1204

Sarma BK, Mugesh G (2005) Glutathione peroxidase (GPx)-like antioxidant activity of the organoselenium drug ebselen: unexpected complications with thiol exchange reactions. J Am Chem Soc 127:11477–11745

Schapira AH (2008) Mitochondrial dysfunction in neurodegenerative disease. Neurochem Res 12:2502–2509

Schewe T (1995) Molecular actions of ebselen—an antiinflammatory antioxidant. Gen Pharmacol 26:1153–1169

Shobha SR, Hofmann LA, Shakil A (2006) Parkinson's disease: diagnosis and treatment. Am Fam Physician 74:2046–2054

Sies H (1993) Ebselen, a selenoorganic compound as glutathione peroxidase mimic. Free Radic Biol Med 14:313–323

Souza AC, Stangherlin EC, Ardais AP, Nogueira CW (2010) Diphenyl diselenide and diphenyl ditelluride: neurotoxic effect in brain of young rats, in vitro. Mol Cell Biochem 340:179–205

Spencer JP, Jenner P, Daniel SE, Lees AJ, Marsden DC, Halliwell B (1998) Conjugates of catecholamines with cysteine and GSH in Parkinson's disease: possible mechanisms of formation involving reactive oxygen species. J Neurochem 71:2112–2122

Stefanis L, Keller JN (2006) The proteasome in neurodegeneration. Springer, New York

Stone JR, Yang S (2006) Hydrogen peroxide: a signaling messenger. Antioxid Redox Signal 8:243–270

Talas ZS, Ozdemir I, Yilmaz I, Gok Y, Orun I (2008) The investigation of the antioxidative properties of the novel synthetic organoselenium compounds in some rat tissues. Exp Biol Med 233:575–579

Wang H, Joseph JA (1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 27:612–616

Wendel A (1981) Glutathione peroxidase. Methods Enzymol 77:325–333

Xie HR, Hu LS, Li GY (2010) SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. Chin Med J (Engl) 123:1086–1092

Xu JH, Hu HT, Liu Y, Qian YH, Liu ZH, Tan QR, Zhang ZJ (2006) Neuroprotective effects of ebselen are associated with the regulation of Bcl-2 and Bax proteins in cultured mouse cortical neurons. Neurosci Lett 399:210–214

Yamaguchi T, Sano K, Takakura K, Saito I, Shinohara Y, Asano T, Yasuhara H (1998) Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Ebselen Study Group. Stroke 29:12–17

Zeevalk GD, Bernard LP, Song C, Gluck M, Ehrhart J (2005) Mitochondrial inhibition and oxidative stress: reciprocating players in neurodegeneration. Antioxid Redox Signal 7:1117–1139

RESULTADOS SUPLEMENTARES II: Padronização da transfecção através da superexpressão da proteína verde fluorescente (GFP) em células diferenciadas da linhagem de neuroblastoma humano SH-SY5Y.

As células SH-SY5Y são amplamente utilizadas para experimentos de manipulação gênica através da transfecção de genes de interesse (Biedler *et al.,* 1973). No entanto este parâmetro não está estabelecido no modelo de célula diferenciada. Por isso, nós realizamos a padronização da transfecção em células diferenciadas através da superexpressão da proteína GFP.

A transfecção e a superexpressão foram feitas de forma transiente utilizando a técnica da Lipofectamina. As células foram transfectadas com plasmídeo contendo cDNA de GFP ou MOCK (sem vetor) e após o tempo de captação (6 horas), nós iniciamos o protocolo de diferenciação com 10 µM de AR em meio de cultura com 1% de SFB.

A eficiência da superexpressão foi determinada através de análise da fluorescência do GFP em Microscópio de Fluorescência Olympus durante 1, 4 e 7 dias. Nós verificamos que a expressão da proteína GFP é mantida durante todo o processo de diferenciação e que a morfologia celular não foi alterada, como ilustra a Figura 1.

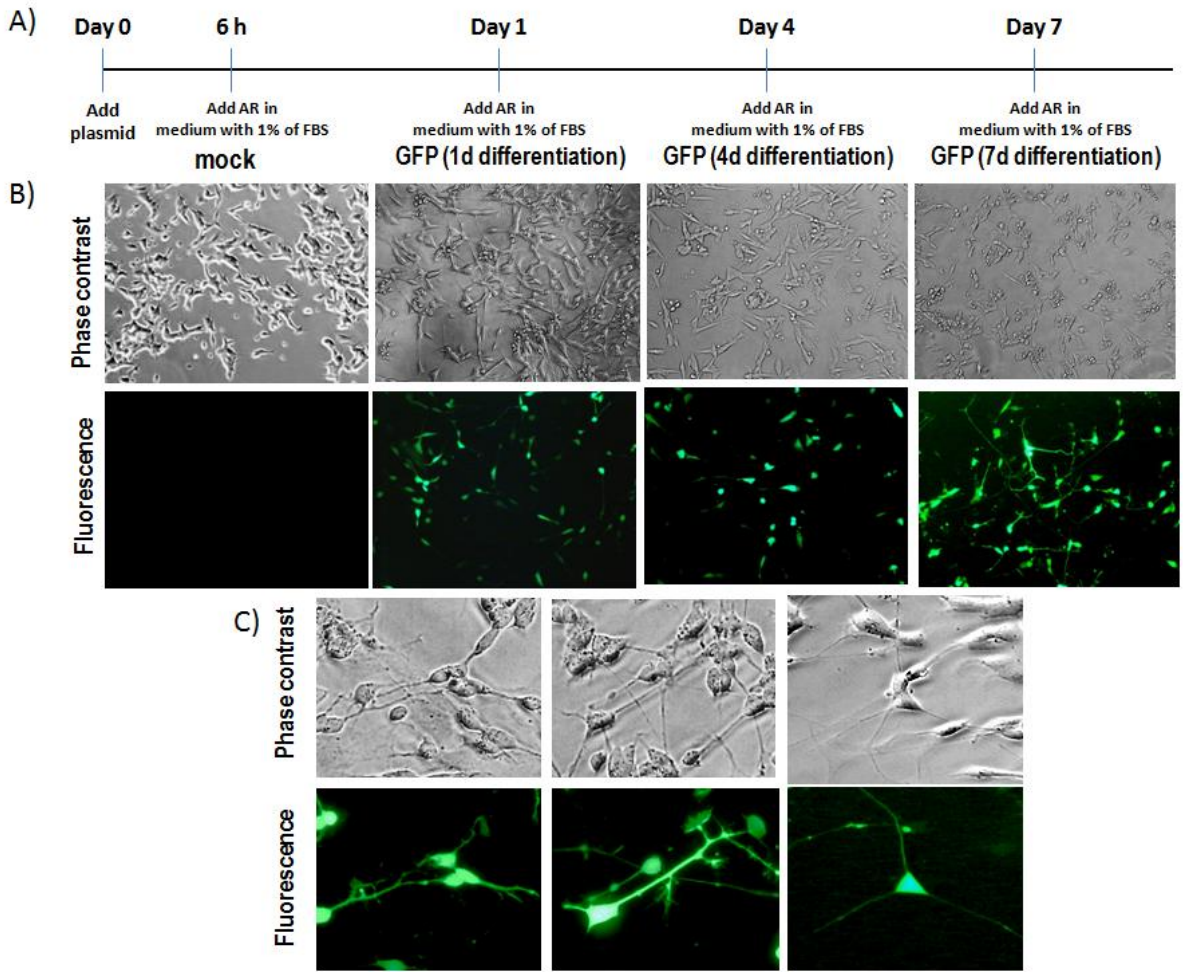


Figure 1: (A,B) Transient transfection and overexpression of GFP. SH-SY5Y cells were transfected with MOCK or plasmid containing GFP cDNA and the differentiation protocol were performed. (C) Note that differentiated cells for 7 days presents the stellate morphology with intense neurites and are still positive for GFP (200X magnification).

