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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

TESE DE DOUTORADO

O papel dualístico de retinóides na neurodiferenciação e neurodegeneração catecolaminérgica

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“Learn from yesterday, live for today, hope for tomorrow.

The important thing is not to stop questioning."

Albert Einstein

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SUMÁRIO

APRESENTAÇÃO

A presente tese de doutorado está organizada em três partes, conforme a seguir:

Parte I: Resumo, *Abstract*, Lista de Abreviaturas, Introdução e Objetivos;

Parte II: Resultados apresentados na forma de 2 artigos científicos, dentre os quais um aceito para publicação e outro submetido. Também contém 01 capítulo com introdução, metodologia, resultados e discussão.

Parte III: Discussão, Conclusões, Perspectivas e Referências Bibliográficas.

PARTE 1

I. RESUMO

A vitamina A (retinol) exerce papéis fundamentais na regulação de processos celulares, tais como crescimento, divisão e apoptose. Os efeitos do retinol a nível celular são classicamente atribuídos à ativação de receptores nucleares da família dos receptores esteroides, RAR e RXR, ativados por diferentes isômeros do AR, considerado o produto mais biologicamente ativo da metabolização do retinol. Trabalhos recentes vêm identificando que o retinol também exerce funções biológicas por mecanismos independentes da transcrição gênica através da ativação desses receptores, em uma ação não-clássica ou não-genômica da vitamina A. Vários trabalhos propõem um papel antioxidante da vitamina A, embora alguns resultados demonstrem o papel pró-oxidante da mesma. Neste trabalho, nós avaliamos o papel da vitamina A no contexto das doenças neurodegenerativas. O mecanismo de ação do AR no processo de neurodiferenciação, a modulação da expressão de marcadores de neurodegeneração pelo retinol e o potencial papel da vitamina A na prevenção de danos associados à DP em modelo de ratos foram estudados. Observamos que o AR (10μM) induziu a neurodiferenciação de células SH-SY5Y através da produção de espécies reativas e estresse oxidativo. O retinol em concentrações maiores que a fisiológica (7, 10 e 20μM), atuou de maneira pró-oxidante, aumentando a produção de espécies reativas, a citotoxicidade e o conteúdo do receptor para produtos finais de glicação avançada (RAGE) de maneira redox-dependente, bem como o imunoconteúdo de marcadores de doenças neurodegenerativas como α-sinucleína, peptídeo β-amilóide e tau fosforilada indepedentemente de estresse oxidativo. No entanto, em modelo *in vivo,* o pré-tratamento com vitamina A (3000 IU/kg/dia) protegeu o dano neuronal induzido pela injeção do agente parkinsoniano 6-hidroxidopamina, e também reduziu o processo inflamatório. Estes resultados demonstram que as ações biológicas dos retinóides no sistema catecolaminérgico podem variar grandemente de acordo com o tipo celular, contexto fisiológico/patológico e modo de administração, demonstrando um amplo espectro de ações e mecanismos celulares.

II. ABSTRACT

Vitamin A (retinol) exerts fundamental role in cellular processes regulation, such as growing, cell division and apoptosis. Retinoids effects occur through binding to nuclear retinoid receptors, RAR and RXR, activated by different isomers of retinoic acid, considered the most biologically active product of retinol. Recent studies have shown receptors independent binding effects of retinoids, which was named as a non-genomic, or non-classical action mechanisms of vitamin A. In recent years, several studies have been proposing an antioxidant effect to vitamin A, however, series of pro-oxidant results have been shown a redox-state of this molecule. In this study, we evaluated the role of vitamin A in the context of neurodegenerative diseases. The mechanism of action of retinoic acid in the neurodifferentiation process, the modulation of neurodegeneration markers by retinol and the potential role of vitamin A in the prevention of damage associated to Parkinson's disease were evaluated. We observed that retinoic acid (10μM) induced the neurodifferentiation of SH-SY5Y cells through reactive species production and oxidative stress. Retinol in concentrations above the physiological range (7, 10 and 20μM) was able to generate reactive species, induce cytotoxicity and modulate RAGE through a oxidative stress mechanism. Also, retinol was able to modulate neurodegenerative disease's markers such as α-synuclein, β-amyloid and phosphorylation of tau. In our in vivo model, vitamin A (3000IU/kg/dia was able to protect neurons from 6-hydroxydopamine induced degenereration, and also reduced the inflammatory process. These results demonstrate that the biological actions of retinoids in the catecholaminergic system may vary according the cell target, physiopathologic context and administration mode, demonstrating a wide spectrum of actions and cellular mechanisms.

III. LISTA DE ABREVIATURAS

AR = ácido retinóico

DP = doença de Parkinson

DA= doença de Alzheimer

DH= doença de Huntington

ELA= esclerose lateral amiotrófica

LB= corpos de Lewy, do inglês *Lewy Bodies*

APP = proteína precursora amilóide, do inglês, *amyloid precursor protein*

MPTP= (1-metil-4-fenil-1,2,3,6-tetraidropiridina) é uma neurotoxina que provoca sintomas da DP

6-OHDA= 6-hidroxidopamina

ATP= adenosina trifosfato

EROS= espécies reativas de oxigênio

ERNS= espécies reativas de nitrogênio

BDNF= Fator neurotrófico derivado do cérebro, do inglês *brain-derived neurotrophic fator*

RAGE= receptor de produtos finais de glicação avançada, do inglês *receptor for advanced glycation endproducts*

RARE= elementos responsivos a ácido retinóico*, do inglês retinoic acid response element*

IL-1β= interleucina 1β

TNF-α= fator de necrose tumoral- α

DNA= ácido desoxirribonucleico, do inglês *deoxyribonucleic acid*

SNC= sistema nervoso central

TH= enzima tirosina hidroxilase

HNE= 4-hidroxi-2-nonenal

PrP= proteína príon, do inglês *príon protein*

IV. INTRODUÇÃO

Vitamina A

Vitamina A é um termo genérico usado para definir uma classe de compostos que possuem atividade biológica do retinol (retinol, ésteres de retinil e retinaldeído). Os retinóides, termo comumente utilizado para definir análogos do retinol, considerado a "molécula-mãe" da família da vitamina A, bem como o ácido retinóico (AR), que é formando intracelularmente através do metabolismo oxidativo do retinol (Montrone *et al.*, 2009), são compostos lipossolúveis, encontrados em alimentos de origem animal –peixe e carne vermelha- como vitamina A pré-formada e em alimentos de origem vegetal como pró-vitamina A – carotenóides (Takeda *et al.*, 2014). No trato gastrointestinal são absorvidos e biodisponibilizados para utilização em processos fisiológicos, armazenados no fígado ou excretados (Flajollet *et al.*, 2013) (Figura 1).

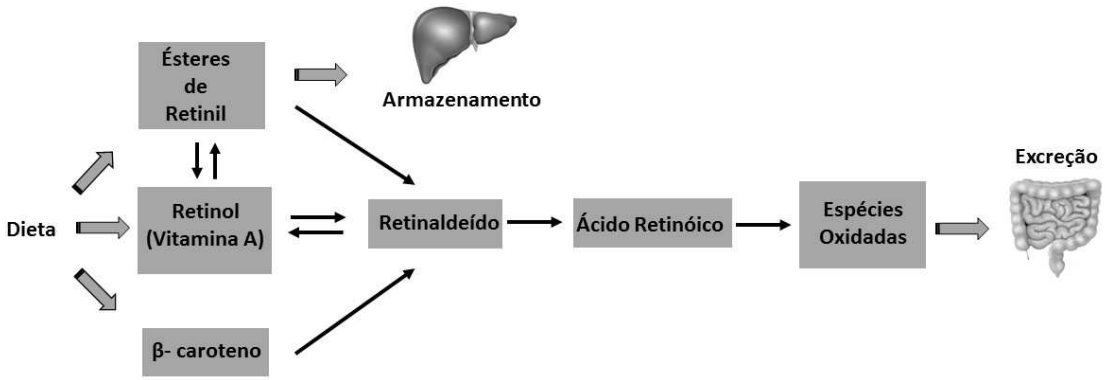

Figura 1: Metabolismo dos retinóides. Uma vez absorvidos no lúmen intestinal, o retinol e o β-caroteno podem ser convertidos a retinaldeído e consequentemente ácido retinóico, e utilizados pelos demais tecidos do organismo. O retinol também pode ser armazenado no fígado na forma de ésteres de retinil. Adaptado de: Libien, J et al. Role of vitamin A metabolism in IIH: Results from the idiopathic intracranial hypertension treatment trial. Journal of the Neurological Sciences 372 (2017) 78–84.

Os retinóides estão envolvidos em uma complexa via de sinalização que regula a expressão gênica em diversos órgãos incluindo o cérebro, controlando em nível de sistema nervoso central (SNC) processos de diferenciação e modelamento neuronal (Maden, 2007). Também exercem um papel essencial em processos de crescimento, e são conhecidos reguladores de funções relacionadas à divisão celular, tais como reprodução, desenvolvimento embrionário e crescimento (Duester, 2008). Além disso, os retinóides estão envolvidos na manutenção de processos fisiológicos tais como a visão e função motoras (Sarah J Freemantle, 2003; Michele Montrone, 2009). O principal metabólito do retinol, o AR (all-*trans*-retinoic acid [ATRA]), é um regulador chave durante o desenvolvimento embrionário do sistema nervoso, estimulando o desenvolvimento de axônios e dendritos levando a uma funcionalidade sináptica (Bo-Tao Tan, 2015).

O AR pode ter sua ação relacionada a duas famílias de receptores nucleares que se ligam ao DNA e podem iniciar a transcrição gênica. Os receptores RA (RARs, isotipos α, β, γ), aos quais se liga a forma abundante de AR, conhecido como AR todo-trans, e os receptores RXR (isotipos α, β, γ), que ligam ao isômero 9-*cis*-AR (Duester, 2008). Na forma de heterodímeros (RAR/RXR), estes receptores controlam a transcrição de genes alvo do AR, contendo a RARE (*retinoic acid-response element*), elementos responsivos a AR. A ligação do AR ao receptor RAR na forma de heterodímero RAR/RXR, conectado a um elemento regulatório do DNA, estimula uma cascata de eventos que resultam no recrutamento de co-ativadores e a consequente iniciação da transcrição (Maden, 2007; Tafti e Ghyselinck, 2007).

Nos últimos anos surgiram trabalhos apontando para uma ação do AR não dependente de *RARE*, e a consequente não ativação dos receptores nucleares RAR e RXR. Estes

mecanismos do AR foram denominados de não-nuclear, não-genômico ou não-clássico dos retinóides. Nosso grupo observou em diversos trabalhos evidências de mecanismos não-genômicos envolvendo retinóides. Em cultura de células de Sertoli, foi observado que o AR induziu uma rápida estimulação de ERK1/2 (Zanotto-Filho *et al.*, 2008), o retinol induziu a ativação de ERK1/2 Src-dependente (Gelain *et al.*, 2006), rápida ativação da fosforilação de ERK1/2, JNK e Src (Gelain *et al.*, 2012), ativação do RAGE via p38 e Akt (Gelain *et al.*, 2011), ativação da catalase (Gelain *et al.*, 2008) e aumento do imunoconteúdo da catalase (Pasquali *et al.*, 2008) através do aumento da geração de espécies reativas intracelulares. Também foi observado em células PC12 que o retinol induz um rápido aumento nas espécies reativas intracelulares (Gelain e Moreira, 2008), e em cultura de células cromafins adrenais o tratamento com retinol levou a uma aguda ativação da enzima tirosina hidroxilase através da indução do influxo extracelular de cálcio e ativação da proteína cinase C (Gelain *et al.*, 2007). Esta forma de ação foi proposta devido à ineficiência de regulação da atividade através do bloqueio dos receptores e devido à rápida resposta observada, que sugeria um mecanismo independente de receptores, inclusive através da produção de espécies reativas (Gelain e Moreira, 2008).

Doenças Neurodegenerativas

Doenças neurodegenerativas são patologias em que ocorre a morte progressiva de neurônios do sistema nervoso central ou periférico, levando a deficiências motoras, fisiológicas ou cognitivas. Estas doenças podem afetar diversas áreas do cérebro, e tem como principais representantes a doença de Parkinson (DP), doença de Alzheimer (DA), Esclerose Lateral Amiotrófica (ELA) e a doença de Huntington (HT). Estas doenças apresentam apenas tratamentos paliativos, e por não apresentarem cura definitiva tornaram-se intenso foco de

estudos, a fim de compreender os mecanismos celulares e moleculares causadores da citotoxicidade. De acordo com a Organização Mundial de Saúde, em 2015, as doenças neurodegenerativas foram a sétima causa de morte no mundo, tendo mais do que dobrado sua incidência desde o ano 2000 (Organização Mundial da Saúde, 2017).

A DP é uma desordem neurodegenerativa que leva a perda progressiva dos neurônios dopaminérgicos da substância negra *pars compacta*. Os pacientes portadores podem apresentar uma combinação de sintomas motores e não-motores. Sintomas motores típicos incluem hipocinesia, bradicinesia, tremor de repouso e/ou rigidez, e são utilizados como fatores no diagnóstico da patologia (Dietrichs e Odin, 2017). Apesar dos inúmeros estudos tentando compreender a doença, os tratamentos disponíveis são apenas sintomáticos e não inibem a progressão da patologia. Acredita-se que a perda neuronal dopaminérgica é responsável pelas alterações motoras encontradas na doença, uma vez que os níveis da enzima tirosina hidroxilase, envolvida na síntese de dopamina, assim como transportadores dopaminérgicos, são severamente reduzidos na DP (Toulorge *et al.*, 2016). Além da perda neuronal, diversos estudos demonstraram desregulação de fatores neurotróficos, como redução do fator neurotrófico derivado de célula da glia e redução da expressão do fator neurotrófico derivado de cérebro. Outro marcador da DP é a presença de agregados fibrilares intracitoplasmáticos denominados corpos de Lewy. Apesar de ser um agregado heterogêneo, os principais constituintes dos corpos de Lewy são ubiquitina e a proteína de terminal nervoso pré-sináptico α-sinucleína. Esta possui propensão à agregação, e em pacientes com DP é encontrada modificada através de oxidação, nitração ou clivagem (Wakabayashi *et al.*, 2013). A DP pertence a uma classe de doenças neurodegenerativas denominada sinucleinopatias, as quais compartilham o acúmulo e agregação de α-sinucleína como quadro central da morte neuronal. Além da DP, encontram-se nessa classe a demência com Corpos de Lewy e

demência da DP (Spencer *et al.*, 2017). Outro grupo de doenças neurodegenerativas em que a citotoxicidade celular é causada pela agregação protéica são as taupatias, nas quais ocorre a hiperfosforilação e agregação da proteína tau, responsável pela regulação da estrutura e função dos microtúbulos em neurônios. Os mecanismos de citotoxicidade tau dependentes são encontrados na DA, ocorrendo também na encefalopatia traumática crônica, doença de Pick, degeneração corticobasal e paralisia progressiva supranuclear (Hasegawa, 2006; Rojas e Boxer, 2016).

A DA é a causa mais comum de demência entre as doenças neurodegenerativas na população acima de 60 anos, caracterizada clinicamente pela progressiva perda de memória e declínio cognitivo (Lee *et al.*, 2010). O cérebro de pessoas afetadas pela doença apresenta significativa perda de sinapses e neurônios resultando em forte atrofia do córtex e hipocampo (Grimm *et al.*, 2016). A citotoxicidade na DA está associada à hiperfosforilação da proteína estabilizadora de microtúbulos - tau, que resulta na formação dos agregados protéicos (placas neurofibrilares), característicos da doença. Estudos relacionam que alterações no número e forma dos dendritos ocorrem antes da morte neuronal (Kocahan e Dogan, 2017). Outra característica da doença é o aumento de formação do peptídeo β-amilóide, e sua liberação extracelularmente no SNC, com a consequente ativação de receptores extracelulares que desencadeiam respostas neurotóxicas. O peptídeo β-amilóide é um polipeptídeo contendo 39-42 resíduos de aminoácidos derivado do processamento proteolítico da APP, uma proteína transmembrana tipo I pertencente à família multigênica que inclui as proteínas amilóides precursoras-*like* 1 e 2. A APP exibe uma variedade de funções biológicas, que incluem regulação do cálcio intracelular, crescimento e adesão celular, transporte axonal de vesículas e homeostase de íons metálicos (Smith *et al.*, 2007).

A Esclerose Lateral Amiotrófica é outra doença neurodegenerativa severa caracterizada pela progressiva perda de neurônios motores superiores do córtex e perda de neurônios motores inferiores do tronco cerebral e espinha vertebral. Esta perda celular resulta em espasticidade, perda muscular e fraqueza, progredindo até paralisia, dificuldades na fala, deglutição e respiração. O marcador patológico da doença é a formação de agregados citoplasmáticos nos neurônios motores em degeneração e oligodendrócitos circundantes, e estas inclusões encontram-se em outras áreas cerebrais como lobos frontal e temporal, hipocampo e cerebelo (Al-Chalabi *et al.*, 2016; Niedzielska *et al.*, 2016b).

Ao contrário da DP, DA e ELA, que apresentam fatores genéticos, mas principalmente fatores ambientais em sua origem, a doença de Huntington possui como origem principal a familiar, sendo herdada geneticamente. A DH é uma patologia caracterizada pela progressiva perda das funções cerebrais e musculares, que ocorre devido à programação genética de degeneração de neurônios, e causa movimentos descontrolados, perda da habilidade intelectual e distúrbios emocionais. A DH é causada pela expansão do trinucleotídeo CAG no éxon 1 do gene *Huntingtin*, localizado no cromossomo 4 (4p63). Enquanto indivíduos normais possuem de 6-35 CAG, portadores da doença apresentam 36 ou mais repetições. O acúmulo da proteína mutada que contém uma longa região de poli glutamina causa morte neuronal e degeneração das redes neurais (Manoharan e Guillemin, 2016; Ochalek *et al.*, 2016).

A doença de Creutzfeldt-Jakob (DCJ) também possui a agregação protéica como causa de citotoxicidade, ocorrendo o acúmulo e agregação da proteína príon mutada (Prp – *prion protein*). Em sua forma esporádica, a doença apresenta origem rara com eventos aleatórios

como início da alteração protéica, ao contrário da forma familiar em que os eventos são desencadeados por mutações no gene que codifica a PrP. As doenças causadas por príons também são classificadas como neurodegenerativas e incuráveis, além se serem infecciosas e afetarem tanto humanos como animais (Karapetyan *et al.*, 2013; Brandner e Jaunmuktane, 2017).

Grande parte das doenças neurodegenerativas compartilha um mecanismo de citotoxicidade comum, a agregação de formas aberrantes de proteínas. Quando recém-sintetizadas, as proteínas possuem uma rede de proteostase, formada por diversas proteínas chaperonas que auxiliam contra interações inespecíficas e atuam no controle da conformação. Estímulos externos podem induzir a perda da conformação protéica nativa, juntamente com a perda de sua função, e mais ainda proteínas em estado desenovelado tendem a entrar em vias que resultam em agregação, as quais fornecem um estado de energia mais baixo do que a conformação nativa (Radwan *et al.*, 2017). Vários estudos apontam o estresse oxidativo como mecanismo de toxicidade inicial e fator contribuidor para a formação de proteínas aberrantes (Squier, 2001).

Estresse Oxidativo no Contexto de Doenças Neurodegenerativas

Diversos estudos ao longo dos anos vêm tentando identificar fatores que contribuem para o processo de neurodegeneração no cérebro, considerado um dos grandes desafios da medicina moderna. Atualmente existem diversas hipóteses relacionadas aos mecanismos que induzem dano e morte celular dos neurônios nas doenças neurodegenerativas, como efeitos excitatórios causados por aminoácidos, perturbação do metabolismo energético celular e estresse oxidativo (Niedzielska *et al.*, 2016b).

A fim de obter energia para o desempenho das funções fisiológicas, as células convertem energia para a forma de ATP, em um processo denominado fosforilação oxidativa que ocorre nas mitocôndrias. Esse processo acaba por produzir subprodutos, espécies reativas de oxigênio (EROS), espécies reativas de nitrogênio (ERNS), entre outros. Além da mitocôndria, outras fontes endógenas produzem espécies reativas, e incluem retículo endoplasmático, peroxissomos, enzimas da família NADH oxidase e outras monoamino oxidases (Tonnies e Trushina, 2017). Em concentrações moderadas ou baixas as espécies reativas são essenciais para o desenvolvimento e função neuronal, sendo inclusive, consideradas importantes barreiras de defesa contra infecções, possuindo um importante papel na inflamação. O sistema antioxidante é composto por dois tipos de barreiras, uma enzimática, constituída pelas enzimas superóxido dismutase, glutationa peroxidase, glutarredoxinas, tiorredoxinas e catalase, e outra não-enzimática, constituída, por exemplo, por vitaminas. Os problemas surgem quando a produção de espécies reativas excede a capacidade de resposta do sistema antioxidante, ocorrendo, assim, oxidação protéica, do DNA e lipoperoxidação, levando a dano oxidativo, degeneração celular, podendo evoluir para um declínio funcional e morte celular (Birben *et al.*, 2012). Este desequilíbrio entre a geração de espécies reativas e a capacidade do sistema antioxidante de neutralizar as espécies recebe o nome de estresse oxidativo. Além de fontes intracelulares, estímulos externos também podem contribuir para a geração de espécies reativas. O cérebro se torna um alvo fácil do excesso de dano oxidativo, já que possui em sua composição grande conteúdo lipídico, grande demanda energética, dependência do metabolismo aeróbico, acesso abundante a metais de transição e fraca capacidade antioxidante. Assim, o estresse oxidativo induz o aumento da permeabilidade da barreira hematoencefálica, ocorrendo desta forma maior infiltração de compostos indesejáveis no SNC causando neuroinflamação e morte neuronal (Halliwell,

2006; Di Domenico *et al.*, 2016; Niedzielska *et al.*, 2016b; Salim, 2017; Tonnies e Trushina, 2017).

Embora a etiologia da maioria das doenças neurodegenerativas seja multifatorial, com participação gênica e fatores ambientais, diversos estudos apontam para o papel do estresse oxidativo no seu desenvolvimento. O principal efeito deletério das espécies reativas como ânion superóxido, radical hidroxil, peróxido de hidrogênio e óxido nítrico é a indução de disfunção mitocondrial, geração de espécies reativas e agregação protéica na neurodegeneração (Losada-Barreiro e Bravo-Diaz, 2017).

