DEBORAH ARIZA

COMPARAÇÃO COMPORTAMENTAL E NEUROQUÍMICA ENTRE DIFERENTES MODELOS DE PARKINSONISMO EM RATOS

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Dissertação apresentada como requisito parcial para a obtenção do título de Mestre em Farmacologia, departamento de Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná.

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Dedico esse trabalho a meus pais, Jeremias Ariza

e Maria Aparecida P. F. Ariza

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NOTA EXPLICATIVA

Esta dissertação é apresentada em formato alternativo – artigo para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná.

Constando de um artigo abordando os experimentos realizados e a discussão dos resultados, formatado conforme as normas propostas pela revista escolhida.

RESUMO

O presente trabalho visou investigar o comportamento motor, e os níveis de dopamina (DA) e glutationa (GSH) em três diferentes modelos de parkinsonismo. Ratos foram infundidos bilateralmente na substância negra com três diferentes toxinas: MPTP, 6-OHDA ou LPS. Para a análise comportamental foi utilizado o teste do campo aberto e os parâmetros freqüências de locomoção e levantar, e tempo de imobilidade foram avaliados. Os níveis de DA estriatal e seus metabólitos foram analisados através de HPLC, e os níveis de GSH na substância negra através de reação colorimétrica. Os animais infundidos com MPTP mostraram diminuição na freqüência de locomoção e de levantar no primeiro dia após a cirurgia, em comparação com grupos controle e sham. Os animais do grupo infundido com 6-OHDA mostraram uma redução em todos os parâmetros motores 1, 3 e 7 dias após a cirurgia. Os animais lesados com LPS não mostraram alterações nos parâmetros motores. A análise neuroquímica mostrou que o grupo infundido com MPTP apresentou uma redução significativa dos níveis de DA estriatal 3 e 7 dias após cirurgia. Os ratos que receberam a 6-OHDA apresentaram um aumento significativo nos níveis de DA no primeiro dia seguido de redução desses níveis no terceiro e sétimo dias. O grupo lesado com LPS exibiu uma diminuição nos níveis de DA no sétimo dia após a cirurgia. Os níveis de GSH mostraram-se reduzidos no grupo MPTP no terceiro dia e no grupo LPS em todos os dias analisados. Os níveis de GSH do grupo 6-OHDA não diferiram do grupo controle. Os modelos induzidos pelas toxinas MPTP e 6-OHDA, mas não LPS, foram capazes de causar alterações motoras conforme o esperado para um bom modelo animal da DP e os três modelos foram capazes de alterar os níveis de DA. Os níveis de GSH foram reduzidos nos modelos MPTP e LPS. Estes dados sugerem que todos os modelos são capazes de mimetizar características da DP, mas cada um com suas particularidades e limitações.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AD - Doença de Alzheimer