PARTE III

4. DISCUSSÃO

Diversas doenças neurodegenerativas, incluindo a DP, tem como base fisiopatológica a disfunção mitocondrial, o estresse oxidativo e a agregação protéica. No entanto, os mecanismos moleculares que levam a estes processos ainda devem ser elucidados (Henchcliff & Beal, 2008; Saiki *et al.,* 2011). Para estudos destes mecanismos, utilizamos modelos experimentais tanto *in vitro* como *in* vivo. Entretanto, a escolha do modelo experimental para DP é um assunto controverso devido à dificuldade de mimetizar a sintomatologia do distúrbio *in vivo* e das limitações dos modelos *in vitro* (Beal, 2010). Para o estudo de mecanismos celulares e moleculares da DP, os modelos experimentais mais indicados são os *in vitro* (Schüle *et al.,*2009).

Este assunto é de muita importância visto que um dos principais fatores para o fracasso de muitos estudos clínicos terapêuticos da DP, além do não conhecimento da sua patologia, é a ausência de modelos experimentais que representem a doença de forma correta (Olanow *et al.,* 2008). Dessa forma, o delineamento para pesquisas em neurociências deve ser voltado para o desenvolvimento de modelos experimentais para estudar a fisiopatologia da doença e, posteriormente, buscar alvos terapêuticos (Jenner, 2008). A DP ainda é incurável e sua terapia é apenas paliativa. Após 50 anos da descoberta do *L*-DOPA como tratamento para DP, nenhuma outra droga se mostrou eficaz e capaz de substituí-lo como terapia-padrão (Jankovic *et al.,* 2005; Hauser *et al.,* 2007).

O modelo experimental *in vitro* mais utilizado é a linhagem de neuroblastoma humano SH-SY5Y (Xie *et al.,* 2010). Apesar de inúmeros estudos mostrarem a diferenciação do neuroblastoma a neurônio

dopaminérgico, bem como o aumento de marcadores bioquímicos dopaminérgicos durante a diferenciação, há poucos estudos que exploram as diferenças entre os dois fenótipos (Tieu *et al.,*1999; Cheung *et al.,* 2009), bem como não há estudos mostrando a aplicação do modelo de célula diferenciada para estudo da fisiopatologia da DP. Dessa forma, ainda há muita discussão sobre a escolha do melhor modelo experimental (vide anexo – Mesa Redonda do Society for Neuroscience: discussão sobre modelos *in vitro* para estudo de neurociências).

Além disso, um fato importante é que não há um consenso nos protocolos utilizados para a diferenciação das células SH-SY5Y induzida por AR. A diferenciação dopaminérgica envolve um conjunto de fatores: o AR, que é a neurotrofina responsável pela estimulação de receptores nucleares, que ativam genes responsáveis pela diferenciação e pela maturação neural; e a diminuição do soro fetal bovino (SFB), que induz a parada da proliferação celular (Edjsö *et al.,* 2007).

Nos estudos sobre a diferenciação celular da linhagem de neuroblastoma humano SH-SY5Y, as concentrações de AR podem variar de 1 a 20 µM e o tempo de duração do tratamento varia de 3 a 14 dias. Ainda, a maior heterogeneidade encontrada é referente à quantidade de SFB. A manutenção do fenótipo proliferativo dá-se pela adição de 10% SFB em meio de cultivo. Nos protocolos de diferenciação geralmente há a diminuição desse componente, variando de 0 a 3% (Tieu *et al.,*1999; Miloso *et al.,* 2004; Presgraves *et al.,* 2007; Cheung *et al.,* 2009; Alghome *et al.,*2010; Schneider *et al.,* 2011).

Dessa forma, no capítulo I da dissertação, nós padronizamos o protocolo de diferenciação utilizando uma concentração fixa de AR (10 µM) em meio de cultura com 1% SFB. Após isso, nós avaliamos em 4, 7 e 10 dias de tratamento as diferenças morfológicas, neuroquímicas e citotóxicas entre células proliferativas e diferenciadas da linhagem de neuroblastoma humano SH-SY5Y.

A morfologia foi avaliada através da presença de neuritos (projeções citoplasmáticas). Já no parâmetro neuroquímico, verificamos marcadores de diferenciação neuronal DAT (resultados suplementares I, Figura 1C), TH, ENS e NeuN e um marcador de célula indiferenciada, a nestina (capítulo I). Dessa forma, nós observamos que já no quarto dia de diferenciação há o aumento do imunoconteúdo dos marcadores de diferenciação neuronal testados. No entanto, o marcador de célula indiferenciada só diminui de forma significativa no sétimo dia de diferenciação (Lopes *et al.,* 2010). Nossos resultados corroboram com estudos anteriores onde demonstraram que as células SH-SY5Y devem ser diferenciadas pelo menos durante 7 dias. Além dos parâmetros avaliados neste estudo, sabe-se que este tempo de tratamento também propicia o estabelecimento e a propagação do potencial de ação e a formação de vesículas sinápticas (Sarkanen *et al.,* 2007).

Muitos estudos utilizam a TH como marcador dopaminérgico clássico, visto que ela é enzima limitante da síntese das catecolaminas (Kume *et al.,* 2008). A diferenciação induzida por AR causa o aumento do imunoconteúdo e da atividade da TH. O AR ativa a fosfatase 2A que defosforila e ativa TH (Dunkley *et al.,* 2004). Entretanto, alguns estudos mostraram que a diferenciação não é capaz de mudar o imunoconteúdo de marcadores

dopaminérgicos, divergindo com os resultados encontrados em nosso estudo (Cheung *et al.,* 2009). Devido a isso, a mudança de fenótipo causada pela diferenciação induzida por AR é ainda muito controverso. Como a TH é uma enzima catecolaminérgica, alguns estudos mostraram que a diferenciação das células SH-SY5Y não aumenta só os níveis de dopamina, mas também de noradrenalina (Encina *et al.,* 2000; Xie *et al.,* 2010).

Trabalhos anteriores mostraram que a diferenciação induzida por AR das células SH-SY5Y tende a ser adrenérgica e não dopaminérgica e por isso não seria o melhor modelo para DP (Encina *et al.,* 2000). No entanto, outros estudos mostraram níveis insignificantes de liberação de noradrenalina pela SH-SY5Y quando comparadas com a liberação de DA (Balasooriya & Wimalasena, 2007). Portanto, esta variabilidade de dados pode estar relacionada com os diferentes protocolos de diferenciação supracitados.

Neste trabalho, nós estudamos dois modelos com dois fenótipos diferentes. A célula proliferativa com fenótipo tumoral e a célula diferenciada como fenótipo neuronal. Dessa forma, após estabelecer as diferenças morfológicas e neuroquímicas entre os modelos, nós avaliamos a suscetibilidade ao tratamento com 6-OHDA. Nós verificamos que células diferenciadas são mais sensíveis à neurotoxina. Esse dado vai de encontro com alguns dados da literatura (Tieu *et al.* 1999; Cheung *et al.,*2009). Estudos anteriores mostraram que células diferenciadas apresentam um aumento da expressão de vias de sobrevivência, como a Akt (Cheung *et al.,* 2009; da Frota-Jr *et al.,* 2011). Esse dado pode estar correlacionado com a resistência a toxinas em células diferenciadas.