A disfunção mitocondrial causada por estresse oxidativo observada nas doenças neurodegenerativas ocorre devido a dano oxidativo ao DNA mitocondrial e acúmulo de mutações. O DNA mitocondrial é menos protegido que o DNA nuclear, apresentando menos mecanismos de reparo e falta de histonas, ficando mais suscetível à constante geração de espécies reativas. Uma vez que o DNA mitocondrial codifica grande parte das enzimas da cadeia transportadora de elétrons, mutações em sua sequência causam sérias consequências nas células, especialmente neurônios que possuem alta demanda energética, podendo levar à apoptose. Também decorre do estresse oxidativo redução da atividade da NADPH desidrogenase da cadeia transportadora de elétrons na substância negra de pacientes com Parkinson, e de enzimas dos complexos piruvato desidrogenase, α-cetoglutarato desidrogenase e citocromo C oxidase em pacientes com DA (Filosto *et al.*, 2011; Federico *et al.*, 2012; Ciccone *et al.*, 2013).

O sistema nervoso central é altamente sensível às espécies reativas devido à alta concentração de ácidos graxos poli-insaturados nas membranas dos neurônios. A peroxidação destes ácidos graxos leva a geração de aldeídos tóxicos como o 4- hidroxi 2-nonenal (HNE), que altera a função celular através da sua ligação com proteínas, e malondialdeído. Altas concentrações de HNE foram encontradas no cérebro e em fluidos de pacientes com Parkinson, Alzheimer, Huntington e Esclerose Lateral Amiotrófica. Estudos apontam o estresse oxidativo, especialmente a produção de HNE como causa de modificações pós-transcricionais que induzem a agregação de α-sinucleína (Xiang *et al.*, 2013; Di Domenico *et al.*, 2016). Outros estudos relacionam o estresse de retículo endoplasmático com a hiperfosforilação da proteína tau (Ho *et al.*, 2012) e o estresse oxidativo com a inibição da autofagia de proteínas mutadas na doença de Huntington (Vidoni *et al.*, 2016).

A presença de metais de transição no cérebro influencia o estado redox, sendo relatado acúmulo de ferro no cérebro de pacientes com DA, aumentando a peroxidação lipídica através da geração do radical hidroxil pela da reação de Fenton. Homeostase anormal de outros metais incluindo cobre, zinco, magnésio, manganês e alumínio também estão relacionados com a geração de estresse oxidativo e agregação do peptídeo β-amilóide e da proteína tau. Foi relatado, por exemplo, que o zinco se liga à APP e afeta o seu processamento, assim como o zinco, alumínio, ferro e cobre se ligam diretamente ao peptídeo β-amilóide e promovem a sua agregação. Similarmente, estes metais podem estar envolvidos na fosforilação da proteína tau e sua consequente liberação dos microtúbulos, com a formação das placas neurofibrilares. Outros estudos indicam que o próprio peptídeo β-amilóide pode gerar espécies reativas na presença de ferro e cobre (Bush *et al.*, 1994; Sayre *et al.*, 2000; Smith *et al.*, 2007; Tonnies e Trushina, 2017). A ligação de metais divalentes como cobre e manganês em resíduos

terminais da α-sinucleína também induz a sua agregação (Berrocal *et al.*, 2015; Rokad *et al.*, 2016).

Além do estresse oxidativo, a inflamação também parece estar relacionada com a neurodegeneração na DP, através da liberação de citocinas e prostaglandinas (Niranjan, 2014). Modelos experimentais da DP utilizando a 6-hidroxidopamina como neurotoxina demonstraram ocorrer um aumento na liberação de citocinas pró-inflamatórias como TNF-α e IL-1β, aumento da lipoperoxidação e redução do nível de GSH (Farbood *et al.*, 2015; Kheradmand *et al.*, 2016). As vitaminas são potentes anti-inflamatórios naturais, e seu uso também é indicado na prevenção de doenças neurodegenerativas (Sutachan *et al.*, 2012).

A presença de marcadores inflamatórios em cérebros de pacientes com doenças neurodegenerativas aponta para uma relação entre estresse oxidativo e inflamação na etiologia das doenças. Estudos relatam que a morte celular com liberação de α-sinucleína induz a ativação da micróglia aumentando assim o dano dopaminérgico na DP. A ativação da micróglia induz a ativação da enzima NADPH oxidase, e assim mais espécies reativas são geradas aumentando a neurotoxicidade. Análises também indicam um aumento na liberação de citocinas pró-inflamatórias como IL-1β e TNF-α na substância negra de pacientes com PD e em soro de pacientes com AD (Zhang *et al.*, 2005; Niranjan, 2014; Demirci *et al.*, 2017).

O RAGE, ou receptor para produtos finais de glicação avançada, é um receptor extracelular multi-ligante, que desencadeia a transcrição NF-κB dependente de citocinas, e também a produção de espécies reativas comumente associada à diabetes e outros estados

inflamatórios crônicos. Um dos ligantes descritos atualmente de RAGE é o peptídeo β-amilóide, liberado no processo de desenvolvimento da DA, mas também pode se ligar a demais produtos oriundos da glicação oxidativa de carboidratos (Cai *et al.*, 2016; Ray *et al.*, 2016). A ativação do RAGE parece estar relacionada com o eixo neuroinflamatório, visto que este receptor estimula a expressão de citocinas pró-inflamatórias, e é também ativado pelas mesmas, contribuindo assim para a formação de um quadro inflamatório crônico. O RAGE é capaz de se ligar não somente em AGEs (*advanced glycation end-products*), como também em anfotericinas, calgranulinas e no peptídio β-amilóide. Em uma situação de desequilíbrio redox, a ativação deste receptor está relacionada com a ativação e progressão de inflamação e vias pró-apoptóticas (Piras *et al.*, 2016). Recentemente, estudos indicam uma forte participação do RAGE na DA, visto que o peptídeo β-amilóide foi descrito como seu ligante, e observou-se a ativação de muitas vias de sinalização neurotóxicas associadas com degeneração celular a partir da ligação do peptídeo ao RAGE (Yan *et al.*, 2012). Outros estudos apontam o RAGE como mediador para o transporte do peptídeo β-amilóide pela barreira hematoencefálica, aumentando assim a sua deposição no tecido neuronal, e também um aumento no imunoconteúdo de RAGE foi associado com o aumento de outros marcadores para a DA (Candela *et al.*, 2010; Valente *et al.*, 2010). Desde que estudos mostrando a capacidade antioxidante da vitamina A surgiram, vários autores começaram a sugerir o seu uso como forma de prevenção ou tratamento contra doenças relacionadas à neurodegeneração (Reinhardt *et al.*, 2016).

Vitaminas, Estado redox e Doenças Neurodegenerativas

Devido a importante relação entre o estresse oxidativo e as doenças neurodegenerativas, pesquisas sugerem o uso de antioxidantes para o tratamento ou prevenção destas doenças. Vitaminas são compostos naturais, e por apresentar características de neutralização das espécies reativas, seu uso tem sido amplamente proposto contra doenças neurodegenerativas (Bhatti *et al.*, 2016).

A vitamina C, ou ácido ascórbico, é um importante antioxidante solúvel em água, que protege os neurônios do dano oxidativo, por restaurar a forma reduzida da vitamina E, ou α-tocoferol. A vitamina E, por sua vez, é um antioxidante lipossolúvel que protege as membranas celulares da lipoperoxidação. Juntamente com a vitamina A o uso dessas vitaminas já foi sugerido como forma de prevenção e/ou tratamento da DP, e foi relatado também uma correlação entre deficiência de vitamina C e o estabelecimento de morte neuronal oxidativa, e seu efeito neuroprotetor contra DH e ELA. Baixos níveis plasmáticos e baixa ingesta de vitamina D estão associados a DP, embora não tenha sido estabelecido se é a causa ou consequência da doença devido a redução da mobilidade em pacientes (Paraskevas *et al.*, 2003; Covarrubias-Pinto *et al.*, 2015; Wang *et al.*, 2015).

A vitamina A e seus derivados influenciam na diferenciação celular, proliferação, apoptose e também possuem um papel fisiológico fundamental em uma série de processos biológicos. Pela sua propriedade redox ativa - protegendo contra a ação de espécies reativas em algumas situações, e promovendo o dano oxidativo em outras-, a vitamina A, e os retinóides em geral, foram postulados como compostos antioxidantes, capazes de influenciar o metabolismo de espécies reativas, sinalização redox e equilíbrio do estresse oxidativo. Alguns

efeitos relatados dos retinóides foram relacionados com a habilidade dessa vitamina em neutralizar formas tóxicas de oxigênio e outros radicais livres (Burton e Ingold, 1984). A administração de retinol ou carotenoides tem sido amplamente demonstrada inibir o crescimento e desenvolvimento de diversos tipos de tumores, incluindo pele, pulmão e hepático (Doldo *et al.*, 2015). Outros trabalhos indicam o uso de AR na indução de apoptose e modulação de enzimas antioxidantes em modelo de câncer de mama (Hong e Lee-Kim, 2009). A partir destas observações, também foi sugerido o uso de retinóides em terapias baseadas em antioxidantes para tratar ou prevenir doenças neurodegenerativas mediadas por radicais livres. Outros modelos experimentais confirmaram a habilidade da vitamina A em alterar o ambiente redox, mas de forma pró-oxidante, aumentando a produção do radical ânion superóxido ($O^{2-\cdot}$) e dano oxidativo ao DNA, modulando a atividade de enzimas antioxidantes (Dal-Pizzol *et al.*, 2000; Dal-Pizzol *et al.*, 2001). Além disso, a vitamina A induz morte por apoptose através de uma desregulação redox em modelo *in vitro*. Devido a sua capacidade de induzir a diferenciação celular, o AR vem sendo proposto para terapia no tratamento de neuroblastoma humano através da indução de apoptose (Qiao *et al.*, 2012), e também como indutor de neurodiferenciação em modelos *in vitro*, a fim de melhor compreender eventos moleculares e vias de sinalização em diversas patologias neurodegenerativas (Alberio *et al.*, 2012).

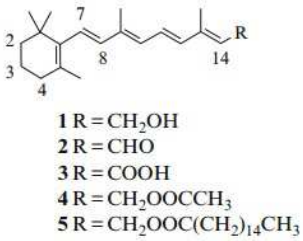

Figura 2: Estrutura dos Retinóides. 1 = retinol, 2 = retinaldeído, 3 = ácido retinóico, 4 = acetato de retinil, 5 = palmitato de retinil. Adaptado de: Harrison, E.H.; Curley, R.W. Carotenoids and Retinoids: Nomenclature, Chemistry, and Analysis. Subcell Biochem. 2016;81:1-19.

V. OBJETIVOS

Justificativa e apresentação dos objetivos

Considerando que: 1) o ácido retinóico tem papel essencial no desenvolvimento neural embrionário, mas seus mecanismos de ação não-clássicos e o seu papel como antioxidante ou pró-oxidante na neurodiferenciação ainda não está evidente; 2) o papel da vitamina A (retinol) na neurotoxicidade e modulação de marcadores neurodegenerativos é incerto; 3) a suplementação alimentar da vitamina A como prevenção e/ou tratamento de doenças neurodegenerativas ainda é debatida visto sua ação redox-dependente, o objetivo central desta tese foi o de **estabelecer a ação e mecanismos de diferentes retinóides sobre processos de neurodiferenciação e neurotoxicidade no sistema catecolaminérgico em modelos *in vitro* e *in vivo***, tendo como objetivos específicos:

a) investigar o papel de espécies reativas e estresse oxidativo na neurodiferenciação catecolaminérgica de células SH-SY5Y induzida por AR;

b) analisar o efeito da suplementação de retinol no estresse oxidativo, viabilidade e acúmulo de marcadores neurodegenerativos células SH-SY5Y;

c) averiguar o efeito da suplementação oral vitamina A *in vivo* na prevenção do parkinsonismo induzido por 6-hidroxidopamina.

PARTE 2 – RESULTADOS

CAPÍTULO I

Changes in Cell Cycle and Up-Regulation of Neuronal Markers During SH-SY5Y Neurodifferentiation by Retinoic Acid are Mediated by Reactive Species Production and Oxidative Stress

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Changes in Cell Cycle and Up-Regulation of Neuronal Markers During SH-SY5Y Neurodifferentiation by Retinoic Acid are Mediated by Reactive Species Production and Oxidative Stress

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Abstract Human neuroblastoma SH-SY5Y cells have been used as an in vitro model for neurodegenerative disorders such as Parkinson's disease and can be induced to a mature neuronal phenotype through retinoic acid (RA) differentiation. However, mechanisms of RA-induced differentiation remain unclear. Here, we investigate the role of reactive species (RS) on SH-SY5Y neuroblastoma cells under RA differentiation, using the antioxidant Trolox® as co-treatment. We found that RA treatment for 7 days reduced the cell number and proliferative capacity and induced the expression of adult catecholaminergic/neuronal markers such as tyrosine hydroxylase (TH), β-III tubulin, and enolase-2. Evaluation of intracellular RS production by DCFH oxidation assay and quantification of cell non-enzymatic antioxidant activity by TRAP demonstrated that RA increases RS production. Furthermore, mitochondrial NADH oxidation showed to be inhibited under differentiation with RA. Cells subjected to co-treatment with antioxidant Trolox® demonstrated a remaining proliferative capacity and a decrease in the pro-oxidant state and RS production. Besides, antioxidant treatment restores the mitochondrial NADH oxidation. Importantly, Trolox® co-treatment inhibited the appearance of morphological characteristics such as neurite extension and branching, and decreased the expression of TH, β-III tubulin, and enolase-2 after a seven-day differentiation with RA, indicating that RS production is a necessary step in this process. Trolox® also inhibited the phosphorylation of Akt and ERK1/2, which are involved in differentiation and survival, respectively, of these cells. Altogether, these data indicate the presence of a redox-dependent mechanism in SH-SY5Y RA-differentiation process and can be a useful insight to improve understanding of neuronal differentiation signaling.

Keywords Retinoic acid · Neuronal differentiation · SH-SY5Y · Oxidative stress · Tyrosine hydroxylase

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Introduction

Parkinson's disease (PD) is the most common movement disorder, affecting approximately six million people worldwide [1] and is highly connected to oxidative stress, with increased lipid peroxidation, decreased glutathione, abnormalities in iron homeostasis, and protein aggregation [2]. Cellular models are often used to understand pathways and molecular events of pathological processes. In vitro research emerged as first choice for preliminary studies on the molecular action, cytotoxic and genetic events of neurodegenerative diseases, including PD [1]. The human neuroblastoma SH-SY5Y cell line has been largely used in neuroscience studies, particularly to generate different PD cell models [3–6]. Originally derived as subclone of the neuroblastoma cell line SK-N-SH, SH-SY5Y

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cells have been frequently used either in an undifferentiated condition or in a neuron-like state after inducing the cells into a functional and morphological neuronal phenotype. Retinoic acid (RA) is a signaling molecule and exerts a major role in neuronal differentiation and patterning [7]. Differentiation with RA has been shown to induce morphological and metabolic changes that confer neuronal-like features [5] and also induces cell cycle arrest, neurite outgrowth, and differentiation into catecholaminergic phenotype [6, 8–10]. Administration of RA in therapeutic protocols for neuroblastoma treatment had promising clinical results [10, 11]. Also, neurons derived from SH-SY5Y treatment with RA for 7 to 10 days express specific neuron markers, such as tyrosine hydroxylase (TH), enolase-2, and β-III tubulin, enabling this model for molecular studies on cell replacement therapy, neuronal differentiation, disease modeling, and drug screening [8, 12].

RA is the main active metabolite of retinol, from the retinoid family of related compounds (which includes vitamin A) and may influence the oxidant status of the cell due to intrinsic redox-active properties [13, 14]. Classically, RA effects are mediated by the nuclear retinoic acid receptors (RAR) and retinoid X receptors (RXR) [9, 10, 14]. More recently, new mechanisms of action triggering cellular events not related to retinoid receptor activation were identified, and these events have been referred to as non-genomic, non-classical, or extra nuclear effects of retinoids [15, 16]. Several studies reported non-genomic actions by retinoids, and some of these are dependent on reactive species (RS) production and redox modifications [17–21]. Although it is known that RAR-/RXR-mediated gene transcription exerts an important role in neuronal differentiation, the necessity of high concentrations (5 to 10 μmol/L) of RA to induce SH-SY5Y differentiation [5] suggests the participation of RAR-/RXR-independent mechanisms.

RA-induced differentiation of SH-SY5Y cells has been related with resistance to oxidants and xenobiotics, possibly due to modulation of RS production and oxidative stress responses [8, 22, 23]. In previous works, it was observed that SH-SY5Y cells expressing Nrf2, a transcription factor responsible for the activation of key genes in antioxidant response and xenobiotic detoxification, enhanced the expression of markers associated to neurite outgrowth, such as neurofilament-M and microtubule-associated protein 2 (MAP2) when treated with RA for up to 4 days [24]. This result indicated that up-regulation of antioxidant defense was essential to RA-induced differentiation in SH-SY5Y cells. Besides, the observation that RA enhances NADPH oxidase expression in SH-SY5Y cells and that pharmacological inhibition of this enzyme inhibits both MAP2 expression and morphological differentiation [25] indicates that RS production may be an essential step in RA-induced neuronal differentiation.

Here, we evaluated the role of RS production and oxidative stress during RA differentiation using the neuroblastoma SH-SY5Y cell line. Using both undifferentiated (proliferative) and differentiated SH-SY5Y cells, we characterized the redox-dependent effects of RA on SH-SY5Y cell viability, proliferation and cell cycle, cellular morphology, expression of specific neuronal proteins, and activation of signaling pathways. Synthetic antioxidant Trolox®, a vitamin E analog, was used to evaluate the effect of oxidative stress during differentiation. Therefore, in the present study, we provide valuable insight into the mechanisms of action of RA on SH-SY5Y cells, specifically on the role of RS and oxidative stress as necessary events taking place in the signaling cascade evoked by RA during neuronal differentiation.

Materials and Methods

Cell Culture and Treatments

Human SH-SY5Y neuroblastoma cell line was obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in 1:1 mixture of Ham's F12 and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 0.28 mg/mL of gentamicin, 250 μg amphotericin B, and 10 % heat inactivated fetal bovine serum (FBS) in a 5 % CO_2-humidified incubator at 37 °C. Cells were seeded at an initial density of 10^4 cells/cm^2 in culture dishes for 24 h (TPP Techno Plastic Products AG). Differentiation was induced in 1 % FBS medium by 10 μM all-*trans*-retinoic acid (RA). The culture medium was replaced every 3 days, and the cells were maintained under differentiation for 7 days. Antioxidant treatment was carried by adding Trolox® (100 μM). Two different control groups were considered for different purposes: proliferating cells cultured in 10 % FBS (proliferative cells) were used as standard for proliferative, undifferentiated cells; and cells exposed to 1 % FBS medium (non-proliferative cells) were used as control in relation to the cells submitted to differentiation with RA 10 μM in 1 % FBS. Besides, a zero time group was used as control of the starting point of differentiation, in which cells were briefly exposed to RA in 1 % FBS and incubation was promptly terminated (Day 0) (Fig. 1). For phosphorylation experiments, cells were changed to free-serum medium for 1 h, followed by pre-incubation of 2 h with Trolox® (AR+T and T groups). After this time, cells were treated with RA or vehicle (Trolox® group) for 0, 15, 30, 45, and 60 min.

Parameters of Cellular Viability and Neuronal Morphologic Phenotype

The sulforhodamine B (SRB) incorporation assay was used for determination of cellular density, as it is directly related to cellular protein biomass. This assay relies in the high affinity of SRB to protein components of the cell [26]. The absorbance

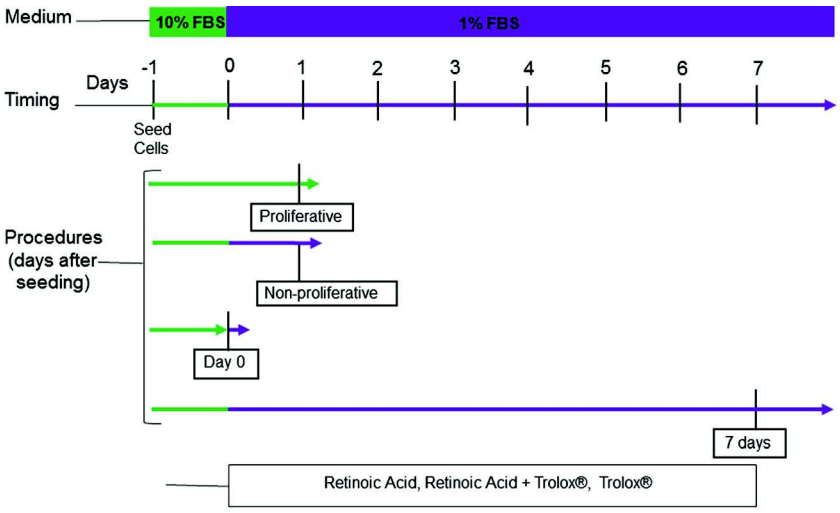


Fig. 1 Schematic overview of experimental design. Proliferative cells consist in cells under 10 % FBS medium for 48 h with medium replacement after 24 h. Non-proliferative cells consist in 24 h under 1 % FBS medium and Day 0 consist of cells briefly exposed to 1 % FBS medium with RA. The differentiation protocol consisted in reducing medium FBS concentration from 10 to 1 % and then adding RA (10 μM) 24 h later. RA plus Trolox® (100 μM) and Trolox® alone were the other groups to evaluate the role of antioxidant treatment in RA differentiation. The culture medium was replaced every 3 days, and after 7 days under treatment, cells were collected and prepared for the experiments

was determined spectrophotometrically at 515 nm, and results were expressed as percentage of SRB incorporation. Lactate dehydrogenase (LDH) activity in the incubation medium was assessed to estimate cell membrane leakage, using a commercial kit from Labtest SA (MG, Brazil). To evaluate cellular morphology at daily basis, phase-contrast micrographies were taken by using an inverted microscope (Nikon Eclipse TE300) connected to a digital camera.

Flow Cytometry Analyses of Cell Proliferation and Differentiation

To determine the cell cycle state of cells, first was used Click-iT EdU® Alexa Fluor 647 flow cytometry assay kit (Molecular Probes). Briefly, SH-SY5Y cells were cultured in 6-well plates and differentiated for 7 days. After this, the cells were pre-incubated for 3 h with EdU® (5-ethynyl-2′-deoxyuridine) to allow it to incorporate into DNA. The cells then were fixed in 4 % formaldehyde followed by incubation with rabbit anti-tyrosine hydroxylase (Cell Signaling Technology, #9664, 1:800). Cells were analyzed by flow cytometry (BD FACS Calibur flow cytometer, BD Biosciences); data were analyzed by FlowJo (cytometric data analysis and presentation software). Ten thousand cells were analyzed per sample, and data were also reported as the percentage of proliferative (EdU®-positive) and differentiated cells (TH-positive cells). We also performed a cell cycle progression assessment in which differentiated and proliferative cells were stained with PI (propidium iodide) followed by flow cytometry sorting.