ATP - Adenosina tri-fosfato

Ca^{++} - Cálcio

COX-2 - Ciclooxigenase-2

DA – Dopamina

DNA - Ácido desoxirribonucléico

DOPAC - Ácido dihydroxyphenylacetic

DP – Doença de Parkinson

GPx - Glutationa peroxidase

GR - Glutationa redutase

GSH - Glutationa

GSSG - Glutationa oxidada

HD - Doença de Huntington

H_2O_2 - Peróxido de hidrogênio

$HO^{\bullet}$ - Radical hidroxila

HVA - Ácido Homovanílico

IL - Interleucina

iNOS - Óxido nítrico sintase induzida

LPS - Lipopolissacarídeo

MAO - Monoamina oxidase

MPDP - 1-metil-4-fenil-2,3-dihidropiridium

MPP^{+} - 1-metil-4-fenil-piridinium

MPTP - 1-metil-4-phenyl-1,2,3,6-tetrahidropiridina

$NO^{\bullet}$ - Óxido nítrico

NOS - NO sintase

O_2 - Oxigênio

$O_2^{\bullet -}$ - Ânion radical superóxido

$ONOO^-$ - Peroxinitrito

Prx - Peroxirredoxinas

RNS - Espécies reativas de nitrogênio

ROS - Espécies reativas de oxigênio

SN - Substância negra

SNpc - Substância Negra *pars compacta*

SOD - Superóxidos dismutases

TH - Tirosina hidroxilase

TNF-α - Fator de necrose tumoral

Trx - Tiorredoxina

TrxR - Tiorredoxina redutase

VMAT-2 - Transportador vesicular de monoaminas 2

6-OHDA - 6-hydroxydopamine

SUMÁRIO

1. INTRODUÇÃO

A Doença de Parkinson (DP) é a segunda mais freqüente doença neurodegenerativa após Doença de Alzheimer (AD) (Przedborski, 2005, Dunnet e Bjorklund, 1999; Dauer e Przedborski, 2003; Emborg, 2004). As manifestações clínicas ou denominados sinais cardinais da DP incluem bradicinesia, tremor de repouso, rigidez, instabilidade postural e anormalidades na marcha. A principal característica neuroquímica e neuropatológica da DP é a perda progressiva de neurônios dopaminérgicos da Substância Negra *pars compacta* (SNpc) associada a inclusões citoplasmáticas denominadas de corpos de Lewy (Hassler, 1938; Hornykiewicz, 1966; Przedborski, 2005; Dauer e Przedborski, 2003; Mattson et al., 2008; Truong et al., 2009; Peng et al., 2009).

A via dopaminérgica nigroestriatal é composta por neurônios dopaminérgicos cujos corpos celulares se localizam na SNpc e projetam seus axônios para o caudado-putâmen, ou corpo estriado (Przedborski, 2005). A redução na densidade neuronal gera uma diminuição na projeção de fibras dopaminérgicas que se projetam para o estriado dorsal (Figura 1) causando redução dos níveis de dopamina (DA) e de seus metabólitos. Também se observa a redução da atividade de enzimas envolvidas na síntese de DA, como a tirosina hidroxilase (TH) e DOPA-descarboxilase (Gerlach e Riederer, 1996; Rubio-Osornio et al., 2009).

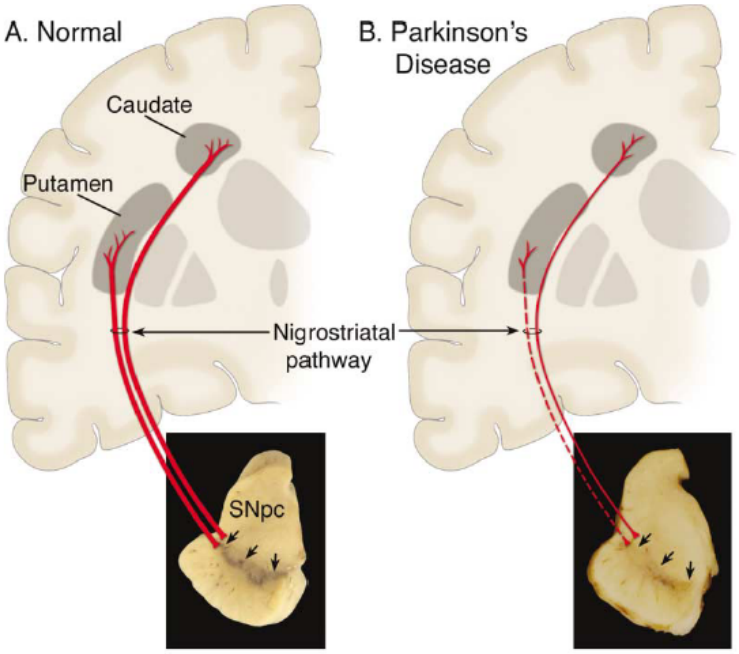

Figura 1: Representação esquemática da via nigroestriatal normal (A) e da via nigroestriatal na Doença de Parkinson (B). Retirada de Dauer e Przedborski, 2003.

Os sinais parkinsonianos aparecem quando a morte neuronal ultrapassa o limiar crítico que acarreta a diminuição de 70-80% de DA nos terminais nervosos do estriado (Hornykiewicz, 1975; Meissner et al., 2004; Warraich et al, 2009)

Os corpos de Lewy são inclusões citoplasmáticas eosinofílicas arredondadas, compostas de várias proteínas, como α-sinucleína, parkina e neurofilamentos. Apresentam um diâmetro aproximadamente de 15 μm e um centro repleto de hialina (Dauer e Przedborski, 2003; Schulz e Falkenburger, 2004). A deposição dessas proteínas pode ser tóxica aos neurônios, visto que essas causariam danos celulares, seqüestrando outras proteínas importantes para a sobrevivência da célula nervosa (Schulz e Falkenburger, 2004, Mattson et al., 2008). Os neurônios alterados pela formação de corpos de Lewy permanecem funcionais durante certo tempo, entretanto, morrem

prematuramente, sendo que neurônios com axônios longos mostram-se mais vulneráveis que neurônios de circuitos locais (Kidd, 2000). Os corpos de Lewy não são achados específicos da DP, são também encontrados em outras doenças como na AD e na Doença de Huntington (HD) (Schulz e Falkenburger, 2004). Segundo Braak et al. (2003), a deposição dos corpúsculos de Lewy na DP parece ocorrer anteriormente ao aparecimento dos sinais e sintomas motores.