Entretanto, outros trabalhos verificaram que as neurotrofinas secretadas pelos astrócitos podem ativar a via da Akt (Skyper, 2008). Assim, a fosforilação desta proteína encontrada em células diferenciadas pode mimetizar este ambiente. Portanto, este dado aumenta a validade do modelo de célula diferenciada (Lutchman & Song, 2010).

As diferenças no estado redox entre os dois modelos também contribuem para explicar as diferentes suscetibilidades à 6-OHDA (resultados suplementares I, Tabela 1). Primeiramente estes dados parecem controversos. As células diferenciadas apresentam uma maior atividade de enzimas antioxidantes (CAT e GST) bem como um maior conteúdo de tióis reduzidos em relação às células proliferativas. Esses dados podem indicar que estas células não são um bom modelo de neurônio dopaminérgico, porque tanto a SNpc como o SNC em geral apresentam baixos níveis de antioxidantes (Jenner *et al.,* 2003; Halliwell *et al.,* 2006). Dessa forma, as células diferenciadas não representariam a fisiologia de neurônios dopaminérgicos. Ainda, o aumento da atividade dos sistemas antioxidantes poderia conferir resistência ao dano causado pela 6-OHDA no fenótipo neuronal.

No entanto, a literatura mostra valores absolutos de atividade enzimática discrepantes dos encontrados no nosso estudo. Estes trabalhos utilizaram culturas primárias, modelos com roedores e também estudos em humanos (Sanchez-Ramos *et al.,* 1997; Halliwell & Gutteridge, 2007; Khan *et al.,* 2010). Por exemplo, no nosso trabalho, o fenótipo proliferativo apresenta aproximadamente 0.3 U/mg de proteína da enzima CAT enquanto o fenótipo neuronal apresenta 1 U/mg de proteína. Apesar das diferenças serem significativas entre os fenótipos, a atividade encontrada ainda é muito baixa em

relação aos outros trabalhos. Além disso, a pouca atividade de enzimas antioxidantes bem como a diminuição dos níveis de tióis reduzidos podem estar relacionados com a característica proliferativa do fenótipo tumoral (Stone & Yang, 2006; Schneider *et al.,* 2011). As células tumorais produzem grande quantidade de oxidantes, como o H_2O_2 que estão relacionados com crescimento celular. Um aumento da atividade da CAT, por exemplo, causa a inibição da proliferação (Policastro *et al.* 2004).

Ainda, nós verificamos que o modelo de célula proliferativa apresenta uma maior atividade da enzima TrxR e um maior conteúdo de GSH. Esses resultados podem estar envolvidos com a resistência da célula proliferativa perante insulto com 6-OHDA. O tratamento com inibidor da TrxR potencializou a morte causada pela 6-OHDA, sendo que isso não foi observado no tratamento com inibidor da GR (resultados suplementares do capítulo I, Figura 2C). Assim, a TrxR pode estar relacionada com detoxificação da 6-OHDA. Somado a isso, estudos anteriores mostraram que o *down-regulation* da TrxR em células SH-SY5Y levam a uma maior suscetibilidade a neurotoxina (Ding *et al.,*2007). Já os baixos níveis de GSH encontrado em células diferenciadas podem validar o modelo (Tabela 1). Os baixos níveis de GSH, principal antioxidante do tecido nervoso, em neurônios dopaminérgicos contribuem para o estresse oxidativo e para a disfunção mitocondrial encontrada na DP (Schulz *et al.,* 2000). Esta característica da célula diferenciada também mimetiza a fisiologia de células dopaminérgicas (Tarozzi *et al.,* 2009), sendo mais um indicativo de adequação do modelo.

Outro dado interessante é a citotoxicidade do H_2O_2 nos dois modelos. Neste caso, as células diferenciadas se mostraram mais resistentes ao insulto

oxidante mediado pelo H_2O_2, que pode estar relacionado ao aumento da atividade de enzimas antioxidantes, como o CAT, e dos níveis de tióis reduzidos. Outro estudo mostrou que a citotoxicidade de outro oxidante, o 4-hidroxinonenal, tem um perfil semelhante com o do H_2O_2 nos dois modelos (Schneider *et al.,* 2011). Ainda, o tratamento com inibidores da GR e da TrxR potencializaram a morte causada pelo oxidante e por isso ambos sistemas enzimáticos contribuem com a detoxificação ao H_2O_2. Este dado está de acordo com a literatura visto que ambas as enzimas participam da remoção do H_2O_2 (Halliwell, 2006).

Assim, as diferentes suscetibilidades à 6-OHDA nas células diferenciadas também podem estar relacionadas como outros processos, como o aumento da expressão de DAT (resultados suplementares I, Figura 1C). Estudos anteriores mostraram que a diferenciação induzida por AR aumenta o imunoconteúdo desse transportador (Presgraves *et al.,* 2007; Lutchman & Song, 2010). Além disso, estes mesmos estudos verificaram que células proliferativas e diferenciadas desta linhagem apresentam suscetibilidades diferentes a outra neurotoxina indutora da DP, o MPTP, sendo que as células diferenciadas se mostraram mais sensíveis à droga. Ainda, o tratamento com inibidor de DAT levou a uma diminuição dos danos causados pela neurotoxina.

A 6-OHDA também entra na célula dopaminérgica através deste transportador (Lehminesk *et al.*, 2006). Dessa forma, as células diferenciadas apresentam um maior número de DAT e por isso, é necessário menos droga para ter o mesmo efeito observado nas células proliferativas. Abaixo segue uma figura referente aos sistemas antioxidantes e a via de auto-oxidação da 6-OHDA para o entendimento da contribuição das enzimas antioxidantes para a

detoxificação das espécies reativas produzidas pala auto-oxidação da 6-OHDA (Figura 5).

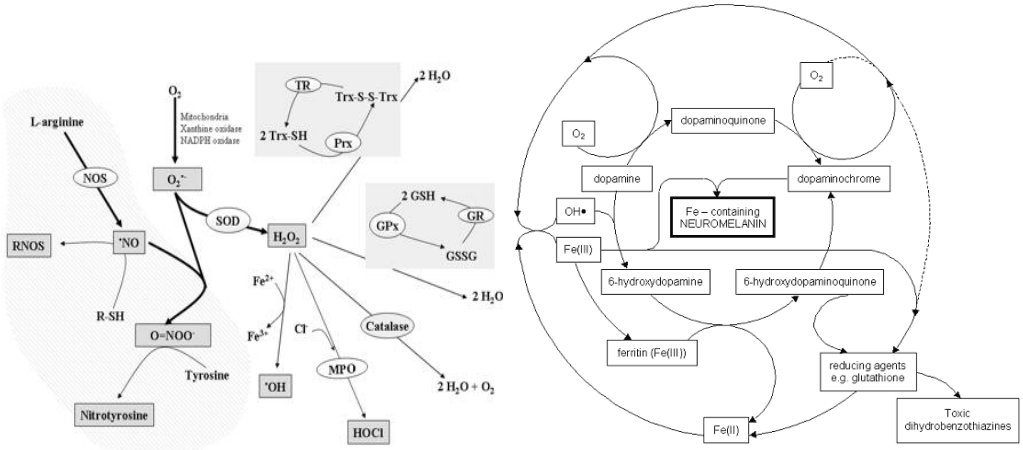


Figura 5: Integração do sistema de defesa antioxidante enzimática e a via de auto-oxidação da 6-OHDA. Em destaque os locais de ação dos inibidores utilizados nos resultados suplementares do capitulo I. (retiradas de Zhao *et al.,* 2007 e de *Institute of Applied Synthetic Chemistry*).