Confocal Immunofluorescence Microscopy

Cells were washed with phosphate-buffered saline (PBS) and fixed on chamber slides with 4 % paraformaldehyde (PFA) in 4 °C for 15 min. Samples were sequentially treated with 0.15 % Triton X-100 and 5 % FBS for 1 h and 30 min. Cells were immunostained with specific antibodies, the primary antibodies were utilized as follows: rabbit anti-TH (Cell Signaling Technology, 9664, 1:800), mouse anti-β-III tubulin (Novex, 480011, 1:1000), and fluorescent-labeling Alexa Fluor 488 goat anti-mouse (Molecular Probes, A11001, 1:800) and fluorescent-labeling Alexa Fluor 488 goat anti-rabbit (Molecular Probes, A11008, 1:800). For nuclear staining, Prolong Gold Antifade Reagent with DAPI (Molecular Probes, P36931) was used. Images were taken with an Olympus FluoView 1000 confocal microscope and subsequently analyzed by using Olympus FluoView FV1000 Software, ver. 3.0. Thresholds discriminating between signal and background were selected by utilizing cells that were only stained with secondary antibodies in order to discriminate any false positive result.

Immunoblotting

To perform immunoblot experiments, cells were lysed with 2 % sodium dodecyl sulfate (SDS) and was resolved in standard SDS-polyacrilamide gel electrophoresis and were transferred to an nitrocellulose membrane (Millipore, Bedford) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Protein loading and electroblotting efficiency was verified through Ponceau S

staining, and the membrane was washed with TTBS (Tris 100 mM, pH 7.5, 0.9 % NaCl and 0.1 % Tween-20). The membrane was blocked in TTBS containing 5 % of non-fat/skim dry milk for 60 min at room temperature. Primary antibody was incubated overnight at 4 °C and subsequently washed with TTBS. Anti-rabbit or mouse IgG peroxidase-linked secondary antibody was incubated for 2 h at room temperature, and the membrane was washed again with TTBS. Blots were finally developed and immunoreactivity was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent kit (Thermo Scientific). Densitometric analyses were carried using a CCD camera (GE ImageQuant LAS 4000) and the quantitative analysis was performed using Image J. software. Anti-enolase-2 (Cell Signaling Technology, 9664, 1:500), anti-β-actin (Sigma-Aldrich®, A1978, 1:500), anti-phospho-Akt (Thr308) (Cell Signaling Technology, #13038S, 1:1000), anti-Akt (Cell Signaling Technology, #9272S, 1:1000), anti-phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology, #9101, 1:1000), and anti-ERK1/2 (Cell Signaling Technology, #9102, 1:1000) were used.

Quantitative RT-PCR

Total RNA was extracted from cells according to the manufacturer's instructions (PureLink® RNA mini kit - 12183018A–Life Technologies™). The cDNA was synthesized with the Super Script III Reverse Transcriptase kit (Invitrogen) using 1 μg of total RNA. Quantitative RT-PCR reactions were performed in triplicate for each sample using the Step One Plus thermocycler (Applied-Biosystems) and Power SYBR® Green PCR Master Mix (4367659, Life Technologies™). Reactions were carried out in 25 μL following manufacturer's instructions. Primer sequences are as follows: forward sequence (5'CAGAGGCCATCATGTCCCCG 3') and reverse (5'CTTCCCCTCCTTCTCCTCAA3') for tyrosine hydroxylase; forward sequence (CCATGTTC GTCATGGGTGTGAACCA) and reverse sequence (GCCAGTAGAGGCAGGGATGATGTTG) for endogen gene GAPDH. Quantification was performed using the $2^{-\Delta\Delta CT}$ method.

Total Reactive Antioxidant Potential (TRAP)

TRAP was measured and calculated as previously described [27]. Briefly, TRAP represents the non-enzymatic antioxidant capacity of the cells. This is determined by measuring the luminol chemiluminescence intensity of emission induced by thermolysis of 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) as free radical source. The system was left to stabilize for 2 h, then samples were added and the readings monitored for 60 min in a liquid scintillator counter (Wallac 1409, Perkin–Elmer, Boston, MA, USA). Results were transformed into a percentage, and the area under the curve (AUC) was calculated by utilizing the GraphPad software (SanDiego, CA, USA). Results express the extent by which the non-proteic fraction of the cells inhibits the free radical-derived luminescence.

Intracellular Reactive Species Production (DCFH-DA)

Intracellular reactive species production was measured by the DCFH-DA oxidation assay, as previously described [28]. This technique is based on the capability of DCFH to be oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of reactive species (RS) and can be used as an index to quantify the overall RS production in cells. Briefly, SH-SY5Y cells were seeded in 96-well plates and 100 μM DCFH-DA dissolved in medium containing 1 % FBS was added in each well. Cells were incubated for 2 h in order to allow cellular incorporation, and DCF fluorescence was read at 37 °C in a fluorescence plate reader (Spectra Max M2, Molecular Devices, USA) with an emission wavelength of 535 nm and an excitation wavelength of 485 nm. The results were expressed as percentage of DCF fluorescence in relation to untreated control.

Assessment of Mitochondrial Activity by NADH Oxidase Assay

The NADH segment comprises the electron transport from Complex I to Complex IV. The mitochondria from SH-SY5Y cells were isolated according to Voss et al. (1961) [29]. For complete lysis of the mitochondria, several freeze-thaw cycles were applied. The membrane fragments were used to evaluate enzymatic activity of NADH oxidase segment through spectrophotometrical assay as described by Singer (1974) [30]. Briefly, NADH oxidase activity was measured at 28 °C in phosphate buffer 80 mmol/L, pH 7.4, EDTA 50 μmol/L, NADH 0.2 mmol/L, and 0.1 mg/mL of mitochondrial protein in a total volume of 1.0 mL. The reaction was initiated by the addition of the substrate and was followed by the decrease in absorbance at 340 nm.

Protein Assay

Total protein was quantified by Lowry assay and used to normalize all data [31].

Statistical Analysis

Results were expressed as the mean ± SEM of three independent experiments ($n = 3$). Data were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Data were analyzed by using GraphPad software® (San Diego, CA; version 5.00). Differences were considered to be

significant when $*p < 0.05$, $** p < 0.01$, or $*** p < 0.001$ and between groups ($\#p < 0.05$, $\#\#p < 0.01$, or $\#\#\#p < 0.001$).

Results

Effects of Antioxidant Treatment on Morphologic Differentiation, Cell Viability, and Proliferation

SH-SY5Y cells were differentiated for 7 days with RA in the presence or absence of the antioxidant Trolox®. Proliferative (grown at 10 % FBS) and non-proliferative (grown at 1 % FBS) cells were used as controls for differentiation, while cells under RA at day zero (brief addition of RA in 1 % FBS and termination at time zero of differentiation) were used to compare with the effects of RA at 7 days of differentiation. The neuroblastoma SH-SY5Y cell line includes at least two morphologically and biochemically distinct phenotypes: neuroblastic (N-type) and substrate adherent (S-type) (Fig. 2a, c). Proliferative cells present typical rounded cell bodies and few short projections. In non-proliferative cells, increased formation of neurite projections, that were generally small, was observed. Prolonged exposure to RA (7 days) caused substantial morphologic differentiation, with a considerable proportion of neuroblastic (N-type) cells being differentiated into a neuronal phenotype by extending long neuritic processes, whereas the S-type cells did not undergo apparent morphological changes (Fig. 2g, h). Besides the length of neurites, cells differentiated with RA also presented a higher number of elongations and branching and evident fewer cells/area than observed in other treatments. Co-treatment with Trolox® led to a smaller proportion of the N-type cells undergoing differentiation, and the apparent number of S-type cells increased compared to cells treated with RA (Fig. 2i, j). Cells treated only with Trolox® presented significantly increased cells/field (Fig. 2k, l), and although many of them had minor neuritic projections, we observed a significantly higher proportion of cells displaying morphologic characteristics resembling proliferative or apoptotic states. Cells maintained in 1 % FBS without RA for 7 days presented extensive cell death and detachment and were not evaluated in further assays (not shown).

In order to understand the effect of antioxidant treatment on RA-induced neurodifferentiation, we performed assays to evaluate parameters of cell viability and cytotoxicity, proliferation and cell cycle progression. First, we assessed SRB incorporation and medium LDH activity to evaluate protein biomass and viability/cytotoxicity. As expected, non-proliferative cells had a decreased rate of SRB incorporation compared to proliferative cells, and RA treatment for 7 days further decreased this effect (Fig. 2m). This effect is probably related to the decrease in the number of proliferating and undifferentiated cells observed along the differentiation treatment with RA. Antioxidant treatment with Trolox®, however, had no significant effect on RA-induced SRB incorporation. Cells subjected to Trolox® alone for 7 days displayed a similar SRB incorporation to proliferative cells. As SRB incorporation is related to the protein biomass and cultured cells undergoing differentiation may present less protein biomass than in proliferative or undifferentiated conditions, we assessed LDH activity in the incubation medium to detect cytosolic leakage. We did not observe significant differences between groups, except for cells treated with Trolox® in the presence or absence of RA, which presented a small increase in medium LDH activity (Fig. 2n). A positive control group for cytotoxicity with Triton X-100 1 % was used, and medium LDH activity was approximately 100-fold higher than in proliferative cells (not shown).

Next, we determined the number of cells incorporating EdU® to evaluate the proportion of cells under active mitotic activity in each treatment. An equal number of cells from each group was analyzed, and the number of cells incorporating EdU® was decreased in non-proliferative cells compared to proliferative cells (Fig. 3a, b). Differentiation with RA substantially decreased the number of EdU®-incorporating cells and this effect was not inhibited by co-treatment with Trolox®. Treatment with Trolox® alone also decreased EdU®-incorporation in relation to the proliferative cell control, but not to the extent of RA treatment (Fig. 3a, b). FACS analysis of cell cycle by PI staining revealed that differentiation with RA increased the proportion of cells in G0/G1 phase and decreased the amount of cells in G2/M phase (Fig. 3c). Co-treatment with Trolox® induced an increase in sub-G1 phase cells and also decreased the proportion of G0/G1 cells (Fig. 3c). Trolox® alone increased the number of cells in G2/M phase and reduced the amount in G0/G1 phase. Altogether, these results are suggestive that RA-induced changes in proliferation and cell cycle progression during the process of differentiation are inhibited by antioxidant treatment, which causes cell cycle arrest with cell death as possible consequence.

Effects of Antioxidant Treatment on Neuronal Markers Expression

Tyrosine hydroxylase (TH) is considered a marker of SH-SY5Y cell differentiation into an adult catecholaminergic phenotype, and all treatments increased the proportion of cells containing TH as seen in FACS analysis (Fig. 3b, quantification at Fig. 4a). The proportion of TH-positive, differentiated cells was decreased by co-treatment with Trolox® (Figs. 3b and 4a). The quantification of TH mRNA by RT-PCR confirmed these observations (Fig. 4b). Enolase-2, another neuronal specific protein [8], was evaluated by WB and

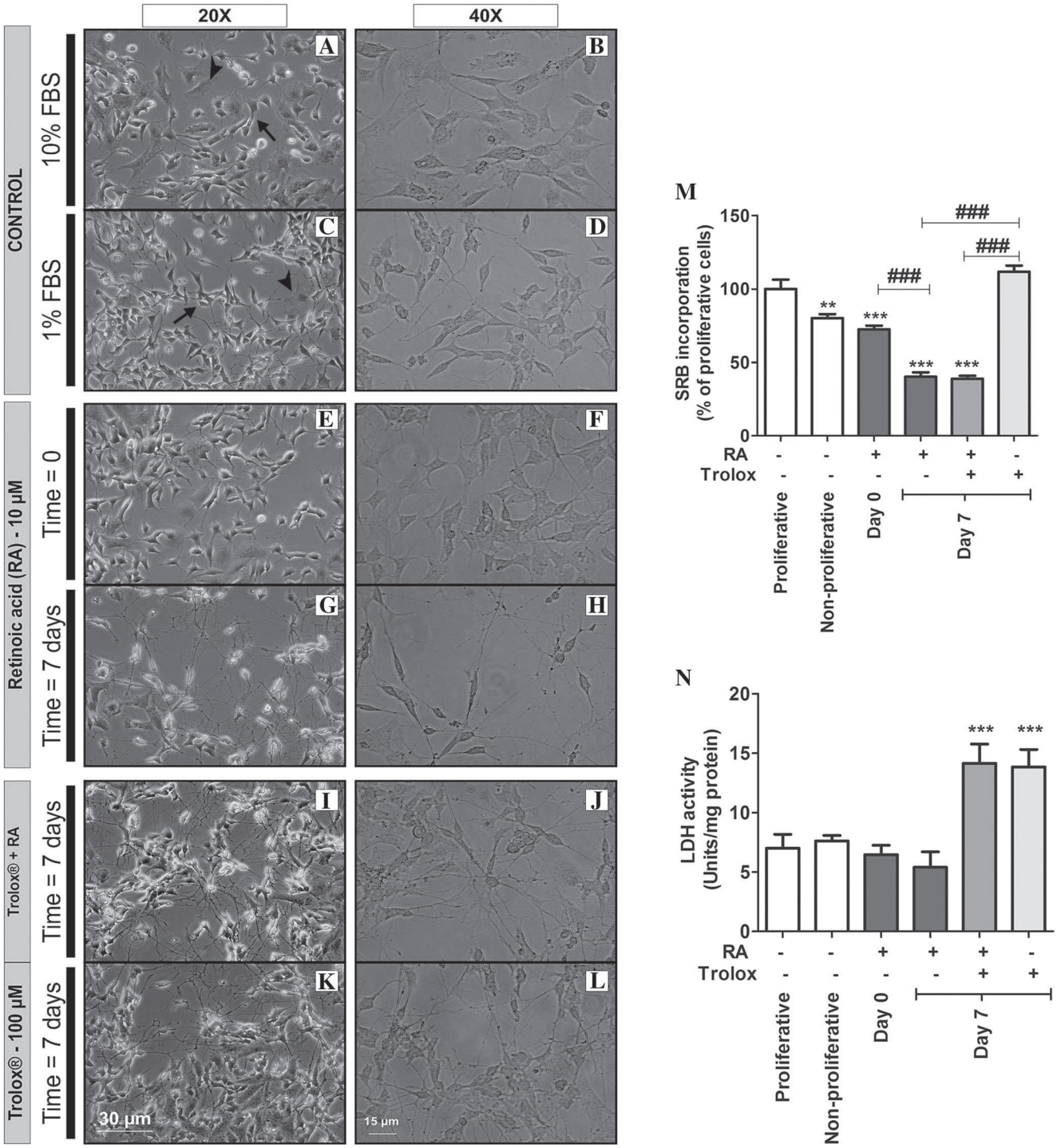


Fig. 2 Parameters of cellular viability and neuronal morphologic phenotype. **a**–**l** Effect of antioxidant co-treatment with Trolox® on morphologic differentiation induced by RA. SH-SY5Y cells are mainly neuroblastic N-type (*arrows*), but some substrate-adherent S-type cells appear (*arrowheads*) and present morphological alterations after differentiation, with neurite elongation and branching. *Scale bars* represent 30 µm (20×) and 15 µm (40×). **m** Cellular protein biomass assessed by SRB incorporation. **n** LDH leakage after RA, RA+ Trolox®, and Trolox® treatment for 7 days. Data are mean ± S.E.M. from three independent experiments. Analyzed by one-way ANOVA followed by post hoc Tukey's test, *triple asterisk* indicates $p < 0.05$ vs control 10 % and *triple octothorpe* indicates $p < 0.05$ vs respective groups

followed a similar pattern (Fig. 4c). We also evaluated the effect of antioxidant treatment on β-III tubulin and TH by confocal immunofluorescence microscopy which allows observing the expression of these neuronal markers along with the differentiation into the neuronal morphologic phenotype (Fig. 4d). All of the treatments increased the content of TH in

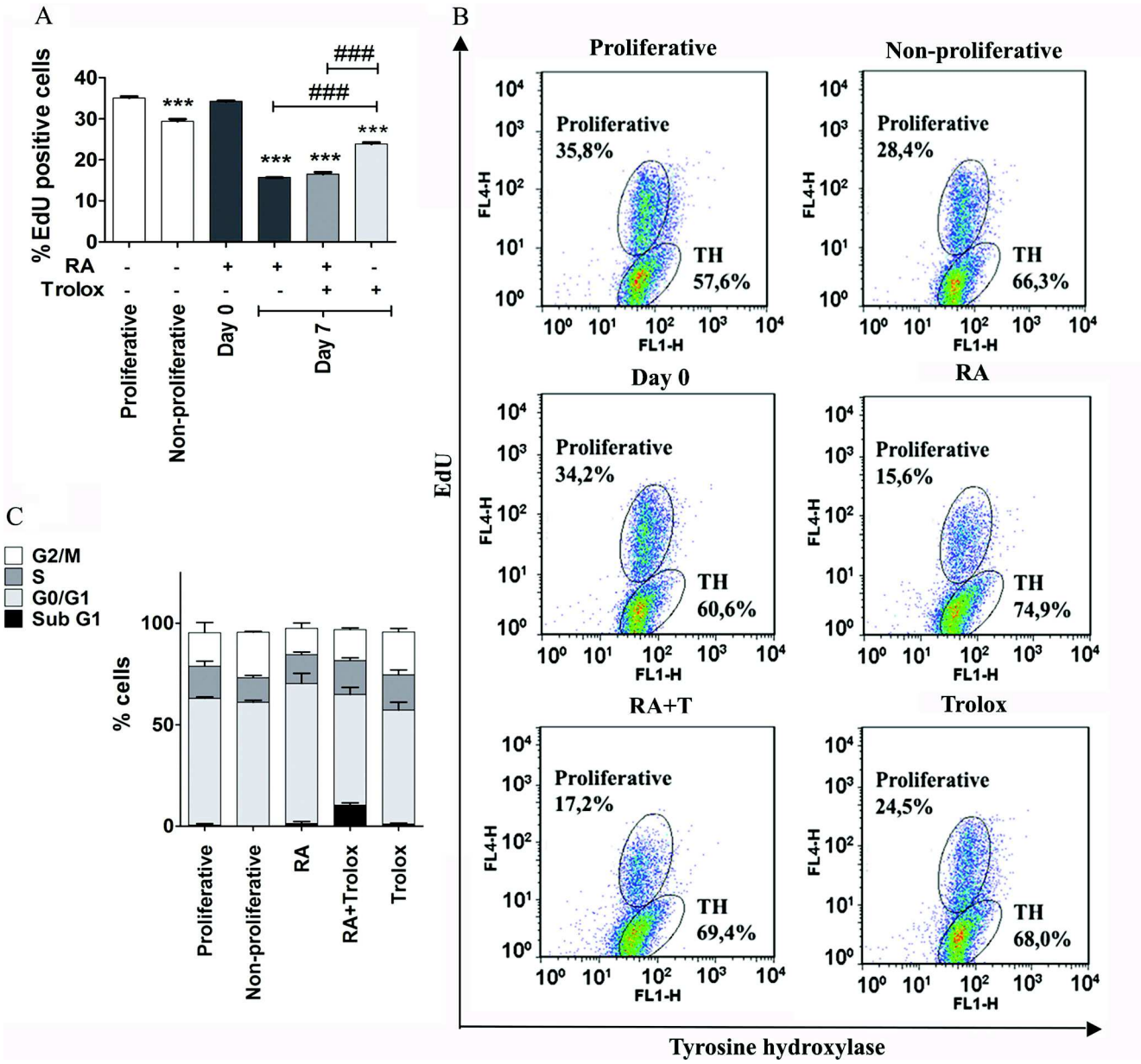


Fig. 3 Flow cytometry analyses of cell proliferation and differentiation. **a** Cell proliferation was assessed by flow cytometry to detect and count cells incorporating EdU®. **b** Quantification of EdU® incorporation in cells treated with RA, RA+Trolox®, and Trolox® for 7 days, with respective controls. **c** Cell cycle analysis of SH-SY5Y cells was performed with detection and sorting of cells with different profiles of incorporated PI. $p < 0.05$ G0/G1 phase RA vs RA+T; $p < 0.001$ SubG1 phase control 10 % vs RA+T; $p < 0.001$ SubG1 phase RA vs RA+T

the same overall pattern as previously seen, with the cells treated with RA for 7 days showing the largest increase in TH immunostaining along with neurite projection and branching per cell body (Fig. 4d13–d16). Co-treatment with Trolox® decreased TH immunostaining along with neurite formation and branching (Fig. 4d17–d20). A similar pattern of neuronal marker expression in response to RA and Trolox® was observed with β-III tubulin (Fig. 4). Cells treated with Trolox® alone also showed a substantial increase in β-III tubulin immunofluorescence, but probably due to the higher number of cells per field.