Além da perda neuronal dopaminérgica e do aparecimento dos corpos de Lewy, outros sistemas como o noradrenérgico, através da perda de neurônios do *locus coeruleus*, serotonérgico, através da redução de serotonina no Núcleo da Rafe, e do sistema colinérgico, através da redução de acetilcolina nos Núcleos Basais de Meynert, estão relacionados com os achados neuroquímicos e neuropatológicos na DP (Stoof et al., 1999; Schulz e Falkenburger, 2004; Frisina et al., 2009).

A etiologia da DP, parkinsonismo primário ou idiopático, ou ainda chamado de parkinsonismo esporádico, permanece desconhecida (Mattson et al., 2008) o que a diferencia de outras formas de parkinsonismo como o secundário, que pode ser causado por hidrocefalia, hipóxia, infecções, trauma, tumor, por exposição a drogas e parkinsonismo associado a outras doenças neurodegenerativas, como AD e HD (Dauer e Przedborski, 2003; Fahn e Sulzer, 2004). Ainda, há a forma familiar da doença, 5 a 10%, dos casos, na qual se observam ligações entre a ocorrência da doença e mutações de genes (Vila e Przedborski, 2003; Hald e Lotharius, 2005). A DP familiar e a DP esporádica se diferenciam clinicamente e patologicamente, mas ambas apresentam degeneração dopaminérgica (Moore et al., 2005).

A DP idiopática deve ser diferenciada dos demais tipos de manifestações, visto que qualquer processo que danifique as estruturas dos gânglios da base e suas conexões, ou prejudique suas funções, pode ser capaz de induzir a diferentes tipos de parkinsonismo (Ahlskog, 2001).

Um melhor entendimento sobre a etiologia da DP esporádica, que representa a grande maioria dos casos de parkinsonismo, se faz necessária, sendo alvo do presente trabalho.

Nas últimas décadas, estudos clínicos, *pos mortem* e experimentos *in vivo* e *in vitro* com modelos animais, têm possibilitado algum entendimento sobre a patogênese da doença. As hipóteses incluem: defeitos conformacionais nas estruturas de proteínas citoplasmáticas e disfunções mitocondriais com conseqüente aumento do estresse oxidativo, resultando na morte neuronal (Dauer e Przedborski, 2003; Singh e Dikshi, 2007; Reale et al, 2009). Outros fatores considerados são a excitotoxicidade, neuroinflamação e eventos apoptóticos (Singh e Dikshi, 2007; Reale et al, 2009).

A disfunção mitocondrial tem sido amplamente relacionada com a patogênese da DP (Abou-Sleiman et al., 2006; Mattson et al., 2008). Aproximadamente 100% do oxigênio molecular é consumido pela respiração mitocondrial formando como subprodutos espécies oxidantes (Dauer e Przedboski, 2003). Segundo Barbosa et al. (2006), o metabolismo aeróbio apresenta uma série de reações que podem formar espécies reativas de oxigênio (ROS) e nitrogênio (RNS). Elétrons podem vazar da cadeia de transporte de elétrons reduzindo o oxigênio (O_2) a ânion radical superóxido ($O_2^{\bullet -}$). O superóxido também pode ser formado pela enzima NADPH oxidase, presente em diversas células. Em níveis elevados, o superóxido pode mobilizar

o ferro da ferritina. A dismutação do $O_2^{\bullet-}$ catalisada pelas superóxidos dismutases (SOD) produz peróxido de hidrogênio (H_2O_2). Níveis elevados de H_2O_2 podem liberar íons de ferro das proteínas do heme. O ferro reduz H_2O_2 a radical hidroxila ($HO^{\bullet}$) (reação de Fenton) e catalisa a formação de $HO^{\bullet}$ pela reação de Haber-Weiss. O $HO^{\bullet}$ é o radical mais reativo encontrado *in vivo*. Esse radical é capaz de oxidar carboidratos, lipídios, proteínas e DNA (Barbosa et al., 2006; Schulz e Falkenburger, 2004; Goldstein e Merenyi, 2008).