No entanto, a ação da 6-OHDA em células diferenciadas ainda deve ser elucidada e, portanto mais estudos devem ser feitos para investigar estes mecanismos.

Por fim, as células diferenciadas apresentam maiores níveis da proteína DJ-1. Esta proteína é um chaperona sensível ao estresse oxidativo e por isso sua expressão é induzida por espécies reativas (Canet-Aviles *et al.,* 2004). Ainda, mutações nesta proteína estão correlacionadas com uma forma prematura da DP (Bonifati *et al.,* 2003).

Após o estabelecimento das condições da diferenciação, nós avaliamos a potencialidade através da aplicação deste em testes de *screening* de neurotoxicidade/neuroproteção de compostos e possibilidade de manipulação gênica.

O s*creening* de possíveis drogas neuroprotetoras é feito inicialmente em diversos modelos *in vitro,* como o cultivo de células primárias e linhagens, visto

da dificuldade do uso de modelos animais. Isso porque necessitaria de um número muito grande de animais para estes ensaios (Bal-Price *et al.,* 2010). Na cultura celular, pode-se prospectar várias drogas com diversas concentrações e a partir destes dados selecionar os mais promissores para os ensaios com modelos animais. Após a obtenção de resultados em modelo *in vivo*, podemos buscar validação em ensaios clínicos (Radio & Mundi, 2008).

Dessa forma, os modelos *in vitro* são uma importante ferramenta para estudo da neuroproteção e são a base de ensaios clínicos. No entanto, a detecção da neurotoxicidade/neuroproteção de compostos representa um grande desafio para o estudo de neurociências visto que os modelos *in vitro* não mimetizam a complexidade fisiológica e bioquímica do sistema nervoso central (Bal-Price *et al.,* 2008).

A neurotoxicidade/neuroproteção deve ser avaliada de duas formas. Primeiramente, deve ser feito um *screening* de concentrações através de curvas de citotoxicidade a fim de estabelecer concentrações não-tóxicas ou subletais. Após, realizar ensaios de função celular, determinado a interferência da droga na homeostase celular (verificar, por exemplo, o metabolismo energético, estresse oxidativo, mudanças na morfologia celular e em marcadores neuronais) (Clemedson *et al.,* 2007).

Muitos compostos testados *in vitro* mostraram-se neuroprotetores e por isso, um promissor alvo terapêutico. Entretanto, estes resultados não se repetiram em ensaios clínicos (Susuki, 2009; Gutteridge & Halliwell, 2011). Como o cultivo de linhagens celulares apresenta um único tipo celular, não há as interações intercelulares, e isso pode mascarar os resultados de citotoxicidade (Banker & Goslin, 1998).

Um dos parâmetros mais criticados em testes de neurotoxidade/neuroproteção é a morfologia celular, visto que as linhagens neuronais amplamente utilizadas apresentam fenótipo diferente de neurônios (Radio & Mundi, 2008). Dessa forma, o modelo de células diferenciadas é uma alternativa, podendo ser utilizado em testes de neurotoxicidade/neuroprteção de compostos. No capítulo II da dissertação, nós verificamos a potencialidade do modelo para testes neurotoxicidade/neuroproteção de compostos.

Como há uma forte evidência do envolvimento das espécies reativas na fisiopatologia da DP, compostos antioxidantes são muito usados para avaliação da proteção ou reversão ao dano em neurônios. Exemplos disso são a vitamina D, o tocoferol, o ebselen e os flavonóides (Suzuki, 2009). Outro exemplo de compostos neuroprotetores são os organocalcogênios, que apresentam propriedades antioxidantes devido ao fato de sua estrutura química mimetizar o sítio ativo da enzima GPx (Rotruck *et al.,* 1973; Brigelius-Flohe 1999; Sarma and Mugesh 2005).

Para avaliação da neurototoxicidade/neuroproteção foram utilizados ensaios discutidos acima, como a viabilidade celular e ensaios de função celular. Primeiramente, através da determinação da viabilidade celular (MTT), foram feitas curvas de citotoxicidade de nove compostos organocalcogênios em células diferenciadas da linhagem de neuroblastoma SH-SY5Y. Dessa forma, na primeira fase deste trabalho, selecionamos as concentrações dos compostos que apresentaram baixa citotoxicidade (doses subletais).

Atualmente este tipo de estudo é realizado em linhagens celulares. Como relatado acima, elas são mitogênicas e por isso apresentam altas taxas de proliferação. Isso é uma grande desvantagem para técnicas de viabilidade

celular. O crescimento celular afeta o ensaio do MTT. Além disso, alguns compostos podem possuir comportamento citostático e por isso interferir na técnica (Souldner *et al.,* 1999; Luchtmann & Song*,* 2010).

Nós também avaliamos alguns ensaios de função celular como verificar as mudanças na morfologia celular e se os compostos interferem em parâmetros de estresse oxidativo. Uma característica importante do modelo de célula diferenciada é a semelhança morfológica com neurônios maduros. A diferenciação induzida por AR causa mudanças morfológicas, como a presença de neuritos (Pahlmann *et al.,* 1984). Estas estruturas são definidas como projeções citoplasmáticas que se referem tanto a axônios como a dendritos em culturas celulares (Carmaliet *et al.,* 2003). Mudanças nesta estrutura, como diminuição do número e do tamanho são importantes parâmetros de neurotoxicidade (Radio & Mundy, 2009).

Nós observamos que alguns compostos diminuíram a quantidade de neuritos de forma significativa. Dessa forma, a modificação da morfologia da célula é um indicativo de mudança na funcionalidade celular e também de citotoxicidade. Esse dado só pode ser observado em células diferenciadas visto que há poucos neuritos nas células proliferativas (Pahlmann *et al.,* 1984; Edjsö *et al.,* 2007; Lopes *et al*., 2010).

Ainda, nós verificamos as mudanças quanto à atividade de enzimas antioxidantes e estado redox das células tratadas com organocalcogênios. Os compostos foram capazes de aumentar os níveis de tióis protéicos, o potencial antioxidante total bem como a atividade das enzimas antioxidantes nas células diferenciadas. Estes dados podem ser a base da neuroproteção contra a 6-OHDA. Como discutido na introdução desta dissertação, a 6-OHDA é uma

neurotoxina redox ativa, que causa dano oxidativo a biomoléculas (Gomez-Lazaro *et al.,* 2008).

Após estabelecer as condições do modelo e mostrar sua aplicabilidade para ensaios de neurotoxicidade/neuroproteção, nós verificamos outra potencialidade do modelo. Nós padronizamos as condições ideais de transfeção e superexpressão da proteína verde fluorescente (GFP), como gene *reporter*, no modelo de célula diferenciada (resultados suplementares II).

A linhagem de neuroblastoma SH-SY5Y é transfectável tanto de forma transiente como com vetores virais (Biedler *et al.,*1973). Muitos estudos têm usados técnicas de biologia molecular para verificar o papel de algumas proteínas em doença neurodegenerativas, visto que estes ensaios são utilizados como prova de conceito, isto é, são usados para confirmar dados e relacionar de forma mais acurada o papel de algumas proteínas nos distúrbios neurodegenerativos.

Como discutido acima, as cultura primárias apresentam características neuronais adequadas. Entretanto, há uma grande dificuldade no processamento e também na realização de ensaios de transfecção neste tipo de cultura. Por isso, uma alternativa é o uso de linhagens celulares, como a linhagem de neuroblastoma humano SH-SY5Y (Schülle *et al.,* 2009). Este tipo de modelo também apresenta desvantagens que já foram discutidas acima.