Effects of RA on Intracellular RS Production and Mitochondrial Activity in SH-SY5Y Cells

As our results indicated that antioxidant treatment inhibits the action of RA on neuronal differentiation, we next investigated the intracellular reactive species production in SH-SY5Y cells treated with RA. The first assay performed was the monitoring of DCFH oxidation, a technique used to analyze the overall production of intracellular RS (Fig. 5a). Proliferative and non-proliferative cells did not present differences in basal RS production. However, RA induces a significant increase in DCFH

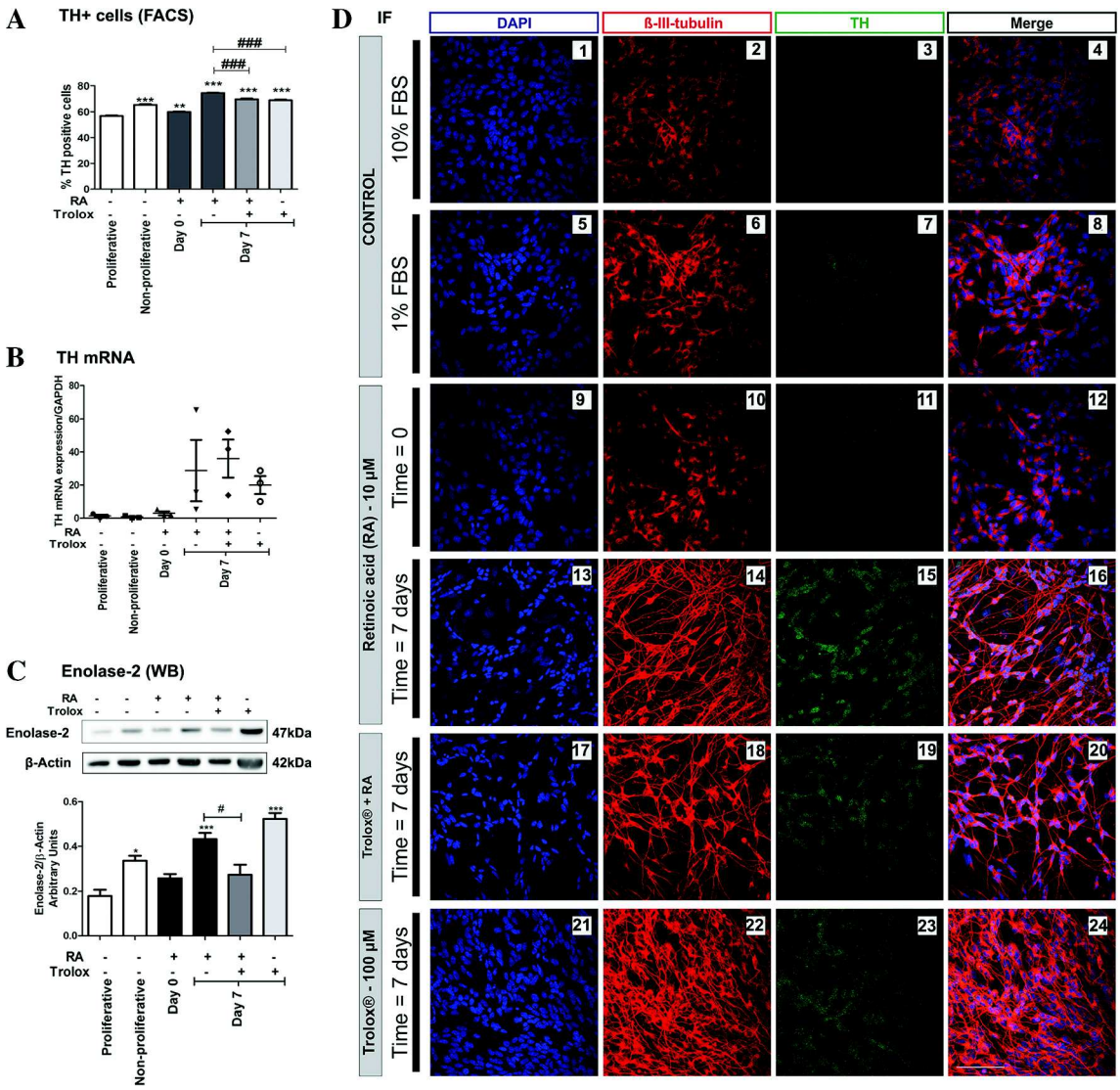
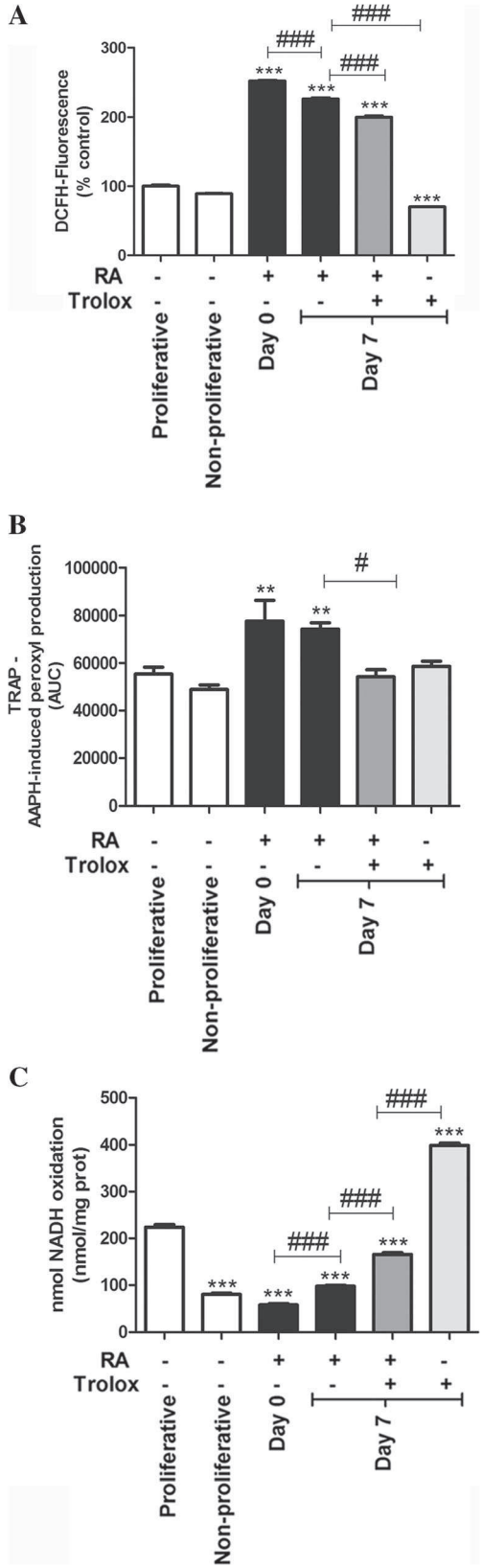


Fig. 4 Effect of antioxidant treatment on expression of mature neuron markers induced by RA differentiation. **a** Mean values quantification of flow cytometry analysis of tyrosine hydroxylase positive cells. Data are mean ± S.E.M. from three independent experiments. **b** Quantification of mRNA expression for catecholaminergic marker tyrosine hydroxylase (TH) after RA differentiation. **c** Western blot analysis of neuron specific marker enolase-2. Values are expressed as mean ± SEM of three independent experiments (n = 3). Statistical difference compared to control was determined by one-way ANOVA followed by Tukey's post hoc test (*single asterisk* indicates different to control 10 % $p < 0.05$; *triple asterisk* indicates $p < 0.001$; *octothorpe* indicates difference between groups $p < 0.05$). **d** Confocal immunofluorescence microscopy of SH-SY5Y cells after RA, RA+Trolox®, and Trolox® treatment for 7 days with respective controls. Tyrosine hydroxylase (*green fluorescence*), DAPI (nuclei), and β-III tubulin (*red fluorescence*) are shown. *Scale bars* represent 10 µm

oxidation rate and this effect was partially inhibited by co-treatment with Trolox®. The second assay used was the total reactive antioxidant potential (TRAP), which gives an indication of the non-enzymatic antioxidant capacity of the cells against RS produced by an artificial generation system. The production of RS by the TRAP system is enhanced by homogenates from cells treated with RA (Fig. 5b), which correlates to the increase in RS production found by DCFH-DA assay. Homogenates from cells co-treated with Trolox® present a significant reversion of this effect, indicating that Trolox® inhibits the pro-oxidant effect of RA. We next evaluated the rate of NADH oxidation by isolated mitochondria, as mitochondrial activity is one of the main sources of RS production in aerobic cells (Fig. 5c). Proliferative cells generally demonstrate higher mitochondrial activity that is related to their increased metabolic demand, and our results confirmed a reduced mitochondrial activity in non-proliferative cells compared to proliferative cells. Cells exposed to RA demonstrated a similar profile of mitochondrial oxidative activity to non-proliferative cells, but co-treatment with Trolox® partially restored the rate of NADH oxidation. Interestingly, Trolox alone® significantly enhanced the rate of NADH oxidation, which may be related to the increased proliferation rate observed in this group earlier. These results, altogether, are



◀ **Fig. 5** Effect of antioxidant co-treatment on reactive species production, non-enzymatic antioxidant profile, and mitochondrial NADH oxidation in cells differentiated with RA. **a** DCF fluorescence monitoring after SH-SY5Y RA, RA+Trolox®, and Trolox® treatment for 7 days and respective controls. **b** The total antioxidant potential (TRAP) evaluation was performed and the area under curve values obtained from kinetic monitoring are demonstrated. Values are expressed as mean ± SEM of three independent experiments (n = 3). **c** SH-SY5Y cells mitochondrial function was analyzed through NADH oxidase assay. Mitochondria were isolated from cells after RA, RA+Trolox®, and Trolox® treatments for 7 days, with respective controls. Statistical difference compared to control was determined by one-way ANOVA followed by Tukey's post hoc test (*single asterisk* indicates different to control 10 % $p < 0.05$; *triple asterisk* indicates $p < 0.001$; *octothorpe* indicates difference between groups $p < 0.05$)

suggestive that the increased RS production observed in cells treated with RA may be a consequence of impaired NADH oxidation by mitochondria, as altered mitochondrial activity is a common source of oxidative stress in cells.

Effect of Antioxidant Treatment on Akt and ERK1/2 Phosphorylation by RA in SH-SY5Y Cells

In previous reports, RA was observed to induce non-genomic, rapid (up to 60 min) activation of Akt and ERK1/2 in SH-SY5Y cells, and neuronal differentiation and cell survival were shown to be dependent on this effect [9, 32]. To investigate whether RS induced by RA influence protein kinases phosphorylation, we evaluated the effect of co-treatment with Trolox®. Cells were pre-incubated with Trolox® for 2 h and then RA was added for different periods. Increases in Akt and ERK1/2 phosphorylation are observed in different periods of RA incubation; Akt phosphorylation was increased 45 min after RA addition while ERK1/2 phosphorylation was enhanced in cells briefly exposed to RA (day zero) and also 15 min later (Fig. 6). The extent of Akt and ERK1/2 phosphorylation with RA was reduced in the presence of Trolox®, thus indicating the participation of RA-induced RS in the mechanism of activation of these protein kinases.

Discussion

For many years, several attempts to transplant neurons and restore the dopaminergic transmission have been performed [12]. The nervous system involves a complex and intrinsic cellular signaling, and our understanding of the molecular mechanisms involved in cell cycle regulation and neuronal differentiation has markedly increased over recent years. In order to elucidate the mechanisms of neuronal differentiation and maturation, different cell models have been used in mechanistic studies. One of these models is the human neuroblastoma SH-SY5Y cell line, which is typically locked in an early neuronal differentiation stage, characterized by low levels of neuronal markers. Upon exposure to appropriate differentiation conditions, SH-SY5Y cells can be driven toward

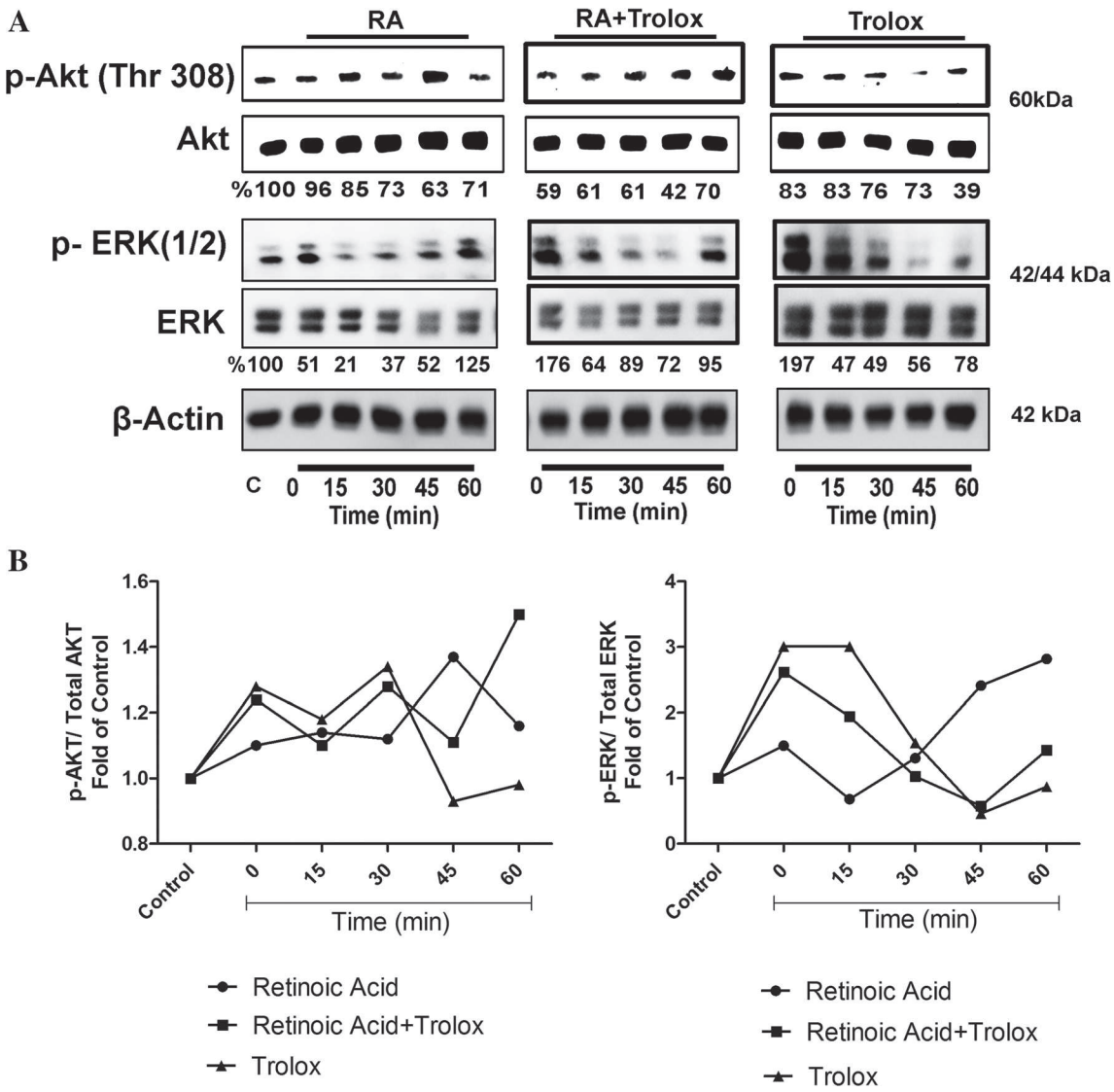


Fig. 6 Effect of antioxidant treatment on Akt and ERK1/2 phosphorylation by RA in SH-SY5Y cells. Cells were pre-incubated for 2 h with Trolox® (AR+T and T groups) and then RA was added (Trolox group). Cells were harvested at 0, 15, 30, 45, and 60 min after RA addition and Akt and ERK1/2 phosphorylation were evaluated by western blot. Mean quantitative values are depicted as **a** percentage of control and **b** fold-induction

differentiated, mature, neuronal phenotypes. In this report, we explored the role of RS during in vitro neurodifferentiation, demonstrating through differentiation of SH-SY5Y cells with RA and the antioxidant Trolox®, that this process is actually dependent on RS production and oxidative stress.

The effects of RA in SH-SY5Y neurodifferentiation are well documented, which include attenuation of proliferative rate and extension of neuritic processes [4]. Nonetheless, proper evaluation of cell cycle progression, cytotoxicity, and cell number are often underestimated, as these parameters rarely are evaluated simultaneously in the same work. In our experiments, we observed considerable changes in cell number and viability, and these features seemed to be conditioned by the concentration of FBS in the medium (which is the major stimulator of proliferation) together with RA, which is the main inducer of biochemical and morphological differentiation in this model. Here, we confirm that long-term exposure to RA markedly reduced proliferation as the cell number decreases, EdU® incorporation (indicative of proliferation) decreases, SRB incorporation (indicative of total cell protein biomass) decreases, and percentage of cells at G0/G1 increases. The SRB incorporation assay, commonly used to assess cell viability, gives an accurate measure of total protein biomass, which is a parameter more closely related to the number of cells under a given condition. Thus, the decrease in SRB incorporation promoted by RA is a consequence of the inhibition in proliferative capacity, but is not related to cytotoxicity/cell death. However, cells co-treated with RA and Trolox® also displayed decreased SRB

incorporation, but increased LDH activity in the medium and this indicates that the decrease in protein biomass is a consequence of cell death. Importantly, cells treated with RA did not show any alterations in morphology or medium LDH activity suggestive of cytotoxicity. However, the co-treatment with Trolox® reduced the proportion of cells under G0/G1 by RA and led to a significant increase in cells at sub-G1 phase, which is associated to cells undergoing apoptotic processes. These observations contribute to the suggestion that antioxidant treatment inhibits the modifications in cell cycle by RA and this process results in cell death. Although LDH activity is commonly associated to necrotic death while cells under sub-G1 state are more associated to apoptosis, these two types of cell death are not mutually exclusive of each other and may take place together in heterogeneous populations of cells. Previous studies demonstrated that RA induction of cellular differentiation is preceded by cell cycle arrest, with a higher percentage of cells in G1 and a reduction of cells in S phase after RA treatment [4, 12]. Our data are consistent with that, as in our model RA treatment maintained SH-SY5Y cells in a post mitotic state, with higher percentage of cells at G0/G1 state, and here we observed that antioxidant treatment did not reverse these cells to proliferative state, as EdU® incorporation was not altered. Altogether, these observations suggest that RS production by RA is a necessary step to drive cells into the differentiated phenotype and that the changes in cell cycle induced by RA may result in cell death if RS production is inhibited.

The capacity of RA to induce differentiation is a well-established protocol in neuroscience research [10]. Here, we confirmed that RA induced morphological alterations to cells, with extension of neuritic processes in N-type cells to form a typical neuronal network phenotype. Importantly, RA induced biochemical changes that are commonly associated to the adult catecholaminergic phenotype, including increased expression of TH, β-III tubulin, and enolase-2, considered markers associated with neurogenesis and neural differentiation [33–35]. Trolox® decreased both the morphological changes and the expression of such markers, indicating that RA-induced differentiation of SH-SY5Y cells requires a pro-oxidant state to take place. In previous works, some evidence that changes in the redox state were required for neurodifferentiation by RA were observed. The induction and activation of Nrf2, which upregulates the transcription of antioxidant enzymes and is considered the major transcription factor in cellular antioxidant response, was demonstrated to be a necessary step in neurite formation and induction of MAP2, a marker of neuritogenesis, in SH-SY5Y cells treated for 4 days with RA [24]. In cells treated with RA for up to 96 h, Nrf2 was shown to exert a prominent role in the promotion of the survival of SH-SY5Y cells against RA cytotoxicity, which suggested that RA induced a pro-oxidant environment during differentiation, causing oxidative stress [36]. Here, we investigated if RS production and consequent oxidative stress induced by RA were necessary events in the full differentiation of SH-SY5Y by evaluating the expression of biochemical markers of adult neuronal phenotype, such as TH (considered the gold standard for catecholaminergic neurons) and enolase-2, as well as β-III tubulin.

Exposure of SH-SY5Y cells to RA substantially increased the level of RS and induced a pro-oxidant state since day zero of incubation, and we found this observation important for several reasons concerning the possible mechanisms of RA action on neuronal differentiation. RA is a byproduct of vitamin A (retinol) metabolism, and it is generally considered to act through the activation of nuclear steroid receptors and retinoid receptors of the RAR/RXR subfamilies. These changes in RS production and pro-oxidant environment occurred well before protein expression changes are likely to have developed and so alternative mechanisms for RA action must be considered. Non-genomic or extra nuclear effects of retinoids have shown that RA can modulate signaling pathways independently of retinoid nuclear receptor activation and gene transactivation [14, 37, 38]. RS production by RA was previously demonstrated to result in increased activity of the antioxidant enzyme catalase [16] and also cause the non-genomic stimulation of MEK1/2-ERK1/2-caspase-3 pathway [15], all events related to oxidative stress responses. Once we observed that RA was creating a pro-oxidant effect in cells and possibly acting through non-genomic mechanisms, we analyzed the phosphorylation of Akt and ERK1/2. Both Akt and ERK1/2 are cell survival pathways and also regulate the differentiation and proliferation. In previous works, the phosphorylation/activation of Akt and ERK1/2 was observed in SH-SY5Y differentiation by RA [9, 32]. Differentiation was completely blocked using inhibitors of PI3K/Akt pathway; however, the use of ERK1/2 inhibitors had no effect on cellular differentiation, although led to cell death [10]. Activation of Akt was demonstrated to be mediated by RAR through a non-classical mechanism of interaction with PI3K [11], confirming the involvement of non-genomic mechanisms of RA action in protein kinase signaling. In our study, brief treatment with RA was able to induce the generation of RS, leading to a pro-oxidant environment that was associated to the increased phosphorylation of Akt and ERK1/2, suggesting that non-genomic actions by RA involving RS production are essential in SH-SY5Y neuronal differentiation. We observed that pre-treatment with Trolox® caused a reduction in Akt and ERK1/2 phosphorylation induced by RA addition. It is important to note that Trolox® alone also increased ERK1/2 phosphorylation in such short periods of incubation and this effect may be related to the increased number of cells in this group. Non-proliferative SH-SY5Y cells, which were exposed only to FBS 1 %, did not show changes in RS production, indicating that serum deprivation did not contribute to the pro-oxidant state observed with RA. Redox-dependent activation of protein kinases including members of the MAPK family and the

PI3K/Akt pathway were observed in other cell types and with different pro-oxidant agents, including retinoids [19, 21, 39, 40].

Long-term exposure of SH-SY5Y cells to RA maintained the level of RS in a pro-oxidant state for 7 days. Retinoids are known to have redox-related properties and influence the oxidant status of the cell [19, 22, 41, 42]. Previous studies showed that RA treatment for 7 days in SH-SY5Y cells alters the redox status of the cell [43], and activates antioxidant enzymes that contribute to endogenous cellular defense (e.g., catalase, superoxide dismutase, and glutathione peroxidase) [22]. RA co-treatment with Trolox® significantly decreased RS production and the pro-oxidant environment generated by RA. The TRAP assay, in which the production of RS (mainly peroxyl radicals) by a synthetic free radical generator system is monitored in the presence of samples depleted from their enzymatic activities, showed that homogenates from cells incubated with RA were able to enhance RS production by this system; however, in DFCH oxidation assay, which is performed with intact living cells, RA also enhances RS production. These assays altogether indicate that both enzymatic and non-enzymatic mechanisms may be involved in RA-induced RS production in our experimental conditions. RA-induced differentiation is therefore dependent on the rapid production and long-term maintenance of RS and pro-oxidant environment, suggesting a redox-dependent mechanism of differentiation. Nonetheless, the exact mechanisms by which RA modulates RS production during neuronal differentiation remain to be better understood. Retinoids and RA itself have been demonstrated to influence the cell redox state by several mechanisms, acting as antioxidants or pro-oxidants in different contexts. Beyond creating a pro-oxidant status in the cell, another consistent feature of exposure to RA is the presence of changes in cellular bioenergetics [23, 44] and mitochondria impairment in response to oxidative stress [45]. Recent evidence indicates that mitochondria might be a key effector in RA-induced differentiation of SH-SY5Y cells [44]. Accordingly, the well-established pharmacological use of RA to treat neuroblastoma is based in its ability to induce cell cycle arrest and apoptosis through Bcl-2 activation [46]. Increased mitochondrial membrane potential, levels of cytochrome c oxidase, MnSOD, and also increased bioenergetic reserve capacity occur after RA-induced differentiation [23, 44]. Furthermore, RA induces the expression of glycolytic pathway proteins, probably due to the increase in energy required for neurodifferentiation [22].