$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + HO^{\bullet} + HO^{-}$ Reação de Fenton

$H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + HO^{\bullet} + HO^{-}$ Reação de Haber-Weiss

O H_2O_2 é eliminado pelas enzimas catalase, glutationa peroxidase e peroxirredoxinas, as quais atuam em conjunto com as SOD (Figura 2).

As peroxirredoxinas (Prx) utilizam a proteína tiorredoxina (Trx) como substrato. A tiorredoxina redutase (TrxR), converte a tiorredoxina oxidada de volta a sua forma reduzida. Por sua vez, o óxido nítrico ($NO^{\bullet}$) é um radical essencial para a vasorregulação e neurotransmissão, formado pela enzima NO sintase (NOS). Em excesso, o $NO^{\bullet}$ pode inibir o citocromo oxidase, levando ao aumento da saída de elétrons e formação de superóxido. O $NO^{\bullet}$ pode reagir com $O_2^{\bullet-}$ formando peroxinitrito ($ONOO^{-}$). A geração de peroxinitrito *in vivo* pode levar à oxidação e nitração de lipídios, DNA e proteínas e conseqüente morte celular (Schulz e Falkenburger 2004; Barbosa et al., 2006; Goldstein e Merenyi, 2008).

A catalase dismuta H_2O_2 à água e oxigênio. A glutationa peroxidase (GPx) remove H_2O_2 pela oxidação de glutationa (GSH) ao dímero GSSG. Este

processo é complementado pela enzima glutationa redutase (GR), a qual recicla GSSG a GSH (Barbosa et al., 2006; Schulz e Falkenburger 2004).

Dessa maneira, a GSH, um antioxidante endógeno, tem um papel importante na proteção das células contra danos causados pelo estresse oxidativo. Segundo Kumar et al. (2008), a redução de GSH em cérebros de pacientes é o primeiro indicador de estresse oxidativo na DP. A depleção de GSH torna as células mais susceptíveis aos efeitos deletérios do estresse oxidativo e da micróglia ativada (Ibi et al., 1999, Chen et al., 2001; Mosley Lee et al., 2006).

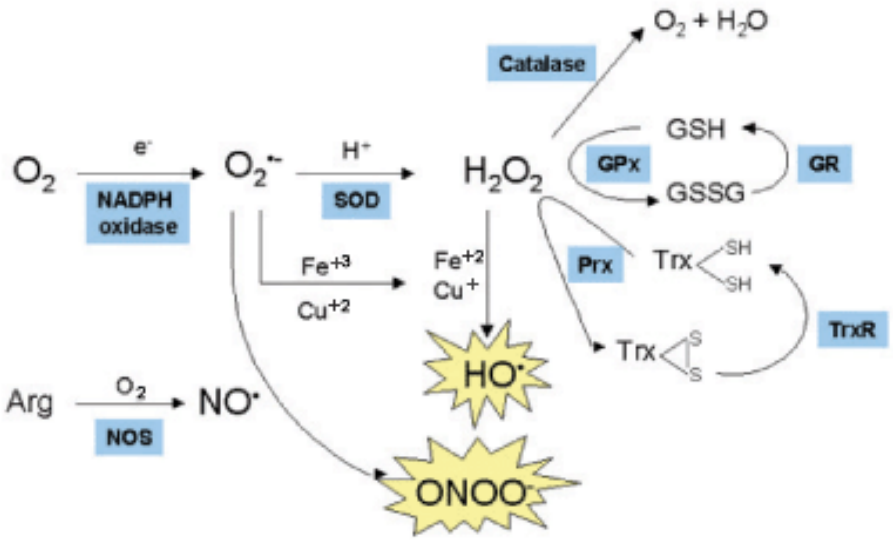

Figura 2: Esquema das reações e respectivas enzimas (em azul) envolvidas na formação de espécies reativas de oxigênio e nitrogênio. NOS: NO sintase; SOD: superóxido dismutase; GPx: glutationa peroxidase; Prx: peroxirredoxinas; GR: glutationa redutase; TrxR: tiorredoxina redutase; Trx:tiorredoxina; GSH: glutaiona reduzida; GSSG: glutationa oxidada (dímero). Retirada de Barbosa et al., 2006.