Dessa forma, apesar das células diferenciadas apresentarem características morfológicas e neuroquímicas adequadas, seria uma grande desvantagem ao modelo a impossibilidade de realizar técnicas de transfecção. Nessa fase do estudo, nós buscamos avaliar se era possível manter a superexpressão de uma proteína e se isso influenciaria no fenótipo da célula.

Os resultados (resultado suplementar II, Figura 1) mostraram que a célula diferenciada é capaz de manter a expressão da proteína GFP visto que a fluorescência se manteve durante todos os dias da diferenciação. O grande problema dos ensaios de superexpressão transiente é a diminuição da expressão da proteína a cada divisão celular (segundo Abcam-protocols). No modelo com fenótipo neuronal, há a parada do ciclo celular, diminuindo este viés (Constantinescu *et al.* 2007).

Este dado é importante, pois mostra que técnicas de transfecção podem ser aplicadas para outras proteínas que podem estar relacionadas com os mecanismos fisiopatológicos da DP, como por exemplo, a α-sinucleína, DJ-1, LRRK2 e parkina (Henchcliff & Beal, 2008). Dessa forma, nossos resultados reforçam o grande potencial que a célula diferenciada apresenta como um modelo experimental *in vitro* para estudos em neurociências.

5. CONCLUSÃO

O nosso trabalho mostrou a eficácia da padronização da diferenciação da linhagem de neuroblastoma humano SH-SY5Y induzido por AR, onde nós verificamos que o fenótipo neuronal apresenta características semelhantes aos neurônios dopaminérgicos (Lopes *et al.*, 2010).

Além disso, o modelo de célula diferenciada pode ser aplicado para estudo de *screening* de neurotoxicidade/neuroproteção de compostos bem como em técnicas de biologia molecular, como a superexpressão de proteínas.

6. PERSPECTIVAS

Nosso trabalho mostrou a potencialidade do modelo para testes de neurotoxicidade/neuroproteção de compostos. Sendo isso, algo importante para a busca de alvos terapêuticos para DP. Ainda, verificamos que estas células podem ser manipuladas geneticamente.

A crítica quanto a modelos experimentais tanto *in vitro* como *in vivo* não se limita somente à DP. Outras doenças neurogenerativas como o Alzheimer também tem limitações de modelos.

A linhagem de neuroblatoma humano SH-SY5Y é também um exemplo de modelo *in vitro* Alzheimer (DA). Além da diferenciação dopaminérgica, a linhagem pode ser diferenciada em outros neurônios colinérgicos através do tratamento com outras neurotrofinas, como o BDNF (Encina *et al.,* 2000). As células adquirem marcadores neuroquímicos e também há o aumento do número de processos neuríticos. Dessa forma, essas diferenciações também modificam o fenótipo, deixando-o mais semelhante com neurônios diferenciados. Isso seria de grande valia para o entendimento dos mecanismos fisiopatológicos de outras doenças neurodegenerativas além de também fazer *screening* de possíveis drogas neuroprotetoras.

7. REFERÊNCIAS BIBLIOGRÁFICAS

Abcam- www.abcam.com

Agholme, L., Lindström, T., Kågedal, K., Marcusson, J., Hallbeck, M. (2010) An in vitro model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons. J Alzheimers Dis 20, 1069-1082.

Balasooriya, I.S., Wimalasena, K. (2007) Are SH-SY5Y and MN9D cell lines truly dopaminergic? Proceedings of the 3rd Annual GRASP Symposium.

Bal-Price, A.K., Sunol, C., Weiss, D.G., van Vliet, E., Westerink, R.H., Costa, L.G. (2008) Application of in vitro neurotoxicity testing for regulatory purposes: symposium III summary and research needs. Neurotoxicology 29, 520–531.

Bain, G., Kitchens, D., Yao, M., Huettner, J. E., Gottlieb, D. I. (1995) Embryonic stem cells expressed neuronal properties in vitro. Dev Biol 168, 342–357.

Banker, G., Goslin, K. (1998) Type of neurvous cells, their advantages and limitations. The MIT Press, Cambridge.

Baumann, C.R. (2012) Epidemiology, diagnosis and differential diagnosis in Parkinson's disease tremor. Parkinsonism Relat Disord 1, S90-S92

Beal, M.F. (2001) Experimental models of Parkinson's disease. Nat Rev Neurosci 2, 325-334.

Beal, M.F. (2010) Parkinson's disease: a model dilemma. Nature 466, S8-S10.

Bernstein, A.I., Garrison, S.P., Zambetti, G.P., O'Malley, K.L. (2011) 6-OHDA generated ROS induces DNA damage and p53-and PUMA-dependent cell death. Mol Neurodegerer 6, 2-13.

Betarbet, T. R., Canet-Aviles, R.M., Mastroberardino, P.G., McLendon, C., Kim, J.H., Lund, S., Na, H.M., Taylor, G., Bence, N.F., Kopito, R., Seo, B.B., Yai T., Yagi, A., Klinefelter, G., Cookson, M.R., Greenamyre, J.T. (2006) Intersecting Pathways to Neurodegeneration in Parkinson's Disease: Effects of the Pesticide Rotenone on DJ-1, α-Synuclein, and the Ubiquitin-proteasome system. Neurobiol Dis 22, 404 – 420.

Biedler, J. L., Roffler-Tarlov, S., Schachner, M., Freedman, L.S. (1978) Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res 38, 3751–3757..

Björklund, A., Dunnett, S.B. (2007) Trends Neurosci 30, 194-202.

Blum, D., Torch, S. , Lambeng, N., Nissou, M., Benabid, A.L., Sadoul, R., Verna, J.M. (2001) Molecular Pathways Involved in the Neurotoxicity of 6-OHDA, Dopamine and MPTP: Contribution to the Apoptotic Theory in Parkinson's Disease. Prog Neurobiol 65, 135–172.

Bove, J., Prou, D., Perier, C., Prezedborski, S. (2005) Toxin-Induced Models of Parkinson's Disease. NeuroRx 2, 484 – 494.

Bove, J., Perier, C. (2011) Neurotoxin-based models of Parkinson's disease. Neurosci. Aceito em 12/2011.

Bonifati, V., Rizzu, P., Squitieri, F., Krieger, E., Vanacore, N., Van Swieten, J.C., Brice, A., Van Duijn, C.M., Oostra, B., Meco, G., Heutink, P. (2003) Mutations in the DJ-1 Gene Associated with Autosomal Recessive Early-Onset Parkinsonism. Science 299, 256 – 259.

Braak, H., Del, T.K., Rub, U., De Vos, R.A., Jansen Steur, E.N., Braak, E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 24, 197–211.

Brigelius-Flohé, R. (1999) Tissue-specific functions of individual glutathione peroxidases. Free Radic Biol Med 27: 951–965.

Canet-Avilés, R.M., Wilson, M.A., Miller, D.W., Ahmad, R., McLendon, C., Bandyopadhyay, S., Baptista, M.J., Ringe, D., Petsko, G.A., Cookson, M.R. (2004) The Parkinson's Disease Protein DJ-1 is Neuroprotective Due to Cysteine-Sulfinic Acide-Driven Mitochondrial Localization. Proc Natl Acad Sci USA 101, 9103 – 9108.

Carmeliet, P. (2003) Blood vessels and nerves: common signals, pathways and diseases. Nat Rev Genet 4: 710-720.

Cavaliere, F., Vicente, E.S., Matute, C. (2010) An organotypic culture model to study nigro-striatal degeneration. J Neurosci Methods 188: 205-212.
Chan, C.S., Guzman, J.N., Ilijic, E., Mercer, J.N., Rick, C., Tkatch, T., Meredith, G.E., Surmeier, D.J. (2007) “Rejuvenation” protects neurons in mouse models of Parkinson’s disease. Nature 447, 1081–86.