Here, we observed that cells submitted to differentiation with RA decreased their rate of mitochondrial NADH oxidation and this was partially reversed by Trolox®. Importantly, mitochondrial NADH oxidation was significantly decreased in all treatments carried in medium with decreased serum (1 % FBS), if compared to proliferative cells, maintained in 10 % FBS. This result is expected due to the presence of oxidizable energetic substrates in FBS, such as glucose; nevertheless, Trolox® also stimulated NADH oxidation in cells maintained in 1 % FBS for 7 days in the absence of RA. These results, altogether, are suggestive that changes in mitochondrial activity in order to adapt the bioenergetic requirements of the cells to the conditions of serum deprivation are a necessary step prior to RA addition in this model of neuronal differentiation. It is known that differentiated cells in a post mitotic state depend primarily on mitochondria to meet their energetic requirements [23, 44], and we found that long-term treatment with RA maintained mitochondrial NADH oxidation decreased. The reduced mitochondrial activity is likely to be associated with the observed increase in RS production by RA, since oxidative stress can result from cellular respiratory changes that lead to the leakage of electrons from donor redox centers to molecular oxygen [45, 47, 48]. Schneider et al. observed that RA treatment actually increased the mitochondrial capacity and bioenergetic reserve along 5 days of differentiation; importantly, in that study, cells at the day zero of differentiation—therefore maintained 24 h in 1 % FBS before RA addition—were considered as undifferentiated cells, compared to cells under 5-day differentiation with RA [23]. Here, we also observed a significant increase in mitochondrial NADH oxidation rate by cells differentiated with RA for 7 days compared to day zero, although cells maintained at 10 % FBS presented much higher rates of NADH oxidation. It was suggested that differentiation by RA increased the cell resistance against oxidative stress [23], but our findings indicate that oxidative stress is actually an essential step of differentiation. Corroborating this, we observed that RA co-treatment with Trolox® reduced the level of oxidative stress and consequently mitochondrial activity was restored; besides, and more surprisingly, treatment with Trolox® alone substantially improved mitochondrial function, consistent with low levels of RS and the continued proliferative status of the cell. This result suggests that maintaining mitochondrial activity at low levels during differentiation may be an adaptation to the pro-oxidant effect of RA, as treatment with Trolox® for 7 days in 1 % FBS without RA-stimulated mitochondrial activity to levels above proliferative cells. Previous works by us and others demonstrated that Nrf2 is an essential step in RA-induced differentiation [24, 36] and the role of Nrf2 activity on mitochondrial activity during RA-induced neuronal differentiation remains to be better understood. Besides, Trolox® is able to exert specific effects in mitochondrial NADH oxidation due to its intrinsic ability to scavenge RS generated by intracellular metabolism. It was previously observed that, when added alone to cultured cells, Trolox® induces mitochondrial thiol reduction, mitochondrial filamentation and, importantly, increased expression of fully assembled mitochondrial complex I, as well as enhanced citrate synthase activity [49].

In conclusion, findings from this study demonstrate that the pro-oxidant effects of RA are required for the differentiation

of SH-SY5Y cells into an adult neuronal phenotype, expressing markers of mature catecholaminergic neurons such as TH, enolase-2, and β-III tubulin. Moreover, RA-differentiation induced cell cycle withdrawn and reduced mitochondrial NADH oxidation. Co-treatment with Trolox® was able to block the neuronal differentiation, maintaining cells into a proliferative state but ultimately leading to cell death after 7 days, probably due to the serum deprivation. Our results confirm previous findings showing that the antioxidant defense system was essential for RA-induced neuronal differentiation in SH-SY5Y cells, but also highlight that the generation of RS and oxidative stress is an essential step in this process. Since RS and oxidative stress affect the activity of several regulatory pathways through redox-dependent protein modulation, this study may bring new insights on the understanding of neuronal differentiation signaling.

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CAPÍTULO II

Retinol (vitamin A) increases α-synuclein, β-amyloid peptide, tau phosphorylation and RAGE content in human SH-SY5Y neuronal cell line

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Neurochemical Research

Retinol (vitamin A) increases α-synuclein, β-amyloid peptide, tau phosphorylation and RAGE content in human SH-SY5Y neuronal cell line

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Abstract

Retinoids (vitamin A and derivatives) are recognized as essential factors for central nervous system (CNS) development. Retinol (vitamin A) also was postulated to be a major antioxidant component of diet as it modulates reactive species production and oxidative stress in biological systems. Oxidative stress plays a major role either in pathogenesis or development of neurodegenerative diseases, or even in both. Here we investigate the role of retinol supplementation to human neuron-derived SH-SY5Y cells over reactive species production and biochemical markers associated to neurodegenerative diseases expressed at neuronal level in Parkinson's disease and Alzheimer's disease: α-synuclein, β-amyloid peptide, tau phosphorylation and RAGE. Retinol treatment (24 hours) impaired cell viability and increased intracellular reactive species production at the highest concentrations (7 up to 20 µM). Antioxidant co-treatment (Trolox 100 µM) rescued cell viability and inhibited RS production. Furthermore, retinol (10 µM) increased the levels of α-synuclein, tau phosphorylation at Ser396, β-amyloid peptide and RAGE. Co-treatment with antioxidant Trolox inhibited the increased in RAGE, but not the effect of retinol on α-synuclein, tau phosphorylation and β-amyloid peptide accumulation. These data indicate that increased availability of retinol to neurons at levels above the cellular physiological concentrations may induce deleterious effects through diverse mechanisms, which include oxidative stress but also include reative species-independent modulation of proteins associated to progression of neuronal cell death during the course of neurodegenerative diseases.

Keywords: vitamin A; neurotoxicity; SH-SY5Y; RAGE; oxidative stress; neurodegeneration.

Introduction

Although retinoids are recognized as essential factors for development of the central nervous system (CNS), the role of retinol (vitamin A) and its derivatives in the adult brain is still a matter of debate. Traditionally, most actions played by retinol were ascribed to nuclear retinoid receptor signaling, probably because this was the first biological mechanism to be understood (Tafti e Ghyselinck, 2007). Later, retinol was postulated to be a major antioxidant component of diet, and then proposed to influence reactive species metabolism, redox signaling and oxidative stress balancing (Elliott, 2005). These observations led various works to suggest the use of retinoids in antioxidant-based therapies to treat or prevent diseases related to free radical-mediated cell cycle alterations (Goodman *et al.*, 2011). Such oxidative stress-evoked perturbations of cell function are observed in different types of cancer, pro-inflammatory conditions and neurodegenerative diseases (Halliwell e Gutteridge, 2007).

It has long been postulated that oxidative stress plays a major role in either pathogenesis or development of neurodegenerative diseases, or even in both (Niedzielska *et al.*, 2016a). Parkinson's disease (PD) is characterized by selective death of dopaminergic neurons of the nigro-striatal axis and accumulation of histological structures known as Lewy's bodies, which are mainly formed by aggregation of α-synuclein and ubiquitin. Reactive species (RS) produced by both endogenous and exogenous sources strongly enhance α-synuclein-mediated protein aggregation, which results in impairment of neuronal function, neuronal death, and microglia activation (Berrocal *et al.*, 2015; Niedzielska *et al.*, 2016a). In Alzheimer's disease (AD), the most common neurodegenerative condition, oxidative stress is also associated to the hyperphosphorylation of the microtubule-stabilizing protein tau, which results in the formation of neurotoxic histological structures known as neurofibrillary tangles, characteristic of this disease (Mondragon-Rodriguez *et al.*, 2013).

Besides the increase of aberrant phosphorylated forms of tau, AD is also characterized by the enhanced formation and accumulation of β-amyloid peptide, which is released to the extracellular environment in the CNS and activates different extracellular receptors that trigger neurotoxic responses (Nalivaeva e Turner, 2013). Recently, the receptor for advanced glycation endproducts (RAGE) was reported to be one of the main mediators of β-amyloid

neurotoxicity (Yan *et al.*, 2012). RAGE is a multi-ligand receptor that triggers NF-kB-dependent transcription of cytokines and reactive species production commonly associated to diabetes and chronic inflammatory states. There is an increasing body of evidence implicating a major role of RAGE in the main cellular processes observed in AD onset and development, which indicates that RAGE may also play a more general role in neurodegenerative processes not only in this disease, but also in other conditions and during ageing (Cai *et al.*, 2016; Ray *et al.*, 2016).

Based on this relationship among oxidative stress and neurodegenerative processes, antioxidant-based protocols for both prevention and treatment of several neurodegenerative diseases have been proposed (Halliwell, 2006; Niedzielska *et al.*, 2016a). Vitamin A, along with vitamins C and E, as well as other antioxidants commonly found in diet – such as plant polyphenols – are generally considered as "non-harmful" compounds. For this reason, different studies propose pharmacological approaches and diet supplementation aiming to increase the circulating levels of these compounds in the body, as strategies to regulate cell redox homeostasis (Halliwell, 2006). However, in the case of vitamin A and other retinoids, this approach has caused negative results when applied to prevention of some types of cancer and cardiovascular diseases, which suggested that retinol also could induce pro-oxidant effects depending on doses and (Goodman *et al.*, 1993; The Abc-Cancer Prevention Study Group, 1994; Omenn, Goodman, Thornquist, Balmes, Cullen, Glass, Keogh, Meyskens, Valanis, Williams, Barnhart, Cherniack, *et al.*, 1996; Mongan e Gudas, 2007). In the present work, we aimed to evaluate the effect of increasing the cellular availability of retinol on the expression of neuronal markers of PD and AD. For this purpose, we evaluated the effect of retinol on the content of α-synuclein, tau phosphorylation, β-amyloid peptide and RAGE using the human SH-SY5Y neuron-derived cell line as an *in vitro* neuronal model. We also investigated the involvement of reactive species in the effects of retinol in the content of these markers of neurodegeneration and over viability parameters.

Material and Methods

Chemicals

All-trans retinol alcohol, Trolox, 2',7'-dichlorohydrofluorescein diacetate (DCFH-DA), sulphorhodamine B (SRB) , Tween-20 and β-mercaptoethanol were from Sigma Chemical Co. (St Louis, MO, USA). Retinol was dissolved in DMSO. Concentrated stocks were prepared immediately before experiments by diluting retinol into DMSO and determining final stock concentration by UV absorption; solution was kept protected from light and temperature during all procedures. Appropriate solvent controls were performed for each condition (DMSO < 0.1%) Treatments were initiated by adding concentrated solutions to reach final concentrations in the well. The final ethanol concentration did not exceed 0.1% in any experiment. Tissue culture reagents were from Gibco (Invitrogen Corporation, Carlsbad, CA, USA) and were of tissue culture grade. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) reagents were from Bio-Rad Laboratories (Hercules, CA, USA), nitrocellulose membrane (Hybond ECL), enhanced chemiluminescence kit (ECL plus), and anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey) were from Amersham Pharmacia Biotech (Amersham, UK).

Cell Culture and Treatments

Human SH-SY5Y neuroblastoma cell line was obtained from European Collection of Cell Cultures (ECACC). Cells grown in a mixture 1:1 of Ham's F12 and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM of glutamine, 0.28 μg/μL of gentamicin and 250 μg/μL of amphotericin B, in a 5 % CO2 humidified incubator at 37 °C. The culture medium was replaced each 2 days and cells were sub-cultured once they reached 90% confluence. For assays, cells were cultured in 6-well culture plates (western blot experiments) or 96-well culture plates (SRB and DCFH-DA assays) until they reached approximately 70% confluence, then the medium was replaced for the same medium with 1% FBS for 2 hours to minimize the effect of FBS endogenous retinoids. After this period, treatments were performed by adding concentrated stock-solutions of retinol and Trolox. Retinol concentrations used were zero (only vehicle, DMSO 0.1%), 1, 2.5, 5, 7, 10 and 20 μM. Trolox concentration used was 100 μM.

Cellular Viability

SH-SY5Y cells viability was assessed by different assays. MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide) reduction is based on the ability of viable cells to reduce MTT and form a blue formazan product. MTT solution (sterile stock solution of 5 mg/mL) was added to the incubation medium in the wells at a final concentration of 0.2 mg/mL. The cells were left for 45 min at 37 ºC in a humidified 5% CO_2 atmosphere, then medium was removed and plates were shaken with DMSO for 30 min. Optical density of each well was measured at 550 nm (test) and 690 nm. Data were expressed as percentage of the formazan formation compared to untreated cells (control). The second method used to analyze cellular viability was SRB incorporation, a colorimetric assay that estimates cell number by staining total cellular protein. Briefly, cells were fixed by layering 100 µL of ice-cold 40% trichloroacetic acid (TCA) on top of the culture medium and incubated at 4°C for 1 h. Plates were then washed five times with cold water. The excess water was then decanted and the plates left to dry in air. SRB stain (50 µL; 0.4% in 1% acetic acid) was added to each well and left in contact with the cells for 30 min. The cells were then washed with 1% acetic acid, rinsed 4 times until only the dye adhering to the cells was left. The plates were then air-dried and 100 µL of 10 mM Tris base pH 10.5 were added to each well. The plates were gently shaken for 20 min on a gyratory shaker and the absorbance of each well was read at 492 nm. Cell survival was measured as the percentage absorbance compared to the absorbance of control (non-treated cells).

Intracellular Reactive Species Production (DCFH-DA)

Intracellular reactive species production was determined by the DCFH-DA assay, as previously described (Gelain *et al.*, 2011). Briefly, 2×10^4 SH-SY5Y cells were seeded in 96-well plates and 100 µM DCFH-DA dissolved in medium containing 1% FBS was added to each well and incubated for 2 h to allow cellular incorporation. After that, the medium was discarded and fresh medium containing retinol was added, and DCF intracellular fluorescence was monitored for 37 °C for 1 hour and read again after 24 hours in a fluorescence plate reader (Spectra Max M2, Molecular Devices, USA) with an emission wavelength set at 535

nm and an excitation wavelength set at 485 nm. The results were expressed as percentage of DCF fluorescence.

Imunoblotting

To evaluate the immunocontent of α-synuclein, tau phosphorylated at Ser396, β-amyloid peptide and RAGE, SH-SY5Y cells treated with retinol and Trolox were lysed with 2% sodium dodecyl sulfate (SDS) and was resolved in standard SDS-polyacrilamide gel electrophoresis and were transferred to an nitrocelullose membrane (Millipore, Bedford) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Protein loading and electroblotting efficiency was verified through Ponceau S staining, and the membrane was washed with TTBS (Tris 100 mM, pH 7.5, 0.9% NaCl and 0.1% Tween-20). The membrane was blocked in TTBS containing 5 % of non-fat/skim dry milk for 60 minutes at room temperature. Primary antibody was incubated overnight at 4°C and subsequently washed with TTBS. Anti-rabbit or mouse IgG peroxidase-linked secondary antibody was incubated for 2 hours at room temperature, and the membrane was washed again with TTBS. Blots were finally developed and immunoreactivity was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent kit (Thermo Scientific). Densitometric analyses were carried using a CCD camera (GE ImageQuant LAS 4000) and the quantitative analysis was performed using Image J. software. Anti- α-synuclein (Cell Signaling Technology, 4179S, 1:1000), anti β-actin (Sigma-Aldrich, A1978, 1:500), anti-phospho-tau (Ser396) (Cell Signalling Technology, 9632S, 1:1000), anti-tau (Cell Signalling Technology, 4019S, 1:1000), anti-β-amyloid (Cell Signalling Technology, 2454S, 1:1000) and anti-RAGE (Sigma Aldrich, SAB2105049, 1:1000) were used.

Quantitative RT-PCR

Total RNA was extracted from cells according to the manufacturer's instructions (PureLink® RNA mini kit - 12183018A–Life Technologies™). The cDNA was synthesized with the Super Script III Reverse Transcriptase kit (Invitrogen) using 1 µg of total RNA. Quantitative RT-PCR reactions were performed in triplicate for each sample using the Step One Plus thermocycler (Applied-Biosystems) and Power SYBR® Green PCR Master Mix (4367659, Life Technologies™). Reactions were carried out in 25 µL following manufacturer's instructions. Primer sequences are as followed: foward sequence (5'ACTGGTGCTGAAGTGTAAGG3') and reverse (5'CCATTCCTGTTCATTGCCT3') for RAGE; forward sequence (5'CCATGTTCGTCATGGGTGTGAACCA3') and reverse sequence (5'GCCAGTAGAGGCAGGGATGATGTTG3') for endogen gene GAPDH. Quantification was performed using the $2^{-\Delta\Delta CT}$ method.

Protein Assay and Statistical Analysis

Protein content was normalized for western blot samples after determination of cellular total content by Bradford method as previously described (Gelain *et al.*, 2011). Results were expressed as the mean ± SEM of three independent experiments with the number of replicates varying from three to eight according the assay. D'Agostino & Pearson test was applied for normality check. Data were analyzed by one-way analysis of variance (ANOVA) followed by Holm-Sidak's post hoc tests. For experiments with Trolox, two-way ANOVA followed by Sidak's post hoc was performed. Data were analyzed by using GraphPad software® (San Diego, CA; version 5.00). Differences were considered to be significant when $*p<0.05$, $**p<0.01$, $***p< 0.0001$, $****p<0.00001$ and between groups (#p<0.05, ##p<0.01, or ###p< 0.001).

Results

Effects of retinol treatment on cell viability and reactive species production

SH-SY5Y cells were incubated with retinol for 24 hours in medium with reduced serum (1% SFB). Retinol concentrations used in this study varied from levels reported to be in the upper physiological limits - 2.5 and 5 µM - up to concentrations of 10 and 20 µM, which are used in pharmacological treatments (Ross *et al.*, 2001). We observed that retinol at 7, 10 and 20 µM significantly reduced cell viability according the MTT- and SRB-based assays (Fig. 1a and b). Concentrations up to 5 µM did not affect either MTT reduction or SRB incorporation in SH-SY5Y cells, but at 7 µM a decrease in these parameters was observed, indicating decreased number of viable cells compared to control. These results indicate that concentrations of retinol beyond the limit of 5 µM may induce deleterious effects in SH-SY5Y neuronal-like cells.

Previous works demonstrated that supplementation with retinol may increase RS production and oxidative stress *in vitro* and *in vivo*, leading to oxidative damage and cell death (Gelain *et al.*, 2006; De Bittencourt Pasquali *et al.*, 2013). In this context, intracellular reactive species (RS) production was evaluated by the DCFH oxidation assay. The rate of DCFH oxidation was enhanced in cells treated with retinol at 7, 10 and 20 µM for 1 h (Fig. 2a). After 24 of retinol exposure, RS production was even more pronounced at 10 and 20 µM compared to other doses (Fig. 2b). These were the same doses that induced significant effects in terms of cytotoxicity in SH-SY5Y cells. To evaluate if the cytotoxic effect of retinol was dependent on RS production, cells were co-treated with the membrane permeable antioxidant Trolox 100 µM, a hydrophilic synthetic analogue of α-tocopherol. Trolox co-treatment inhibited the loss of cell viability induced by retinol at 10 and 20 µM (Fig. 3a and b), confirming the relationship between RS species production and cytotoxicity induced by retinol in SH-SY5Y cells.

Effects of retinol on α-synuclein, tau phosphorylation, β-amyloid peptide and RAGE

To evaluate the cytotoxic effect of retinol on neuronal-like SH-SY5Y cells, we used retinol at 10 µM as, besides having a pro-oxidant and cytotoxic effect, this dose is widely used in cell studies to mimic a condition of increased retinol availability through dietary or pharmacological supplementation (Lengyel *et al.*, 2014; Owusu e Ross, 2016). As mentioned earlier, the increase in α-synuclein is a cellular hallmark of PD, while β-amyloid peptide accumulation and aberrant tau phosphorylation are cellular characteristics of AD and other diseases associated to protein misfolding. Cells treated with 10 µM retinol for 24h had increased levels of α-synuclein and β-amyloid peptide (Fig. 4a and b). To assess whether this was mediated by RS production, the effect of Trolox upon these parameters was evaluated. The antioxidant co-treatment did not affect the increase in α-synuclein and β-amyloid content induced by retinol (Fig. 4a and b). We also evaluated the content of tau phosphorylated at Ser396 in neuronal SH-SY5Y cells. Retinol led to increased phosphorylated tau and antioxidant co-treatment with Trolox did not change this effect (Fig. 4c).

Recently, induction of RAGE expression had been implicated in the pathogenesis and/or progression of AD, PD and other processes through sustained inflammation (Sathe *et al.*, 2012; Teismann *et al.*, 2012; Cai *et al.*, 2016). In the adult neurons, RAGE expression is normally repressed, but oxidative insults and transient inflammation are able to induce RAGE expression, thus triggering a cascade of events that may lead to chronic neuroinflammation and neurodegeneration (Ray *et al.*, 2016). Retinol led to an increase in RAGE content and this effect was prevented by antioxidant co-treatment with Trolox, suggesting the involvement of RS production in this effect (Fig. 5a). Oxidative stress may affect protein expression by several mechanisms, including regulation of mRNA expression and proteolytic degradation; thus RAGE mRNA expression was evaluated by quantitative RT-PCR. Retinol caused an increase in RAGE mRNA levels, but this effect was not inhibited by Trolox (Fig. 5b). This suggests that RS production by retinol is important to maintain RAGE protein levels constantly enhanced, while retinol-mediated RAGE mRNA up-regulation is not dependent on RS production.

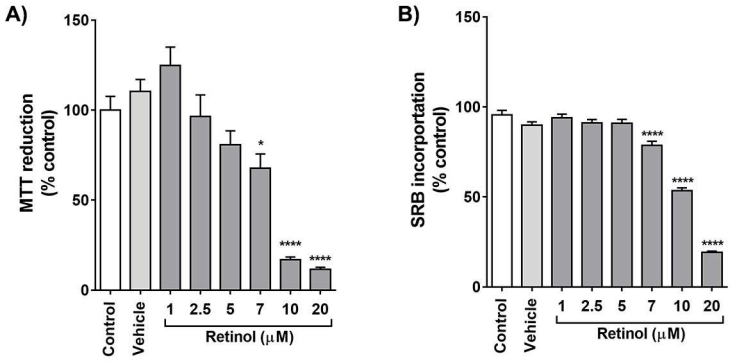

Fig. 1. *Effect of retinol on cell viability.* (A) MTT reduction in cells treated with different concentrations (1 to 20 μM) of retinol for 24 hours. Vehicle is DMSO 0.1%. Data were expressed as percentage of the formazan formation in control (untreated) cells. (B) SRB incorporation in cells treated with increasing concentrations of retinol for 24 hours. Vehicle is DMSO 0.1%. Graphs depict mean ± S.E.M. Data obtained from three independent experiments (8 replicates in each experiment). Data analyzed by one-way ANOVA followed by Holm-Sidak's post hoc test. *p<0.05 and ****p<0.00001 compared to control.