Segundo Schulz e Falkenburger (2004), muitas biomoléculas cruciais para a integridade celular, incluindo lipídios, proteínas e DNA, podem ser danificadas por espécies reativas (ROS e RNS) e desencadear o processo de neurodegeneração.

Além do estresse oxidativo e conseqüente disfunção mitocondrial, diversos estudos têm demonstrado que a inflamação é outro importante mecanismo envolvido no desenvolvimento e progressão da DP (Asanuma et al., 2004; Pieper et al., 2008; Reale et al., 2009). As células da glia e micróglia, a partir de um insulto tóxico, poderiam produzir substâncias nocivas aos neurônios, tais como citocinas pró-inflamatórias, prostaglandinas, ROS e RNS (Fahn e Sulzer, 2004; Tanaka et al., 2006; Pieper et al., 2008)

De acordo com McGeer e McGeer (2004), a ativação microglial pode resultar em aumento da produção de ânions superóxido e, como demonstrado *in vitro*, tal efeito pode contribuir para processos neurotóxicos, incluindo dano às células dopaminérgicas (Hirsch *et al.*, 2005; Pieper et al. 2008).

Corroborando tais evidências, foi encontrado no fluído cerebroespinhal de pacientes parkinsonianos aumento do número de células microgliais ativadas, bem como níveis elevados de citocinas, tais como o fator de necrose tumoral (TNF-α) e interleucinas (IL): IL-1β, IL-2, IL-4, IL-6, dentre outras (Hirsch *et al.*, 2005).

Conforme Emerit et al. (2004), a excitotoxicidade é a principal causa de morte celular, ocorrendo prolongada despolarização do neurônio, aumento na concentração de cálcio intracelular e ativação da morte celular por mecanismos enzimáticos e nucleares (Doble,1999).

O glutamato é o principal neurotransmissor excitatório no cérebro de mamíferos, responsável por um terço de todas as sinapses no SNC. Sob certas condições, como insuficiência na captação de glutamato ou despolarização prolongada da terminação nervosa, o glutamato pode se acumular na fenda sináptica e desencadear um processo excitotóxico. Concentrações excitotóxicas de glutamato causam a despolarização excessiva do neurônio pós sináptico e, conseqüentemente, distúrbios da homeostasia iônica e energética, ativação de enzimas líticas mediada por cálcio (Ca^{++}), geração de radicais livres, lesão mitocondrial podendo levar à lise celular e morte (Barbosa et al., 2006, Rubio-Osornio et al., 2009).

A apoptose é um importante mecanismo celular pelo qual o organismo controla o número de células, o tamanho dos tecidos e a remoção de células, que podem colocar em risco sua homeostasia (Hengartner, 2000, Mattson, et al., 2008; Wang et al., 2009). Sendo assim, o processo de apoptose encontra-se envolvido, além do desenvolvimento normal do cérebro, na perda de neurônios em doenças neurológicas (Becker e Boni, 2004).

Segundo Schulz e Falkemburger (2004), a apoptose tem sido considerada uma via importante para a morte neuronal no cérebro de humanos e parece contribuir para a degeneração dos neurônios dopaminérgicos da SN. Evidências acumuladas através de estudos *in vitro*, *in vivo* e em humanos (*post mortem*), mostram a morte celular por apoptose como um fator etiológico para a DP (Lev et at., 2003; Wang et al., 2009).

Os estudos citados acima nos mostram que ainda há muito a ser esclarecido sobre os mecanismos envolvidos na patologia da DP. O provável seria o envolvimento não apenas de um, mais a participação de vários fatores

na perda neuronal e, consequentemente, a interação desses fatores estariam diretamente relacionados a etiologia da DP.