Chinta, S.J., Andersen, J.K. (2005) Dopaminergic neurons. Int J Biochem Cell Biol 37, 942-946.

Choi, H.K., Won, L., Roback, J.D., Wainer, B.H., Heller, A. (1992) Specific modulation of dopamine expression in neuronal hybrid cells by primary cells from different brain regions. Proc Natl Acad USA 89, 8943-8947.

Cheung, Y.T., Lau, W.K., Yu, M.S., Lai, C.S., Yeung, S.C., So, K.F., Chang, R.C. (2009) Effect of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology 30, 127-135.

Chung, K.K., Dawson, V. L., Dawson, T. M. (2001) The role of the ubiquitin-proteasomal pathway in Parkinson's disease and other neurodegenerative disorders. Trends Neurosci 24, S7-S14.

Clemedson, C., Kolman, A., Forsby, A. (2007) The integrated acute systemic toxicity project (ACuteTox) for the optimisation and validation of alternative in vitro tests, Altern Lab Anim 35, 33–38.

Conroy, W.G., Berg, D.K. (1995) Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. J Biol Chem 270, 4424–4431.

Constantinescu, R., Constantinescu, A.T., Reichmann, H., Janetzky, B. (2007) Neuronal differentation and long-term culture of the human neuroblastoma line SH-SY5Y. J Neural Transm Suppl 72, 17-28.

da Frota Junior, M.L., Pires, A.S., Zeidán-Chuliá, F., Bristot, I.J., Lopes, F.M., de Bittencourt Pasquali, M.A., Zanotto-Filho, A., Behr, G.A.,, Klamt, F., Gelain, D.P., Moreira, J.C. (2011) In vitro optimization of retinoic acid-induced neuritogenesis and TH endogenous expression in human SH-SY5Y neuroblastoma cells by the antioxidant Trolox. Mol Cell Biochem 358, 325-334.

Dauer,W., Przedborski, S. (2003) Parkinson's disease: mechanisms and models. Neuron 39, 889–909.
Dawson, T.M., Dawson, V.L. (2003) Molecular Pathways of Neurodegeneration in Parkinson's Disease. Science 302, 819 – 821.

De Rijk, M.C., Breteler, M.M., Graveland, G.A., Ott, A., Grobbee, D.E., Van Der Meché, F.G., Hofman A. (1995) Prevalence of Parkinson's disease in the elderly: the Rotterdam Study. Neurology 45, 2143–2146.

Ding, H., Gao, J., Zhu, Z., Xiong, Y., Liu, J. (2007) Mitochondrial dysfunction enhances susceptibility to oxidative stress by down-regulation of thioredoxin in human neuroblastoma cells. Neurochem Res 33, 43-50.

Dunkley, P.R., Bobrovskaya, L., Graham, M.E., von Nagy-Felsobuki, E.I., Dickson, P.W. (2004). Tyrosine hydroxylase phosphorylation: regulation and consequences. J. Neurochem. 91, 1025–1043.

Edsjö, A., Holmquist, L., Pahlman, S. (2007) Neuroblastoma as an experimental model for neuronal differentiation and hypoxia-induced tumor cell differentiation. Semin Cancer Biol 17, 248-256.

Encina, M., Iglesias, M., Liu, Y., Wang, H., Muhaisen, A., Ceña, V., Gallego, C., Comella, J.X. (2000) Sequential Treatment of SH-SY5Y Cells with Retinoic Acid and Brain-Derived Neurotrophic Factor Gives Rise to Fully Differentiated, Neurotrophic Factor-Dependent, Human Neuron-Like Cells. J Neurochem 75, 991 – 1003.

Fahn, S. (1996) The case of the frozen addicts: how the solution of an extraordinary medical mystery spawned a revolution in the understanding and treatment of Parkinson's disease. N Engl J Med 335, 2002–2003.

Falkenburger BH, Schulz JB. (2006) Limitations of cellular models in Parkinson's disease research. J Neural Transm Suppl 70, 261-268.

Gasser, T. (2009) Molecular pathogenesis of Parkinson disease: insights from genetic studies. Expert Rev Mol Med 11, e22

Gilany, K., Elzen, R.V., Mous, K., Coen, E., Dongen, W.V., Vandamme, S., Gevaert, K., Timmerman, E., Vandekerckhove, J., Dewilde, S., Ostade, X.V.,

Moens, L. (2008) The proteome of the human neuroblastoma cell line SH-SY5Y: An enlarged proteome. Biochim Biophys Acta 1784, 983-985.

Gomez-Lazaro, M., Bonekamp, N.A., Galindo, M.F., Jordán, J., Schrader, M. (2008) 6-Hydroxydopamine (6-OHDA) Induces Drp-Dependent Mitochondrial Fragmentation in SH-SY5Y Cells. Free Radic Biol Med 44, 1960-1969.

Gutteridge, J.M., Halliwell, B. (2010) Antioxidants: Molecules, medicines, and myths. Biochem Biophys Res Commun 393, 561- 564.

Halliwell, B. (2006) Oxidative stress and neurodegeneration: where are we now? J Neurochem 97, 1634-1658.

Halliwell, B., Gutteridge, J.M. (2007) Free radicals in biology and medicine. Oxford 4th ed.

Han, B.S., Hong, H.S., Choi, W.S., Markelonis, G.J., Oh, T.H., Oh, Y.J. (2003) Caspase- dependent and independent cell death pathways in primary cultures of mesencephalic dopaminergic neurons after neurotoxin treatment. J Neurosci 23, 5069-5078.

Hatcher, J.M., Pennell, K.D., Miller, G.W. (2008) Parkinson's Disease and Pesticides: A Toxicological Perspective. Trends Pharmacol Sci 29, 322-329.

Hauser, R.A., Zesiewicz, T.A. (2007) Advances in the pharmacologic management of early Parkinson disease. Neurologist 13: 126–132.

Henchcliffe, C., Beal, M.F. (2008) Mitochondrial biology and oxidative stress in Parkinson disease pathogenesis. Nat Clin Pract Neurol 4, 600-609.

Howman-Giles, R., Shaw, P.J., Uren, R.F., Chung, D.K. (2007) Neuroblastoma and other neuroendocrine tumors. Semin Nucl Med 37, 286-302.

Institute of Applied Synthetic Chemistry (www.ias.tuwien.ac.at)

Jankovic, J. (2005) Motor fluctuations and dyskinesias in Parkinson's disease: clinical manifestations. Mov Disord 11, S11–S16.

Jankovic, J. (2008) Parkinson's disease: clinical features and diagnosis. J Neurol Neurosurg Psychiatry 79, 368–376.

Jenner, P. (2003) Oxidative stress in Parkinson's disease. Ann Neurol 53, S26-S36.

Jenner, P. (2008) Functional models of Parkinson's disease: a valuable tool in the development of novel therapies. Ann Neurol 2: S16-S29.

Khan, M.M., Ahmad, A., Ishrat, T., Khan, M.B., Hoda, M.N., Khuwaja, G., Raza, S.S., Khan, A., Javed, H., Vaibhav, K., Islam, F. (2010) Resveratrol attenuates 6-hydroxydopamine-induced oxidative damage and dopamine depletion in rat model of Parkinson's disease. Brain Res 1328, 139-151.

Katzenschlager, R., Head, J., Schraq, A. Ben-shlomo, Y., Evans, A., Lees, A.J. (2008) Fourteen-year final report of the randomized PDRG-UK trial comparing three initial treatments in PD. Neurology 71, 474–480.