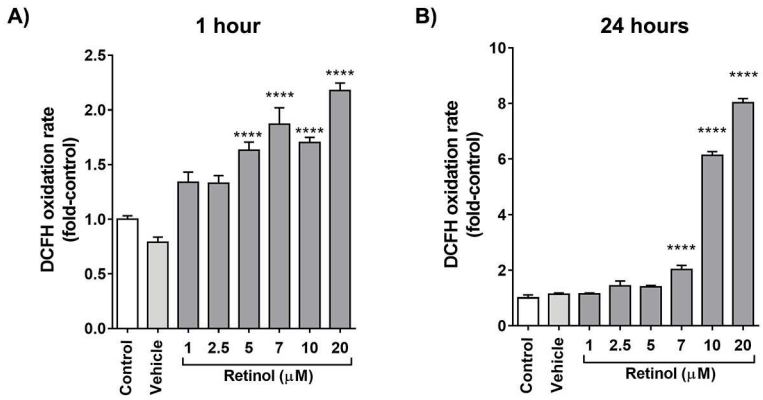

Fig. 2. *Effect of retinol on cell RS production.* SH-SH5Y cells were previously loaded with DCFH and retinol was added later (see material and methods). DCF fluorescence was monitored (A) 1 hour and (B) 24 hours after retinol addition to cells. Vehicle is DMSO 0.1%. Graphs depict mean ± S.E.M. Data obtained from three independent experiments (8 replicates in each experiment). Data analyzed by one-way ANOVA followed by Holm-Sidak's post hoc test. ****p<0.00001 compared to control.

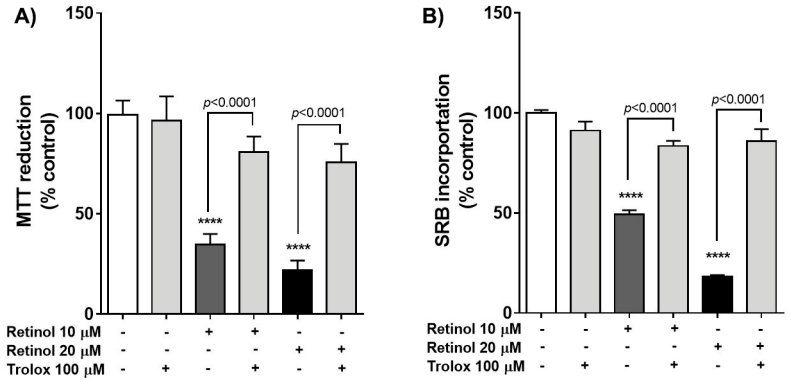

Fig. 3. *Effect of antioxidant co-treatment on retinol induced cytotoxicity*. SH-SY5Y cells were treated for 24 hours with retinol at either 10 or 20 µM in the presence or absence of the cell permeable antioxidant Trolox 100 µM. Cell viability was assessed by (A) MTT reduction and (B) SRB incorporation. Data are mean ± S.E.M from three independent experiments (8 replicates in each experiment). Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ****p<0.00001 vs control; p values from comparison between selected groups are indicated.

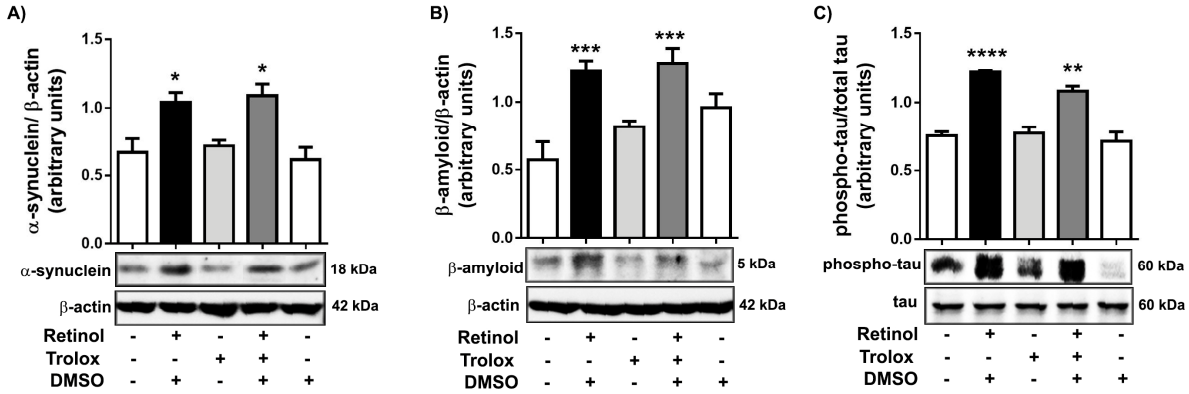

Fig.4. *Content of α-synuclein, β-amyloid peptide and tau phosphorylated at Ser396 in cells treated with retinol.* Cells were treated with retinol 10 µM with or without Trolox 100 µM for 24 hours. The content of (A) α-synuclein and (B) β-amyloid peptide was evaluated by western blot and normalized in relation to β-actin content. (C) Phospho-tau western blots were normalized in relation to total tau. Representative blots are shown below graphs. Values are expressed as mean ± SEM of three independent experiments (3 replicates in each experiment). Data were analyzed by two-way ANOVA followed by Sidak's post hoc test. ***p<0.0001, **p<0.001 and *p<0.005 compared to control.

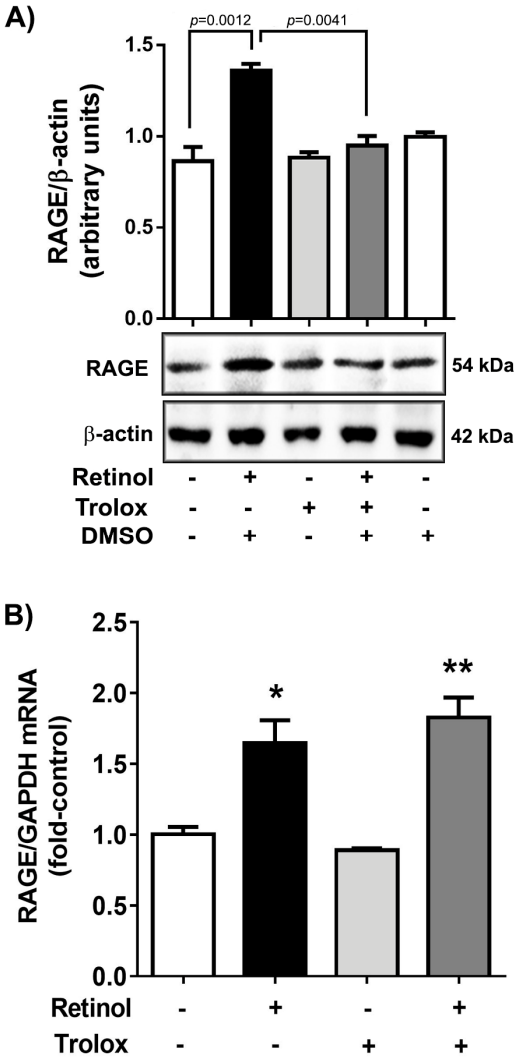

Fig.5. *Effect of retinol on RAGE protein and mRNA expression*. Cells were treated with retinol 10 µM with or without Trolox 100 µM for 24 hours. (A) RAGE protein content was assessed by western blot and normalized in relation to β-actin content. Representative blots are shown in panel below graph. (B) RAGE mRNA expression was assessed by qPCR-RT and GAPDH was used as internal marker. Values are expressed as mean ± SEM of three independent experiments (3 replicates in each experiment). Data were analyzed by two-way ANOVA followed by Sidak's post hoc test. *p<0.05 and **p<0.001 compared to control; p values from comparison between selected groups are indicated.

Discussion

Control of the cellular redox balance became one of the main goals in therapeutic strategies against neurodegenerative diseases. As result, the search for compounds with a potential antioxidant action at CNS level has increased. As mentioned earlier, the focus of most studies on the role of vitamin A on adult brain is on its role as either antioxidant or regulator of gene transcription through retinoid nuclear receptors. However, as observed here, retinol may exert pro-oxidant effects at increasing concentrations. Retinoids, as other compounds commonly referred to as "antioxidants", must be more accurately defined as a "redox-active" molecule. Interaction of redox-active compounds with other electron donors or acceptors may influence the oxidative balance of a given environment in several possible ways, which will vary according the molar ratio between each component, their current oxidation state, and the presence of other redox catalysts. Redox catalysts in brain include catecholamines, quinones, iron, zinc and copper, which in neurodegenerative conditions have increased availability for redox reactions as they are released in protein-free forms from damaged cells (Halliwell, 2006).

Antioxidants present in diet, such as retinol, have been suggested to be included in the development of protocols to treat or prevent neurodegeneration and other oxidative stress-related diseases (Fang *et al.*, 2002; Le Prell *et al.*, 2007; Mongan e Gudas, 2007). On the other hand, our results show that at concentrations above the physiological range observed in cells (0.2 – 5 µM (Ross *et al.*, 2001)), retinol enhances RS production and cytotoxicity to SH-SY5Y neuronal cell line. It is not surprising that compounds generally considered "antioxidants" present a dualistic effect in biological systems. We have previously observed that retinol may act as a pro-oxidant agent in rat Sertoli cells (Gelain *et al.*, 2006), bovine adrenal chromaffin cells (Gelain *et al.*, 2007), PC12 cells (Gelain e Moreira, 2008) and in animal models (Pasquali, Gelain, De Oliveira, *et al.*, 2009; Pasquali, Gelain, Oliveira, *et al.*, 2009). In all these systems, this pro-oxidant effect was observed as increasing concentrations of retinol were available to cells. Retinol availability to cells may be greatly stimulated by supplementation or pharmacological protocols that use administration of "mega-doses" (Imdad *et al.*, 2011; Mayo-Wilson *et al.*, 2011). In the past, clinical studies evaluating oral supplementation with retinol and β-carotene had to be discontinued due to increased lung

cancer incidence and cardiovascular disease, which are conditions strongly related to oxidative stress (Goodman *et al.*, 1993; The Abc-Cancer Prevention Study Group, 1994; Omenn, Goodman, Thornquist, Balmes, Cullen, Glass, Keogh, Meyskens, Valanis, Williams, Barnhart e Hammar, 1996). Long-term effects at CNS were not evaluated in these subjects. However, retinol-based supplements and supplemented foods are still available without any kind of restriction or special recommendations in practically all countries.

The relationship between α-synuclein accumulation and progression of neuronal damage in the course of PD is widely reported (Berrocal *et al.*, 2015). Since early suggestions that oxidative stress plays an important role in PD, some studies observed that free radical-mediated reactions constitute essential steps in α-synuclein aggregate formation (Niedzielska *et al.*, 2016a). Antioxidants were reported to prevent neuronal damage by blocking the conjugation between dopamine to α-synuclein, a process resulting in protofibril formation and neurocytotoxicity (Halliwell, 2006). Therapeutic strategies aiming to maintain the redox balance in cells of the CNS have been proposed to treat or prevent PD and dietary antioxidants such as vitamins C and E are suggested to play a role in PD development (Paraskevas *et al.*, 2003). Nonetheless, we observed that cytotoxic doses of retinol also led to increased α-synuclein in SH-SY5Y cells. Although deleterious effects caused by α-synuclein in PD are commonly associated to its abnormal aggregation into Lewy's bodies, increased expression and accumulation are essential steps prior to this process of aggregation (Berrocal *et al.*, 2015). Interestingly, the effect of retinol on α-synuclein was not prevented by antioxidant treatment with Trolox, which was unexpected, as most deleterious effects of retinol at cellular level observed so far were associated to oxidative stress. This suggests that retinol is able to influence α-synuclein protein turnover by a mechanism that is not associated with RS production, although oxidative stress is present in these experimental conditions. Previously, it was observed that oral supplementation with vitamin A led to oxidative stress and increased α-synuclein in the nigro-striatal axis of rats, and this latter effect was attributed to oxidative stress (De Oliveira, Oliveira, Behr, Hoff, *et al.*, 2009). On the other hand, our data indicate that the effect of retinol upon this parameter is not dependent on RS production. Modulation of α-synuclein gene expression by retinol or other derivatives through genomic mechanisms was not reported so far, but this could be assessed in future studies.

In AD a number of studies reported a relationship between oxidative stress and activation of signaling pathways leading to aberrant tau phosphorylation, a cell hallmark of this disease (Mondragon-Rodriguez *et al.*, 2013). Retinoids were suggested for development of therapeutic strategies against AD (Lee *et al.*, 2009), and retinoic acid was observed to inhibit amyloid deposition in a transgenic animal model of the disease (Ding *et al.*, 2008). Tau is phosphorylated at approximately 25 different sites through the action of diverse protein kinases; nonetheless, Ser396 phosphorylation is located in an epitope recognized by specific antibodies against paired helical filaments and has been referred to as one of the earliest steps leading to neurofibrillary tangles formation in AD and Down syndrome (Mondragon-Rodriguez *et al.*, 2014). We observed here that incubation of SH-SY5Y cells with pro-oxidant concentrations of retinol induced the increase in phosphorylation of tau at Ser396. It is known that aberrant forms of tau disrupt the neuronal homeostasis by enhancing misfolded protein aggregates, thus reinforcing neurofibrillary tangles formation. This further contributes to microglia activation and pro-inflammatory cytokines release, which in turn stimulates RS production and establishes a neurocytotoxic feedback cycle (Smith *et al.*, 2007). The β-amyloid peptide exerts an important role in this process as it may activate membrane receptors (such as RAGE) that trigger cytotoxic signaling pathways, as well as undergo a process of oligomerization and formation of amyloid (senile) plaques, which accumulate in the extracellular space. Both processes result in activation of microglia, which tends to enhance RS production through pro-inflammatory activation and further enhance this cycle (Nalivaeva e Turner, 2013). This neuroinflammatory axis is also thought to be a consequence of RAGE up-regulation, as the expression of this receptor is known to be stimulated by pro-inflammatory cytokines and protein/peptide-carbohydrate aggregates (Maczurek *et al.*, 2008). RAGE activation enhances the NF-kB-mediated up-regulation of pro-inflammatory cytokine expression, thus contributing for chronic neuroinflammation. Recently, overwhelming evidence has been indicating a prominent role for RAGE in AD. The β-amyloid peptide was found to be a RAGE ligand/activator, and many neurotoxic signal pathways associated to cellular degeneration were observed to be triggered by extracellular β-amyloid peptide in a RAGE-dependent fashion (Yan *et al.*, 2012). RAGE was also reported to mediate the transport of systemic β-amyloid peptides across the blood-brain barrier, thus increasing brain amyloid deposition (Candela *et al.*, 2010). Besides, an increase in the immunocontent of RAGE was reported to be associated to the increase of other markers of AD in human brains (Valente *et al.*, 2010).

Oxidative stress is extensively associated to tau aberrant phosphorylation and RAGE up-regulation (De Bittencourt Pasquali *et al.*, 2013; Mondragon-Rodriguez *et al.*, 2013; Mondragon-Rodriguez *et al.*, 2014). As mentioned earlier, oxidative stress exerts an important role in AD-associated neurodegeneration. In turn, aberrant phosphorylation of tau, with consequent formation of paired helical filaments and neurofibrillary tangles, induces a pro-oxidant state by causing the microglia-dependent activation of RS production (Schmitt *et al.*, 2012). Administration of retinoic acid, a metabolite of retinol, decreased the formation of tau aggregates in a triple transgenic AD mouse model (APPswe/PS1M146V/tauP301L) (Watamura *et al.*, 2016). Here, retinol stimulated tau phosphorylation but, surprisingly, this effect was not mediated by the increase in RS production that is responsible for the loss of cell viability. Although the mechanism by which retinol induces this effect is not clear, the induction of neuronal differentiation with retinoic acid and BDNF to SH-SY5Y cells was previously observed to enhance tau expression and phosphorylation (Jamsa *et al.*, 2004). In that study, it was observed that GSK3β and Cdk5 played important roles in the effect induced by retinoic acid. Tau phosphorylation is affected by a myriad of different protein kinases, and retinol may be inducing this effect through a similar mechanism, as it was previously demonstrated to activate different protein kinase-dependent signaling pathways (Gelain *et al.*, 2011; De Bittencourt Pasquali *et al.*, 2013).

RAGE up-regulation in response to oxidative stress was observed in different diseases characterized by increased RS production, including diabetes, atherosclerosis and AD (Pugazhenthi *et al.*, 2016). In AD, RAGE is believed to contribute in maintaining an elevated state of RS production by stimulating NADPH oxidase (Kojro e Postina, 2009), enhance tau phosphorylation via the ERK1/2-GSK3β pathway (Li *et al.*, 2011; Barroso *et al.*, 2013) and amyloid translocation through blood-brain barrier, as mentioned earlier. In the course of AD, RAGE was implicated in the activation of the innate immune system, which is an important component in the progression of neurodegeneration, since local pro-inflammatory stimulation contributes to neuronal death. The involvement of RAGE in the pathogenesis and/or progression of other neurodegenerative conditions is poorly explored. In PD, the expression of RAGE and its ligands has been studied only very recently. The RAGE -429T/C polymorphism was associated with increased PD susceptibility in a Chinese ethnic group

(Han population), and the CC genotype of -429T/C was observed as a protective factor in the same study (Gao *et al.*, 2014). Ablation of RAGE or its ligand S100B showed to be protective in the MPTP-induction of PD model in rodents, together with alterations in the levels of S100B and HMGB1 in PD patients, were also suggestive for a prominent role for RAGE in the course of this condition (Sathe *et al.*, 2012; Teismann *et al.*, 2012; Santoro *et al.*, 2016), Here, we observed that pro-oxidant concentrations of retinol led to an increase in α-synuclein, tau phosphorylation and β-amyloid, but antioxidant treatment did not affect this effect; however, the increase in RAGE was blocked by Trolox, indicating the involvement of RS production in RAGE stimulation. RAGE expression may be induced by several distinct mechanisms, including oxidative stress, increased availability of RAGE ligands and pro-inflammatory mediators (Ray *et al.*, 2016). Our results are suggestive that high concentrations of retinol may induce deleterious effects to neurons via multiple mechanisms, which may (or not) be dependent on RS production. In a previous work, our group showed that incubation of A549 human lung cancer cell line with retinol at similar conditions also increased RS production and oxidative stress, but these effects were responsible for RAGE down-regulation (De Bittencourt Pasquali *et al.*, 2013). In lungs, RAGE is expressed in a constitutive fashion at physiological conditions, and deleterious processes such as oxidative stress or pro-inflammatory stimuli downregulate RAGE expression, as opposite to other cells (Queisser *et al.*, 2008; Creagh-Brown *et al.*, 2010).

Regarding the relationship between retinol supplementation and cellular retinol concentrations, there are some controversies to be pointed. It is a consensus that increased retinol intake results in increased retinol concentrations inside cells, but dynamics of retinol metabolism in mammalian cells turns difficult to establish an accurate relationship between oral intake dosage and plasma levels (Penniston e Tanumihardjo, 2006). Retinol, as a hydrophobic molecule, is carried by serum retinol-binding proteins (SRBP's) between tissues, and may be stored inside cells bound to cellular retinol-binding proteins (CRBP's) or esterified to fatty acids. In situations characterized as hypervitaminosis A, the serum levels of retinol are not different from well-fed, normal individuals, but the levels or other retinoids are markedly increased (Penniston e Tanumihardjo, 2006). For this reason, although it is a consensus that the availability of retinol and its derivatives is increased to cells during hypervitaminosis or supplementation, serum retinol quantification is not considered a reliable method for determination of individual vitamin A status. On the other hand, it is well-

established that dietary vitamin A deficiency causes neurologic deficits in both animal models and humans (Etchamendy *et al.*, 2003; Penniston e Tanumihardjo, 2006), and that oral supplementation at high doses to rats induces oxidative damage to brain and cognitive impairment (Schnorr *et al.*, 2011). Thus, it is clear that both deficit and excess of dietary retinol influence cell homeostasis and function, and that oral supplementation of retinol may increase the cellular concentrations of this vitamin to harmful levels.

Concluding, in the present study we show evidence demonstrating that increasing retinol concentrations to neuron-like SH-SY5Y cells enhanced RS production, cytotoxicity and increased the immunocontent of α-synuclein, tau phosphorylation, β-amyloid peptide and RAGE. The effects of retinol upon biochemical hallmarks of PD (α-synuclein) and AD (tau phosphorylation and β-amyloid peptide) were not dependent on RS production, but RAGE up-regulation and loss of cell viability were rescued by antioxidant treatment with Trolox. These results show that retinol is capable of modulating markers of neurodegeneration at cellular level through different mechanisms, some of which are related to oxidative stress. Altogether, these results indicate that increasing the availability of retinol to neuronal cells may lead to deleterious effects and contribute to neurodegeneration.

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CAPÍTULO III

Resultados de trabalho *in vivo*-

Vitamina A (retinol) como tratamento preventivo na neurodegeneração induzida por 6-hidroxidopamina em ratos

Introdução

A vitamina A é um importante nutriente para o desenvolvimento do sistema nervoso central, devido a suas propriedades de diferenciação, crescimento e proliferação celular (Maden, 2007). Ainda mais, a vitamina A e seus derivados – os retinóides-, são utilizados no tratamento de diversas condições, como problemas de pele e tumores, como câncer de pele, mama, pulmão e hepático (Doldo *et al.*, 2015). Por muitos anos a vitamina A foi postulada como antioxidante a doses baixas. Nos últimos anos efeitos pró-oxidantes dos retinóides demonstraram que a vitamina A é uma molécula redox ativa, e seus efeitos dependem do estado redox do ambiente, como por exemplo, a capacidade de defesa antioxidante celular e a qualidade da função mitocondrial (De Oliveira, Lorenzi, *et al.*, 2011). Como potencial agente antioxidante, a vitamina A, assim como outras vitaminas, vem sendo sugeridas como tratamento ou prevenção de doenças neurodegenerativas (Paraskevas *et al.*, 2003). Nosso grupo demonstrou que doses terapêuticas de vitamina A (1000-9000IU/Kg/dia) tiveram efeito pró-oxidante em algumas regiões do cérebro, com dano mitocondrial e aumento de α-sinucleína na substância negra e estriado (De Oliveira, Oliveira, Behr, Hoff, *et al.*, 2009). No entanto, faltam estudos apontando o efeito da suplementação com vitamina A em doses recomendadas frente ao dano parkinsoniano. Assim, no presente estudo nós investigamos o efeito da vitamina A, em doses diárias recomendadas de 3000IU/Kg dia^{-1} por 28 dias frente ao dano induzido por 6-hidroxidopamina, no soro, LCR e substância negra (SN). Pré-tratamento com vitamina A antes da indução do dano parkinsoniano reduziu o quadro inflamatório e teve efeito protetor no cérebro. Os presentes resultados sugerem o papel das vitaminas lipossolúveis, especificamente retinóides derivados do palmitato de retinol, na prevenção de neurodegeneração induzida ao eixo nigro-estriatal (sistema dopaminérgico) do cérebro.