Kitamura, Y., Taniguchi, T., Shimohama, S., Akaike, A., Nomura, Y. (2003)Neuroprotective mechanisms of antiparkinsonian dopamine D2-receptor subfamily agonists. Neuroche Res 28, 1035-1040.

Kume, T., Kawato, Y., Osakada, F., Izumi, Y., Katsuki, H., Nakagava, T., Kaneko, S.,
Niidome, T., Takada, N., Takada-Takori, Y., Akaike, A. (2008) Dibutyryl cyclic AMP induces differentitation of human neuroblastoma SH-SY5Y cells into a noradrenergic phenotype. Neurosci Lett 443, 199-203.

Lawlor, P.A., During, M.J. (2004) The dopamine synthesis and storage pathway. Rev Mol Med 6, 120-12.

Lee, C.S., Park, W.J., Ko, H.H., Han, E.S. (2006) Differential Involvement of Mitochondrial Permeability in Citotoxicity of 1-methyl-4-phenylpyridinium and 6-hydroxydopamine. Mol Cell Biochem 289, 193 – 200.

Lehmensiek, V., Tan, E.M., Liebau, S., Lenk, T., Zettlmeisl, H., Schwarz, J., Storch, A. (2006) Dopamine Transporter-Mediated Cytotoxicity of 6-Hydroxydopamine In Vitro Depends on Expression of Mutant a-Synucleins Related to Parkinson's Disease. Neurochem Int 48, 329 – 340.

Liu F., Nguyen, J.L., Hulleman, J.D., Li, L., Rochet, J.C. (2008) Mechanisms of DJ-1 europrotection in a Cellular Model of Parkinson's Disease. J Neurochem 105, 2435-2453.

Liss, B., Roeper, J. (2008) Individual dopamine midbrain neurons: functional diversity and flexibility in the healthy and disease. Brain Res Rev 58, 314-321.

Lopes, F.M, Schröder, R., da Frota, M.L. Jr, Zanotto-Filho, A., Müller, C.B., Pires, A.S, Meurer, R.T., Colpo, G.D., Gelain, D.P., Kapczinski, .F, Moreira, J.C., Fernandes, Marilda, C., Klamt, F. (2010) Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain Res 1337: 85-94.

Lopes, F.M., Londero, G.F., de Medeiros, L.M., da Motta, L.L., Behr, G.A., de Oliveira, V.A., Ibrahim, M., Moreira, J.C., Porciúncula, L.O., da Rocha, J.B., Klamt, F. (2012) Evaluation of the neurotoxic/neuroprotective role of organoselenides using differentiated human neuroblastoma SH-SY5Y cell line challenged with 6-hydroxydopamine. Neurotox Res. Aceito em 01/2012.

Luchtman, D.W., Song, C. (2010) Why SH-SY5Y cells should be differentiated. Neurotoxicology 1, 164-165.

Marder, K., Tang, M.X., Mejia, H., Alfaro, B., Côté, L., Louis, E., Groves, J., Mayeux, R. (1996) Risk of Parkinson's disease among firstdegree relatives: A community-based study. Neurology 47, 155–60.

Mark, M., Ghyselinck, N. B., Chambon, P. (2006) Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. Annu Rev Pharmacol Toxicol 46, 451–480.

Malik, M.A., Blusztajn, J.K., Greenwood, C.E. (2000) Nutrients as trophic factors in neurons and the central nervous system: Role of retinoic acid. J Nutr Biochem 11, 2-13.

Marsden, C.D. (1996) Movement disorders. In: Weatherall DJ, Ledingham JGG, Warrell DA, editors. Oxford textbook of medicine, vol. 3. New York: Oxford University Press Inc, 3998–4022.

McNaught, K.S., Olanow, C. W. (2003) Proteolytic stress: a unifying concept for the etiopathogenesis of Parkinson's disease. Ann Neurol 53, S73, S74.

Meissner ,W.G., Frasier, M., Gasser, T., Goetz, C.G., Lozano, A., Piccini, .P, Obeso, J.A., Rascol, O., Schapira, A., Voon, V., Weiner, D.M., Tison, F., Bezard, E. (2011) Priorities in Parkinson's disease. Nat Rev Drug Discov 10: 377-393.
Miloso, M., Villa, D., Crimi, M., Galbiati, S., Donzelli, E., Nicolini, G., Tredici, G. (2004) Retinoic acid-induced neuritogenesis og human neuroblastoma SH-SY5Y is ERK independentad PKC dependent. J Neurosci Res 15, 241-252.

Morais, V.A., De Strooper, B. (2010) Mitochondria dysfunction and neurodegenerative disorders: cause or consequence. J Alzheimer Dis 20, S255-S263.

Muangpaisan W, Mathews A, Hori H, Seidel D. (2011) A systematic review of the worldwide prevalence and incidence of Parkinson's disease. J Med Assoc Thai 94, 749-755.

Olanow, C.W., Kieburtz, K., Schapira, A.H. (2008) Why have we failed to achieve neuroprotection in Parkinson's disease? Ann Neurol 2, S101-S110.

Pahlman, S., Ruusala, A.I., Abrahamsson, L., Mattsson, M.E., Esscher, T. (1984) Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. Cell Differ 14, 135–144.

Parker, W.D., Parks, J.K., Swerdlow, R.H. (2008) Complex I deficiency in Parkinson's disease frontal cortex. Brain Res 1189, 215–218.

Poewe, W. (2008) Non-motor symptoms in Parkinson's disease. Eur J Neurol 15, 14–20.

Policastro, L., Molinari, B., Larcher, F., Blanco, P., Podhajcer, O.L., Costa, C.S., Rojas, P., Duran, H. (2004). Imbalance of antioxidant enzymes in tumor cells and inhibition of proliferation and malignant features by scavenging hydrogen peroxide. Mol Carcinog 39, 103-113.

Presgraves, S.P., Ahmed, T., Borwege, S., Joyce, J.N. (2004) Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. Neurotox Res 5, 579–98.

Radio, N.M., Mundy, W.R. (2008) Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. Neurotoxicology 29, 361-376.

Rao, S.S., Hofmann, L.A., Shakil, A. (2006) Parkinson's disease: diagnosis and treatment. Am Fam Physician 15, 2046-2054.

Reichman, N., Porteous, C.M., Murphy, M.P. (1994) Cyclosporin A Bloks 6-Hydroxydopamine-Induced Efflux of Ca^{2+} From Mitochondria Without Inactivating the Mitochondrial Inner-Membrane Pore. Biochem J 297, 151 – 155.

Ross, S. A., McCaffery, P. J., Drager, U. C., De Luca, L. M. (2000) Retinoids in embryonal development. PhysiolRev 80, 1021–1054.

Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., Hoekstra, W.G. (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science 179: 588-90.

Saiki, S., Sato, S., Hattori, N. (2011) Molecular pathogenesis of Parkinson's disease: update. J Neurol Neurosurg Psychiatry. Aceito em 12/2011.

Sanchez-Ramos, J.R., Song, S., Facca, A., Basit, A., Epstein, C.J. (1997) Transgenic murine dopaminergic neurons expressing human Cu/Zn superoxide dismutase exhibit increased density in culture, but no resistance to methylphenylpyridinium-induced degeneration. J Neurochem 68, 58-67.

Sarkanen, J.R., Nykky, J., Siikanen, J., Selinummi, J., Ylokomi, T., Janolen, T.O. (2007) Cholesterol supports the retinoic acid-induced synaptic vesicle formation in differentiating human SH-SY5Y neuroblastoma cells. J Neurochem 102, 1941-1952.