Metodologia

Animais

Ratos Wistar adultos (280-300g) obtidos da nossa própria colônia foram mantidos em caixas em grupos de 5 animais. Água e comida foram mantidas *ad libidum*, e os animais mantidos em ciclo de 12 horas claro-escuro (7:00- 19:00 h), a temperatura controlada (23±1 °C). Todos os experimentos foram realizados de acordo com o Guia Nacional de Saúde de Cuidados no

Uso de Animais em Laboratório (NIH publication number 80-23 revised 1996), assim como as recomendações de cuidados animais da Sociedade Brasileira de Neurociências e Comportamento. O protocolo de pesquisa foi aprovado pelo Comitê de Ética para experimentação animal da Universidade Federal do Rio Grande do Sul.

Tratamento

Antes de iniciar o tratamento, os animais passaram por um período de 10 dias de habituação à manipulação. Após este período, os animais foram tratados uma vez ao dia durante 28 dias com o uso de uma agulha de gavagem com palmitato de retinol (forma lipossolúvel). Os tratamentos foram realizados à noite (quando os animais estavam mais ativos e consumiam maior quantidade de comida) a fim de garantir uma máxima absorção da vitamina A, uma vez que essa vitamina é melhor absorvida durante uma refeição. Os animais foram tratados com veículo (óleo mineral; n=30 animais) ou 3000 IU/kg de palmitato de retinol (n=30 animais) via oral utilizando gavagem, em um volume máximo de 0,6mL. Medidas adequadas foram tomadas para minimizar a dor e o desconforto (Figura 1).

Cirurgia Estereotáxica

Os ratos foram anestesiados com uma única dose de ketamina e xilasina via intraperitoneal e fixados no aparelho estereotáxico. Uma solução de 2µL de 6-OHDA (Sigma-Aldrich; St. Louis, MO) (10µg, contendo 0,01% ácido ascórbico), foi injetada na substância negra (lado direito), nas seguintes coordenadas AP -5.0mm, DV -8.0mm, ML+2.1mm a partir do bregma. Os animais controles passaram pelo procedimento, mas não foi injetada nenhuma solução após a perfuração com a agulha. A taxa de liberação da 6-OHDA foi mantida por um modulador a 0,5µL/min. Após toda solução ter sido injetada no cérebro, a agulha permaneceu no cérebro por mais 5 minutos.

Análise Comportamental

Duas semanas após a injeção de 6-OHDA o equilíbrio motor foi verificado através do aparelho rotarod. Os ratos foram treinados através de 3 sessões de 5 minutos e ambientados por 2 horas antes da análise. Os animais foram alocados no aparelho (18rpm) e o tempo de latência que o animal permaneceu no rotarod foi automaticamente gravado, e novamente cada

animal teve 3 chances no aparelho. Resultados foram expressos como a média do tempo de latência nas 3 tentativas.

*ELISA (*enzyme linked immunosorbent assay*)*

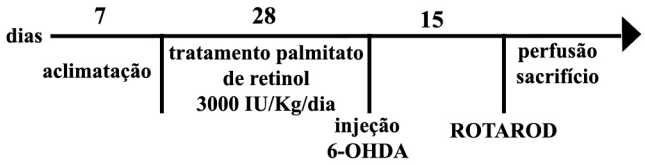
O ensaio ELISA indireto foi realizado para analisar mudanças no conteúdo de TNF-α e IL-1β utilizando soro como amostra. As amostras foram dispensadas em placas de 96 poços e mantidas sob incubação por 24 horas. As placas foram então lavadas com uma solução de lavagem (PBS com 0,05%Tween-20), e então anticorpos específicos foram adicionados e incubados por mais 24 horas (anti-TNF-α e anti-IL-1β; 1:1000). As placas foram novamente lavadas e incubadas com anticorpo secundário por 2 horas (anti-rabbit; 1:1000).

Imunohistoquímica

Ratos foram perfundidos através de *clamp* da aorta do sistema vascular descendente. Foi administrada salina estéril durante 10 minutos, seguidos de 10 minutos com solução de paraformaldeído (PFA) 4% in PBS (7,4). Os cérebros foram cuidadosamente extraídos e mantidos em PFA 4% por 24 horas a 4ºC, depois passados para solução de sucrose 15% por 24 horas a 4ºC e então passados a solução de sucrose 30% e mantidos a 4ºC. Os cérebros foram secos e congelados a -20ºC. Depois de 24 horas a substânia negra foi seccionada em fatias de 20µm utilizando um aparelho criostato a -20ºC (Jung Histoslide 2000R; Leica; Heidelberg, Germany). Um total de 20 a 30 fatias da substância negra foram coletadas e armazenadas em PBS contendo Triton 100x 0,1% (PBS 0,1%). As fatias foram incubadas com albumina 5% durante 2 horas para bloqueio de ligações não-específicas. O anticorpo primário foi incubado durante 48 horas a 4ºC. Os anticorpos foram diluídos em PBS contendo albumina sérica bovina (2%). Depois de 4 lavagens com PBS 0,1%, as fatias foram incubadas com anticorpo secundário diluído em PBS 2% albumina. Depois de uma hora a temperatura ambiente, as fatias foram lavadas várias vezes em PBS 0,1%, transferidas para lâminas gelatinizadas, montadas com FluorSaveTM (345789 – Merck Millipore; MA, USA), e finalizadas com lamínulas. As imagens foram obtidas com Microscópio EVOS_ FL Auto Imaging System (AMAFD1000 –Thermo Fisher Scientific; MA, USA).

Análise Estatística

A análise estatística foi realizada através do software GraphPad 5.0. Para múltiplas comparações foi utilizado ANOVA seguido de Tukey ou Bonferroni *post hoc*. Os resultados foram expressos como média ± erro médio padrão. O conteúdo protéico foi mensurado através da técnica de Bradford em todos os ensaios. As diferenças foram consideradas significativas quando $*p<0.05$, $**p<0.01$, $***p< 0.0001$, ****p<0.00001 and between groups (#p<0.05, ##p<0.01, or ###p< 0.001).



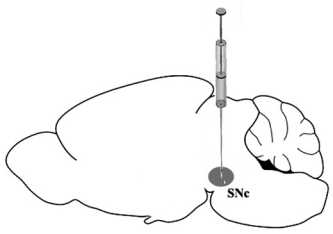


Figura 1: Desenho esquemático da metodologia utilizada. Os ratos foram pré-tratados por 28 dias com palmitato de retinol, logo após foram submetidos ao dano com 6-hidroxidopamina. Após 15 dias o parkisonismo foi avaliado através do teste comportamental rotarod, e após os animais foram sacrificados e os tecidos coletados para posterior análise.

Resultados

Comportamento e Caracterização do Dano por 6-OHDA

A vitamina A conseguiu reverter o efeito neudegenerativo/tóxico da 6-hidroxidopamina através da análise comportamental do rotarod, em que os animais previamente tratados com vitamina A mantiveram-se por mais tempo no aparato, quando comparados com os animais que somente receberam a 6-OHDA. Foi possível confirmar através de análise da tirosina hidroxilase por imunohistoquímica a indução do modelo de dano por 6-OHDA, já que os ratos tratados somente com a toxina tiveram considerável redução no número de neurônios dopaminérgicos.

Quadro Inflamatório Sistêmico

A administração prévia de vitamina A foi capaz de controlar o quadro inflamatório desenvolvido pelo dano, e mais ainda reverteu os níveis de citocinas pró-inflamatórias (TNF-α e IL-1β) no soro de animais quando comparado ao grupo que somente recebeu a injeção de 6-OHDA.

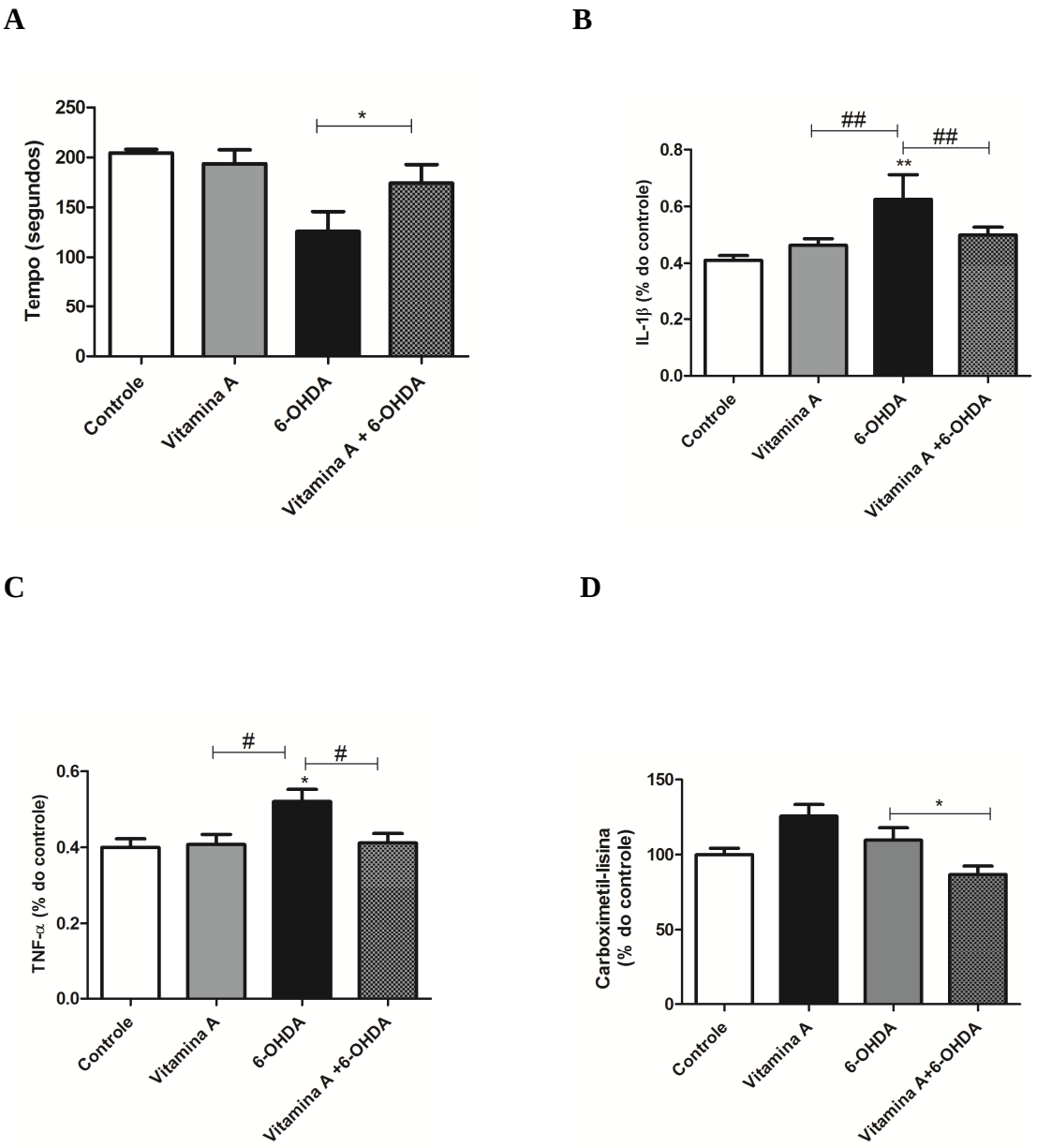


Figura 2: Efeito do pré-tratamento com vitamina A em teste comportamental, citocinas séricas e análise de líquor. A) Comportamento motor dos ratos após a lesão com 6-OHDA analisando através do Rotarod. Foi analisado o tempo de latência dos animais (n=10). B) Análise da citocina pró-inflamatória IL-1β no soro dos animais (n=8). C) Análise da citocina pró-inflamatória TNF-α no soro dos animais (n=8). D) Análise do age Carboximetil lisina no líquor dos animais (n=6).

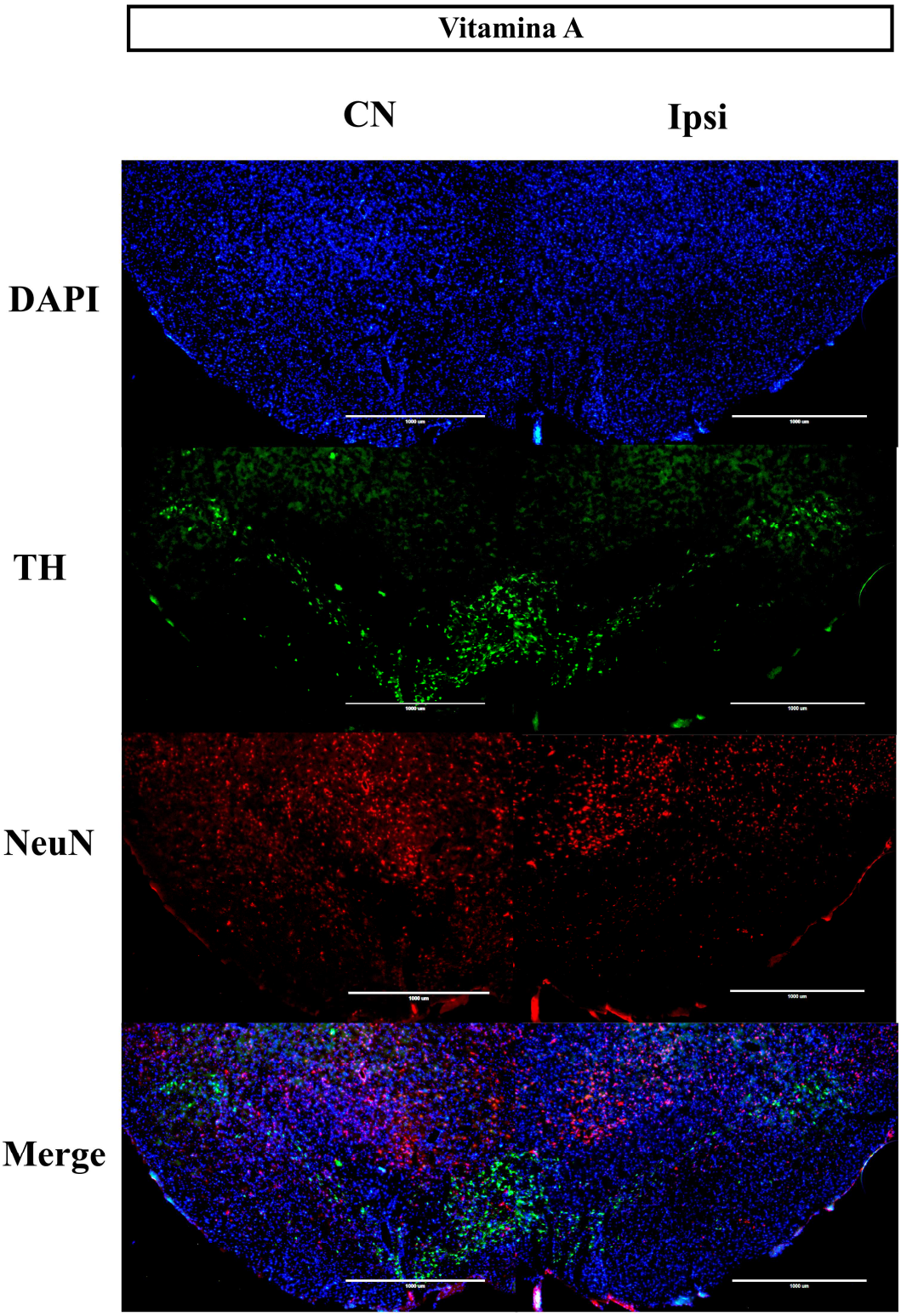


Figura 3: Efeito do pré-tratamento com vitamina A na substância negra de ratos (n=2). Visualização de imunofluorescência de tirosina hidroxilase e NeuN. Dapi foi utilizado para coloração do núcleo.

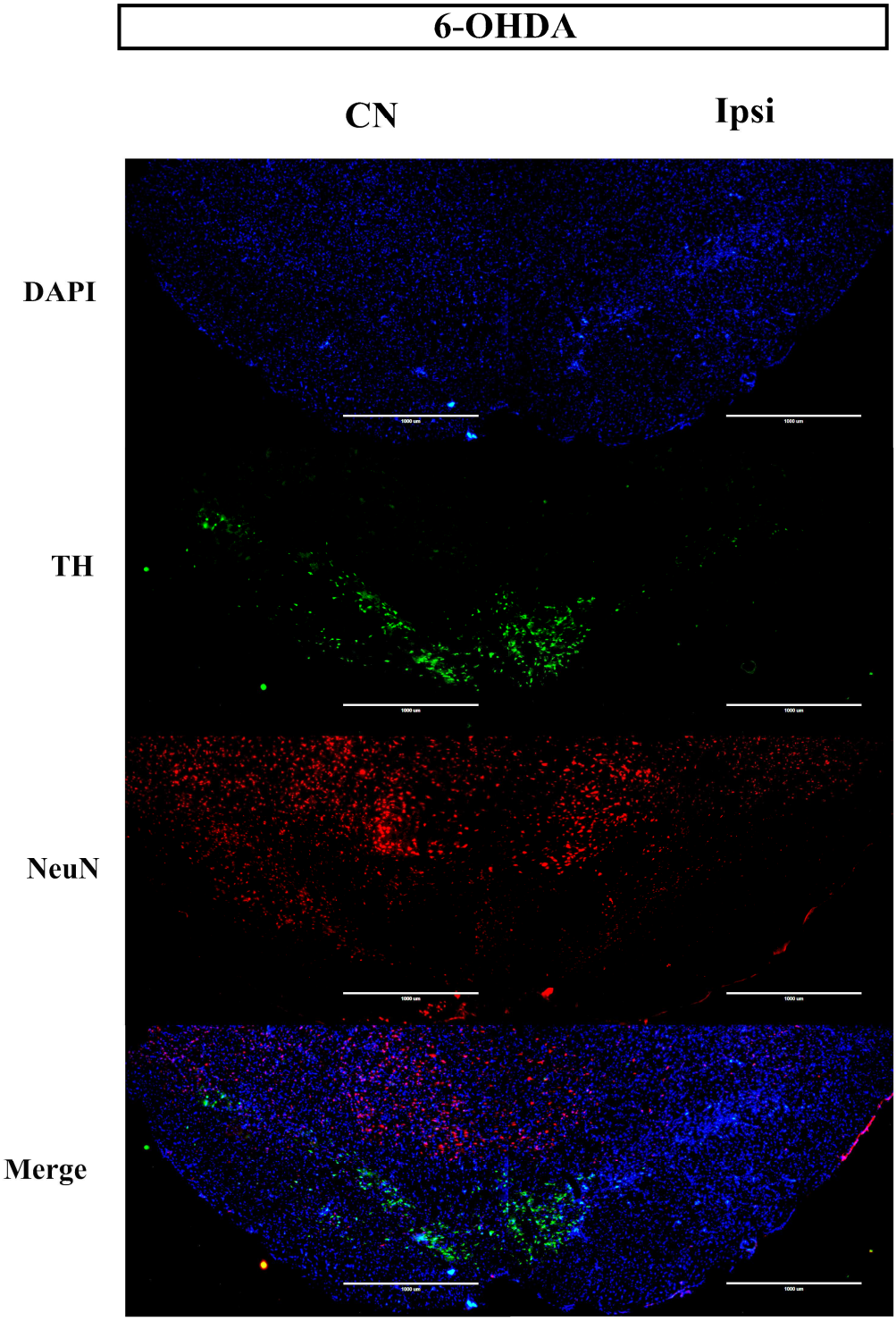


Figura 4: Efeito da indução de dano com 6-hidroxidopamina na substância negra de ratos (n=2). Visualização de imunofluorescência de tirosina hidroxilase e NeuN. Dapi foi utilizado para coloração do núcleo.

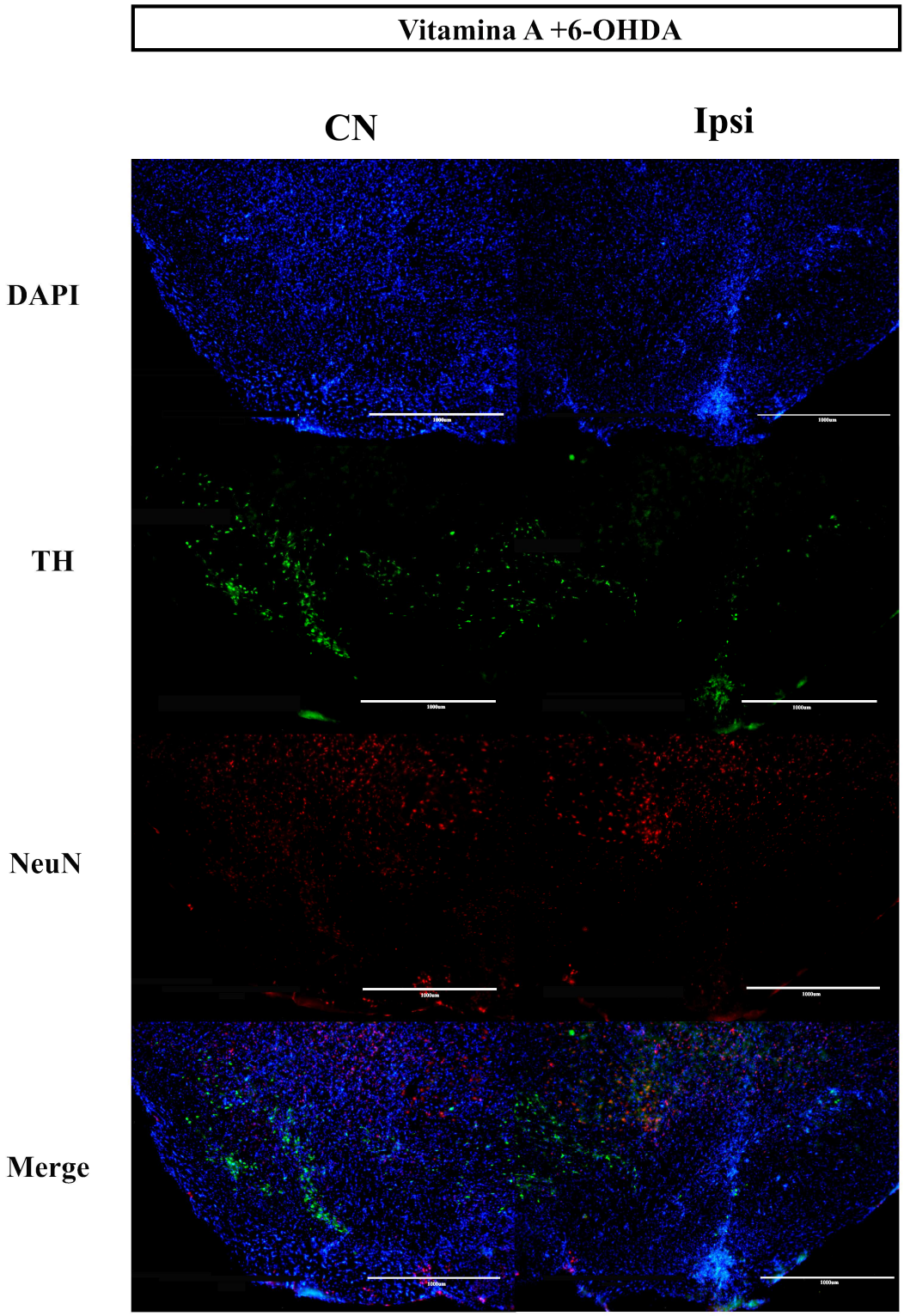


Figura 5: Efeito do pré-tratamento com vitamina A na indução de dano com 6-hidroxidopamina na substância negra de ratos (n=2). Visualização de imunofluorescência de tirosina hidroxilase e NeuN. Dapi foi utilizado para coloração do núcleo.