Sarma, B.K., Mugesh, G. (2005) Glutathione peroxidase (GPx)-like antioxidant activity of the organoselenium drug ebselen: unexpected complications with thiol exchange reactions J Am Chem Soc 127: 11477-11745.

Schapira, A.H., Mann, V.M., Cooper, J.M., Dexter, D., Daniel, S.E., Jenner, P., Clarck, J.B. Marsden, C.D. (1990) Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. J Neurochem 55, 2142–2145.

Schapira, A.H. (2008) Mitochondrial dysfunction in neurodegenerative disease. Neurochemical Research 33, 2502-2509.

Schapira, A.H. (2009) Etiology and pathogenesis of Parkinson Disease. Neurol Clin 27, 583- 603.

Schapira, A.H., Tolosa, E. (2010) Molecular and clinical prodrome of Parkinson disease: implications for treatment. Nat Rev Neurol 27, 583-603

Schapira, A.H., Jenner, P. (2011) Etiology and pathogenesis of Parkinson's disease. Mov Disord 26, 1049-1055.

Schneider, L., Giordano, S., Zelickson, B.R.S., Johnson, M.A., Benavides, G., Ouyang, X., Fineberg, N., Darley-Usmar, V.M., Zhang, J. (2011) Differentiation pf SH-SY5Y cells to a neuronal phenotype changes cellular bioenergetics and the response to oxidative stress. Free Radic Biol Med 51, 2007-2017.

Schober, A. (2004) Classic Toxin-Induced Animal Model of Parkinson's Disease: 6-OHDA and MPTP. Cell Tissue Res 318, 215 – 224.

Schülle, B., Pera, R.A., Langston, J.W. (2009) Can cellular modelos revolutionize drug discovery in Parkinson's disease. Biochim Biophys Acta 1792, 1043-1051.

Schulz, J. B., Lindenau, J., Seyfried, J., Dichgans, J. (2000) Glutathione, oxidative stress and neurodegeneration. Eur. J. Biochem. 267, 4904–4911.

Skper, S.D. (2008) The biology of neurotrophins, signaling pathways, and functional peptide mimetics of neurotrophins and their receptors. CNS Neurol Disord Drug Tarrgets 7, 46-62.

Stahl, K., Skare, O., Torp, R. (2009) Organotypic cultures as a model of Parkinson s disease. A twist to an old model. ScientificWorldJournal. 9, 811-821.

Studer, L. (2001) Culture of substantia nigra neurons. *Curr. Protoc. Neurosci.* 3, Unit 3.3.

Soldner, F., Weller, M., Haid, S., Beinroth, S., Miller, S.W., Wüllner, U., Davis, R.E., Dichgans, J., Klockgether, T., Schulz, J.B.. (1999) MPP+ inhibits proliferation of PC12 cells by a p21(WAF1/Cip1)-dependent pathway and induces cell death in cells lacking p21(WAF1/Cip1). Exp Cell Res 250, 75–85.

Stone, J.R., and Yang, S. (2006). Hydrogen peroxide: a signaling messenger. AntioxidRedox Signal *8*, 243-270.

Suzuki, K. (2009) Anti-oxidants for therapeutic use: why are only a few drugs in clinical use? Adv Drug Deliv Rev 61, 287-289.

Taira, T., Saito, Y., Niki, T., Iguchi-Ariga, S.M., Takahashi, K., Ariga, H. (2004) DJ-1 has a role in antioxidative stress to prevent cell death. EMBO Rep 5, 213 – 218.

Tarozzi, A., Morroni, F., Merlicco, A., Hrelia, S., Angeloni, C., Cantelli-Forti, G., Hrelia, P.(2009) Sulforaphane as an inducer of glutathione prevents oxidative stress-induced cell death in a dopaminergic-like neuroblastoma cell line. J Neurochem 111, 1161- 1171.

Tieu, K., Zuo, D.M., Yu, P.H. (1999) Differential effects of staurosporine and retinoic acid on the vulnerability of the SH-SY5Y neuroblastoma cells: involvement of bcl-2 and p53 proteins. J Neurosci Res 58, 426-435.

Thomas, S.K., Messam, C.A., Spengler, B.A., Biedler, J.L. (2004) Nestin is a Potential Mediator of Malignacy in Human Neuroblastoma cells. J Biol Chem 279, 27994- 27999.

Uversky, V.D. (2004) Neurotoxicant-Induced Animal Models of Parkinson's Disease: Understanding the Role of Rotenone, Maneb and Paraquat in Neurodegeneration. Cell Tissue Research 318, 225 – 241.

Weyer, A., Schilling, K. (2003) Developmental and cell type-specific expression of the neuronal marker NeuN in the murine cerebellum. J Neurosci Res 73, 400–9.

Wirdefeldt, K., Adami, H.O., Cole, P., Trochopoulos, D., Mandel, J. (2011) Epidemiology and etiology of Parkinson's disease: a review of the evidence. Eur J Epidemiol 26, S1-S8.

Xie, H.G. Hu, L.S., Li, G.Y. (2010) SH-SY5Y human neuroblastoma cell line: *in vitro* cell model of dopaminergic neurons in Parkinson's disease. Chin Med J (Engl) 123, 1086-1092.

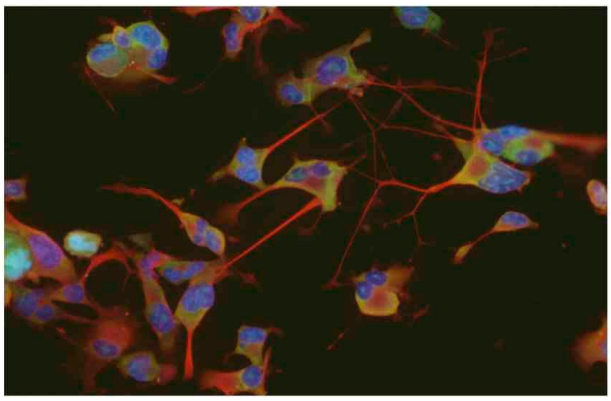
Yokota, T., Sugawara, K., Ito, K., Takahashi, R., Ariga, H., Mizusawa, H. (2003) Down Regulation of DJ-1 Enhances Cell Death by Oxidative Stress, ER Stress, and Proteasome Inhibition. Biochem Biophys Res Commun 312, 1342 – 1348.

Zhang, J., Perry, G., Smith, M.A., Robertson, D., Olson, S.J., Graham, D.G., Montine, T.J. (1999) Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. Am J Pathol 154, 1423–1429.

Zhao, W., Diz, D.I., Robbins, M.E. (2007) oxidative damage pathways in relation to normal tissue injury. Br. J Radiol 80, S23-S31.

8. ANEXO: Discussão sobre modelos experimentais *in vitro* em neurociências no Society for Neuroscience 2010

Pitfalls of Cell-based Screening Assays – Is it the Cell, the Assay or the Question Asked?



November 15, 2010 from 6:30-8:30 PM
Room: Indigo 206
Hilton San Diego Bayfront Hotel

Come hear leading speakers discuss the use of cell-based assays as models in neurological diseases and in studies of neurotoxicity. The goal of this symposium is to open a dialogue concerning how cell-based systems like neuroblastoma and neuroblastoma hybrid cell lines may best be used as tools in understanding neurological diseases and drug discovery.

Speakers:

Dr. Raymond Chang, The University of Hong Kong, China
Dr. Joanne Hackett, Linkoping University, Sweden
Dr. Fabio Klamt, Universidade de Rio Grande do Sul, Brazil
Dr. Nick Radio, ThermoScientific, USA