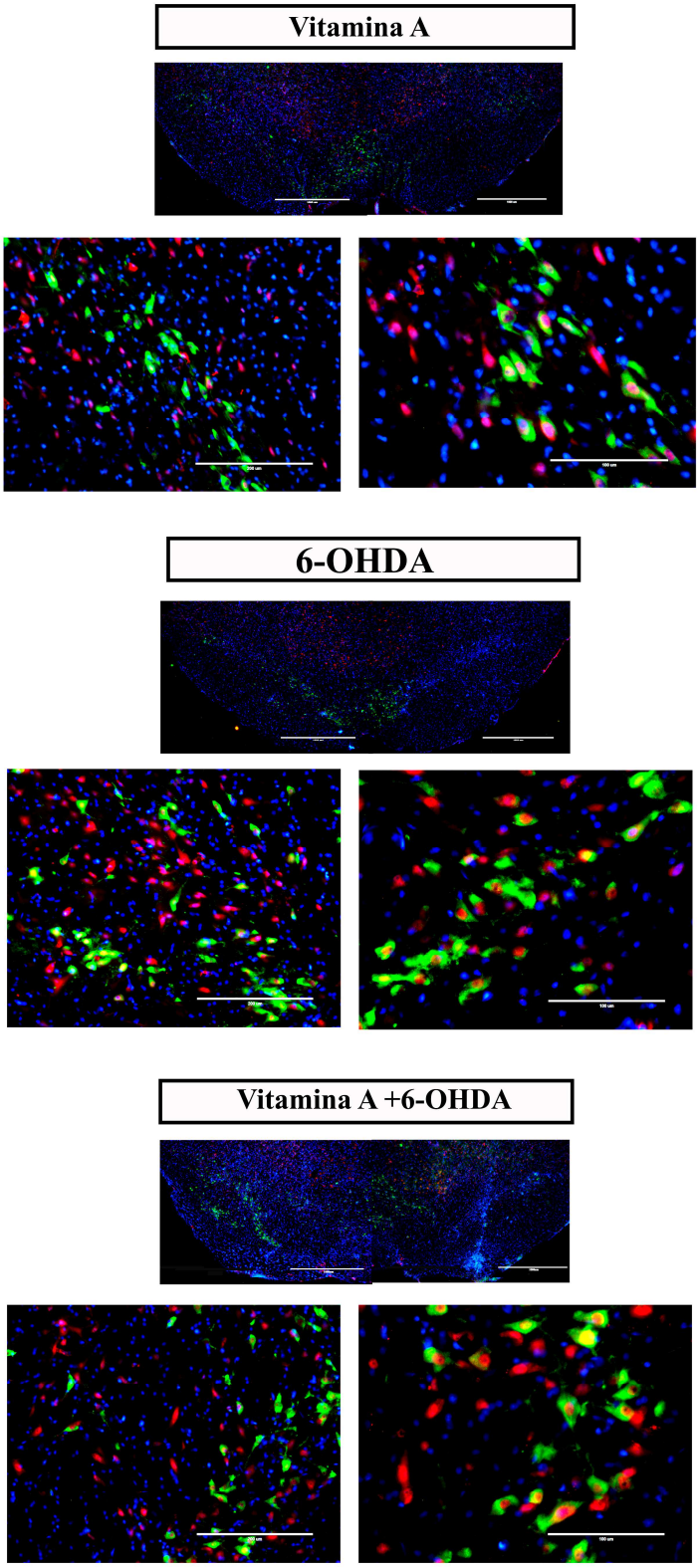


Figura 6: Efeito do pré-tratamento com vitamina A na indução de dano com 6-hidroxidopamina na substância negra de ratos. Visualização de imunofluorescência de tirosina hidroxilase e NeuN. Dapi foi utilizado para coloração do núcleo. Detalhes da morfologia celular em aumentos de 20x e 40x.

Discussão

A fim de explorar se a vitamina A poderia exercer algum efeito benéfico no curso da DP, nós caracterizamos os efeitos de ingestão crônica de palmitato de retinol em doses diárias recomendadas em condições de dano aos neurônios dopaminérgicos. Neste estudo, nós utilizamos o modelo de lesão induzida por 6-hidroxidopamina em ratos, um modelo in vivo extensivamente utilizado em avaliações pré-clínicas de compostos com potencial anti-parkinsoniano (Hansen *et al.*, 2016). A análise de imunohistoquímica revelou que o número total de neurônios TH-positivos foi extensamente reduzido com a aplicação de 6-OHDA na região ipsilateral de rato parcialmente lesados, de acordo com estudos prévios (Kim *et al.*, 2016). Uma relação positiva ocorreu em relação aos resultados da análise comportamental, em que ratos lesionados com 6-OHDA desenvolveram mais deficiências motoras, quando comparados com ratos pré-tratados com vitamina A. Também observou-se uma proteção da vitamina A apresentando maior quantidade de neurônios TH-positivos. Estes dados contrastam estudos prévios realizados com vitamina A, em que a administração da vitamina teve efeito tóxico em diversas áreas do cérebro (De Oliveira, De Bittencourt Pasquali, *et al.*, 2007; De Oliveira, Oliveira, Behr, Hoff, *et al.*, 2009; De Oliveira *et al.*, 2012). Neste estudo, no entanto, contrariando os estudos anteriores que utilizavam doses relativamente altas de retinol, nós utilizamos doses recomendadas de suplementação (3000IU/Kg dia^{-1}). Além disso, nenhum estudo utilizando a vitamina A como pré-tratamento frente ao dano parkinsoniano induzido por 6-OHDA foi desenvolvido anteriormente.

Além de se demonstrar efeito protetor sobre os neurônios dopaminérgicos neste modelo da DP, a vitamina A também exibiu efeitos anti-inflamatórios. A inflamação associada a neurodegeneração no modelo com 6-OHDA já foi descrito em outros trabalhos (Farbood *et al.*, 2015). Análise do soro dos animais revelou que o pré-tratamento com vitamina A foi capaz de reverter o quadro inflamatório desenvolvido pela 6-OHDA. Ratos que receberam a suplementação com vitamina A tiveram menores níveis séricos das citocinas pró-inflamatórias IL-1β E TNF-α.

Muitos estudos demonstram a importância da ativação do receptor para produtos finais de glicação avançada (RAGE) na ativação de vias de sinalização relacionadas a processos patológicos (Abdelsalam e Safar, 2015). Além disso, vários estudos também indicam o papel dos produtos finais de glicação avançada (AGE's) no desenvolvimento de doenças

neurodegenerativas (Santoro *et al.*, 2016). Neste trabalho observamos que a vitamina A aumentou os níveis de carbometil-lisina, mas quando administrada em forma de pré-tratamento frente ao dano com 6-OHDA, se demonstrou ser protetora e reduziu os níveis do AGE no líquido cefalorraquidiano dos animais.

Este estudo fornece novas informações em relação a vitamina A e sua relação com doenças neurodegenerativas, em especial a DP. A administração de forma preventiva de doses recomendadas da vitamina se demonstraram ser protetoras frente à neurodegeneração e também de caráter anti-inflamatório.

PARTE 3

DISCUSSÃO

Grande parte da relação entre vitamina A e sua capacidade de indução de rotas via produção de espécies reativas e geração de estresse oxidativo está relacionada com a sua caracterização redox ativa. Dependendo do microambiente em que se encontra, a vitamina A em doses baixas a moderadas pode atuar de maneira antioxidante, combatendo espécies reativas, ou de maneira pró-oxidante em altas concentrações, sendo a fonte geradora de estresse oxidativo (Pravkin *et al.*, 2013). Em pacientes portadores de alguma condição tóxica ou patológica, os retinóides parecem aumentar os efeitos deletérios, visto que interagem com um microambiente alterado fisiológica e bioquimicamente. Uma vez que a suplementação com retinóides é feita em indivíduos considerados saudáveis, os efeitos encontrados são benéficos, já que desta forma parecem atuar aumentando preventivamente a defesa antioxidante (Goralczyk, 2009).

Por muitos anos, o uso de retinóides foi sugerido como tratamento antioxidante. A suplementação com β-caroteno foi indicada no tratamento de câncer, já que o consumo deste retinóide estava associado com uma menor incidência desta doença. Vários mecanismos de ação foram propostos ao β-caroteno, como neutralização de espécies reativas como o oxigênio *singlet* e consequente redução da lipoperoxidação (Burton e Ingold, 1984). Estudos epidemiológicos realizados utilizando vitamina A e β-caroteno como prevenção na incidência de câncer de pulmão em fumantes apontaram para nenhum efeito preventivo, relatando inclusive maior incidência de câncer no grupo que recebeu a suplementação (Omenn,

Goodman, Thornquist, Balmes, Cullen, Glass, Keogh, Meyskens, Valanis, Williams, Barnhart, Cherniack, *et al.*, 1996). Outros estudos posteriores provaram que os efeitos adversos do β-caroteno estavam diretamente relacionado com o status de fumante dos participantes, e que em circunstâncias normais, em indivíduos não fumantes, existem evidências de efeitos benéficos (Goralczyk, 2009). Da mesma forma, a suplementação com β- caroteno em ratos saudáveis melhorou a defesa antioxidante e foi considerada uma alternativa segura para o consumo de retinóides (Schnorr *et al.*, 2014).

Por outro lado, muitos trabalhos indicam atuação pró-oxidante do retinol, demonstrando que a geração de espécies reativas pode estar relacionada com a disfunção mitocondrial e aumento do radical superóxido ($-O_2\cdot$). A suplementação com palmitato de retinol (1000, 2000 e 10000IU/kg/dia) demonstrou ser pró-oxidante em áreas do cérebro como cerebelo, córtex, hipocampo e estriado, além de induzir alterações comportamentais similares a ansiedade (Schnorr *et al.*, 2015). Análise de suplementação com palmitato de retinol (1000-9000IU/kg/dia) demonstrou ser pró-oxidante com aumento de estresse oxidativo e nitrosativo (4500 IU), além de induzir dano ao complexo IV mitocondrial no hipotálamo de ratos (De Oliveira, Oliveira, Da Rocha, *et al.*, 2009); aumento da atividade da caspase-3 no córtex (De Oliveira *et al.*, 2010); redução da atividade mitocondrial e redução nos níveis de BDNF e receptor D2 no hipocampo (De Oliveira, Da Rocha, *et al.*, 2011); indução de estresse oxidativo no estriado, córtex, cerebelo, hipocampo, substância negra e estriado (De Oliveira, De Bittencourt Pasquali, *et al.*, 2007; De Oliveira e Moreira, 2007; De Oliveira, Silvestrin, *et al.*, 2007; De Oliveira e Moreira, 2008; De Oliveira *et al.*, 2008; De Oliveira, Oliveira, Behr, Hoff, *et al.*, 2009; De Oliveira, Oliveira, Behr e Moreira, 2009) e aumento do imunoconteúdo de RAGE no córtex (De Oliveira, Oliveira, Behr, De Bittencourt Pasquali, *et al.*, 2009). Um estudo realizado com suplementação de palmitato de retinol em ratas gestantes revelou que o

consumo excessivo de retinol durante a gestação induz estresse oxidativo no estriado e hipocampo materno, assim como da prole (Schnorr *et al.*, 2011). Além disso, suplementação utilizando doses baixas de palmitato de retinol por maior período de tempo em ratas também indicou aumento de estresse oxidativo e disfunção mitocondrial no córtex, hipocampo, estriado e cerebelo, e indução de estresse oxidativo na substância negra e estriado de ratos machos (De Oliveira, Lorenzi, *et al.*, 2011; De Oliveira *et al.*, 2012).

O estresse oxidativo vem sendo relacionado com a indução ou progressão de doenças cardiovasculares e inflamatórias há vários anos. Recentemente, a geração de espécies reativas também tem sido apontada como causa de doenças que levam a morte neuronal, nas doenças neurodegenerativas. Dentre estas, estão a DA como a causa mais comum de demência na atualidade, e a DP. Trabalhos relatam a relação entre reações mediadas por espécies reativas e a formação dos agregados protéicos de α-sinucleína na DP, e fosforilação da proteína tau na DA (Halliwell, 2006; Mondragon-Rodriguez *et al.*, 2013; Berrocal *et al.*, 2015; Niedzielska *et al.*, 2016b). O peptídeo β-amilóide também possui um papel importante no processo da DA, e pode sofrer oligomerização com a formação de placas senis (Nalivaeva e Turner, 2013).

O grupo dos retinóides inclui o β-caroteno e a vitamina A e seus metabólitos, sendo o AR o mais ativo componente derivado do retinol. Diversos trabalhos utilizam estes compostos isoladamente, e é possível, por exemplo, identificar diferentes ações para cada um. Estudos comparativos, inclusive, relatam que o retinol é capaz de produzir espécies reativas, efeito que não é observado no tratamento com AR (Gelain e Moreira, 2008).

A deficiência de vitamina A foi apontada como fator de suscetibilidade ao desenvolvimento da DA, promovendo a β-clivagem da APP e a geração de peptídeo β-amilóide com a formação de placas senis, exacerbando também a perda de memória. Suplementação com doses terapêuticas de vitamina A demostrou melhorar a memória e o déficit cognitivo em camundongos, e níveis séricos de vitamina A foi correlacionado com melhora cognitiva em humanos (Zeng *et al.*, 2016; Zeng *et al.*, 2017). A sinalização dos retinóides tem influência no desenvolvimento da DA através da ativação de múltiplas vias, melhorando os sintomas da doença e reduzindo a acumulação amilóide e hiperfosforilação da proteína tau (Sodhi e Singh, 2014; Grimm *et al.*, 2016). Embora muitos autores sugiram a suplementação com vitamina A na prevenção da DP, uma meta-análise revelou ser inconclusiva a relação entre vitamina A, β-caroteno e Parkinson, necessitando mais estudos epidemiológicos para conferir maior validação (Takeda *et al.*, 2014).

O AR, considerado o principal metabólito do retinol, possui papel importante na diferenciação, proliferação, apoptose, podendo atuar como antioxidante, exercendo suas funções através da ligação com os receptores retinóides nucleares. O uso de AR também já foi sugerido no tratamento de câncer de mama e de fígado, assim como em episódios isquêmicos e no tratamento da acne (Hong e Lee-Kim, 2009). Tratamento com AR em células de leucemia mieloblástica revelou que o estado redox basal das células teve influência na ação do AR e estes dois fatores em conjunto influenciaram na resposta ao tratamento, com a presença de ações tanto antioxidantes quanto pró-oxidantes do AR (Mantymaa *et al.*, 2000). Além disso, o AR possui papel importante no tratamento de diversas doenças, entre elas a DA, em que parece reduzir a agregação do peptídeo β-amilóide e ativar funções acetilcolina dependentes (Lee *et al.*, 2009; Chakrabarti *et al.*, 2016). A administração de AR teve efeito protetor contra o dano induzido por MPTP, reduzindo a morte de neurônios dopaminérgicos

na substância negra, assim como suas projeções no estriado (Esteves *et al.*, 2015); e também produziu efeito protetor contra o dano induzido por 6-OHDA, reduzindo a morte celular, a alteração motora e aumentando o número de neurônios tirosina hidroxilase positivos na área afetada (Yin *et al.*, 2012). Em modelo experimental da DP utilizando rotenona como agente indutor do dano, revelou-se que o AR restaurou o déficit locomotor quando utilizado como tratamento pós dano cerebral (Ulusoy *et al.*, 2011).

Observamos neste trabalho que o AR induziu a diferenciação de células de neuroblastoma humano SH-SY5Y para um fenótipo catecolaminérgico maduro através da produção de espécies reativas e indução de estresse oxidativo via sinalização de ERK1/2 e Akt. Células expostas a rápidos tratamentos com AR já exibiram um aumento na produção de espécies reativas, ressaltando o rápido mecanismo de ação do AR, e a importância da geração do estresse oxidativo no processo de neurodiferenciação.

Neste trabalho, além do AR possuir sua atividade neuromoduladora através de ação pró-oxidante, também foi que o retinol em concentrações maiores que a fisiológica, é capaz de modular o RAGE em células SH-SY5Y não diferenciadas através da produção de espécies reativas. O tratamento com retinol foi capaz de aumentar a expressão do receptor através do aumento da produção de espécies reativas. No entanto, o co-tratamento com Trolox® bloqueou o aumento do RAGE, indicando a envolvimento da produção de espécies reativas na estimulação do receptor. Observamos também que o retinol atuou de maneira pró-oxidante, aumentando a produção de espécies reativas e do imunoconteúdo de marcadores de doenças neurodegenerativas como α-sinucleína, peptídeo β-amilóide, tau fosforilada e também do

receptor para produtos finais de glicação avançada (RAGE). Nos trabalhos *in vitro* apresentados aqui, tanto o retinol quanto o AR atuaram de maneira pró-oxidante.

Muitos modelos de estudos para as doenças neurodegenerativas vêm sendo desenvolvidos, modelos que utilizam métodos *in vitro* através do uso de culturas celulares, e modelos *in vivo*, através do uso de animais. A escolha do modelo depende do desenho experimental, e do objetivo do estudo, e tanto modelos celulares quanto animais possuem vantagens e desvantagens (Beal, 2001).

Os modelos celulares se tornam primeira escolha devido ao rápido desenvolvimento experimental. Além disso, tipos celulares específicos, como neurônios dopaminérgicos, podem ser isolados e estudados, detalhe importante para determinar a sua contribuição na DP. Dentre os modelos celulares de estudo da DP se encontram duas linhas principais, uma com o objetivo de estudar a perda dos neurônios dopaminérgicos da substância negra, e outra estudando os agregados protéicos contendo α-sinucleína. Várias linhagens celulares são utilizadas para tanto, como a linhagem de neuroblastoma humano SH-SY5Y e a linhagem de feocromocitoma humano PC12. Estas células produzem e liberam catecolaminas e podem desenvolver características neuronais maduras com o uso de protocolos específicos de diferenciação neuronal. O uso de modelos *in vitro* para o estudo de doenças neurodegenerativas é um importante avanço, visto que a utilização de uma população celular definida possibilita uma melhor compreensão dos mecanismos celulares e moleculares envolvidos na patologia. (Martinez-Morales e Liste, 2012; Falkenburger *et al.*, 2016).

Ao mesmo tempo, muitos passos importantes da DP em relação à patogênese e patofisiologia requerem a interação de diferentes tipos celulares, e, portanto, são mais bem estudados em modelos animais, como por exemplo, o papel das células da glia e inflamação, assim como alterações sinápticas (Falkenburger *et al.*, 2016). A fim de que tenham relevância no estudo da DP, os modelos animais devem conter algumas características específicas básicas, como perda gradual dos neurônios dopaminérgicos e déficits motores facilmente detectáveis. Os modelos animais se classificam como os que utilizam toxinas ou manipulação gênica para mimetizar o quadro patológico (Blesa e Przedborski, 2014). Atualmente existem diversas toxinas que mimetizam o dano semelhante à DP, como o uso de 6-hidroxidopamina, rotenona e MPTP (Gubellini e Kachidian, 2015; Sarath Babu *et al.*, 2016). A 6-hidroxidopamina foi o primeiro agente a ser utilizado em modelos de DP. Depois de injetada na substância negra, a 6-OHDA se acumula seletivamente nos neurônios dopaminérgicos, levando a morte destes neurônios devido a toxicidade e geração de espécies reativas, e é um modelo eficaz em estudos de *screening* farmacológico (Beal, 2001; Tieu, 2011). Atualmente, nenhum modelo animal é capaz de reproduzir perfeitamente a condição encontrada em pacientes com PD, no entanto, ainda são os modelos mais próximos à realidade humana (Blesa *et al.*, 2012). Os modelos que utilizam toxinas reproduzem a perda neuronal, mas falham em não induzir a formação de corpos de Lewy. Já os modelos genéticos embora reproduzam a presença dos corpos de Lewy, não apresentam uma morte neuronal considerável, constituindo essa a maior limitação do modelo (Blesa e Przedborski, 2014). Ainda assim, é importante também ressaltar que não se sabe de maneira concreta todas as causas envolvidas na forma esporádica da DP, e, portanto, não se consegue reproduzir a patogênese inteira encontrada na doença (Falkenburger *et al.*, 2016).

No modelo *in vivo* apresentado neste trabalho, os resultados da suplementação com palmitato de retinol teve efeito protetor contra os efeitos danosos da 6-OHDA sobre a substância negra, e ainda reduziu o quadro inflamatório, possivelmente atuando como antioxidante. Os resultados deste trabalho reforçam a concepção de que a vitamina A é uma molécula redox-ativa, podendo atuar como pró-oxidante ou antioxidante.

CONCLUSÕES

Concluindo com base nos resultados apresentados neste trabalho, é possível identificar mecanismos do retinol e do ácido retinóico envolvendo a geração de espécies reativas tanto na neurodiferenciação quanto na modulação de marcadores neurodegenerativos como o RAGE. Também conclui-se haver outros mecanismos de modulação de marcadores de doenças neurodegenerativas pelo retinol, não envolvendo a geração de ambiente pró-oxidante. A vitamina A também teve efeito neuroprotetor contra o dano induzido pela 6-OHDA, e também parece estar envolvida em mecanismos de inflamação, sugerindo-se um papel anti-inflamatório para o retinol em modelos *in vivo* da doença de Parkinson.

PERSPECTIVAS

Com base nos resultados observados neste trabalho, as perspectivas futuras são:

- Verificar o comportamento do retinol em modelo *in vitro* utilizando 6-hidroxidopamina em células SH-SY5Y;

- Melhor compreender o papel do RAGE na DP, com modelo *in vitro* utilizando 6-hidroxidopamina em células SH-SY5Y.

- Realizar mais análises complementares aos dados obtidos no experimento *in vivo*, tais como: western blotting da substância negra para tirosina hidroxilase e RAGE; PCR da substância negra para interleucinas pró-inflamatórias, IL-1β e TNF-α e análise de HPLC para retinol e metabólitos no soro e LCR.

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