Essa idéia corrobora com Esposito et al. *(*2007), que cita a degeneração neuronal, não apenas pela ação de um único fator deletério, mas da convergência de diversos fatores patogênicos (Figura 3)

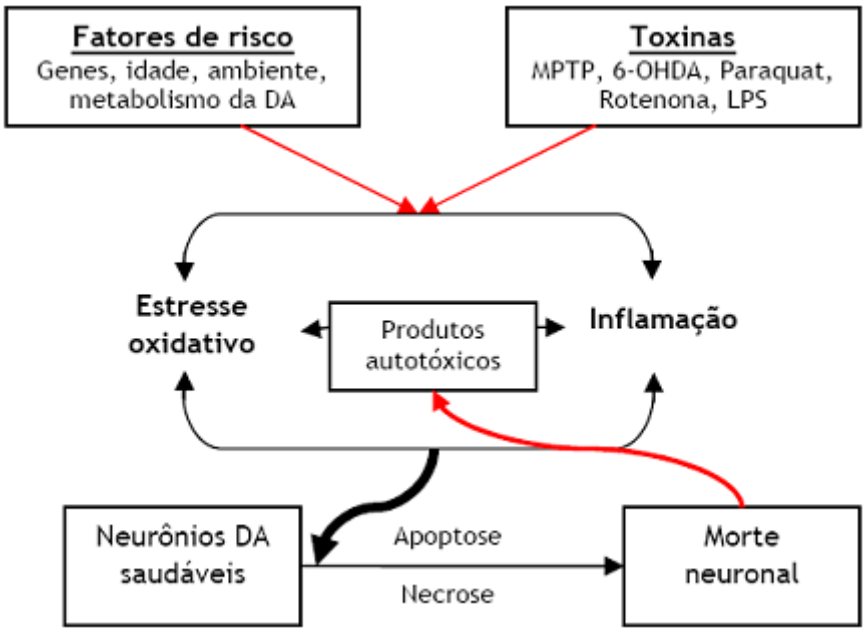

Figura 3. Interação sinérgica de mecanismos envolvidos na morte de neurônios dopaminérgicos na DP (Adaptado de: Esposito *et al.,* 2007).

O tratamento farmacológico da DP é realizado principalmente por drogas que substituem a dopamina, inibidores da dopa descarboxilase de ação periférica, agonistas dopaminérgicos, inibidores da monoamina oxidase (MAO-B), drogas que liberam dopamina, e antagonistas de receptores colinérgicos, destinados a contrabalancear o desequilíbrio neuroquímico provocado principalmente pela degeneração da via nigroestriatal dopaminérgica (Poewe e

Granata, 1996; Kitamura, et al., 2002; Warraich et al, 2009). Apesar de muitas drogas estarem sendo estudadas, a progressão da doença não é ainda bloqueada por nenhum recurso terapêutico disponível e, conforme a doença progride, faz-se necessária a administração de doses cada vez maiores desses fármacos paliativos (Samii, et al., 1998; Dunnet e Björklund, 1999; Kitamura, et al., 2002; Warraich et al, 2009).

Para a investigação de novos recursos terapêuticos eficazes e da fisiopatologia da DP, faz-se necessário o estudo e desenvolvimento de modelos animais que melhor mimetizem a DP. Modelos animais experimentais são ferramentas importantes na ciência médica, permitindo o estudo dos mecanismos patogenéticos e ajudando a compreender os princípios terapêuticos no tratamento dos distúrbios funcionais (sintomas) de doenças humanas (Gerlach e Riederer, 1996).

Infelizmente ainda não existe um modelo que pode, por si só representar fidedignamente todos os sinais e sintomas da DP (Mizuno et al., 1996; Dauer e Przedborski, 2003; Schulz e Falkenburger, 2004; Bové et al., 2005; Da Cunha et al., 2008), no entanto, existem características particulares de cada modelo que facilitam a elucidação da fisiopatologia e permitem estudos sobre a eficácia dos recursos terapêuticos utilizados e de novas abordagens a serem testadas. A comparação entre os modelos utilizados é necessária para fornecer uma visão mais abrangente nesses estudos.

Modelos animais baseados em neurotoxinas têm fornecido importantes provas do envolvimento de disfunções mitocondriais e excitotoxicidade em doenças neurodegenerativas. Várias dessas toxinas foram identificadas, mas

as suas contribuições para a doença ainda não são claras (Mattson e Magnus, 2006)

Segundo a Willner (1986), a validade de um modelo animal pode ser avaliada por três conjuntos de critérios: validade preditiva (*"predictive validity"*), que é avaliada com base na sua capacidade de diferenciar entre drogas que são, ou não são, clinicamente eficazes na patologia humana que está sendo estudada; validade analógica ou por semelhança (*"face validity"*), que é avaliada com base nas qualidades comportamentais e farmacológicas cujas semelhanças com o distúrbio humano podem ser demonstradas; e validade teórica ou por homologia (*"construct validity"*), onde os mesmos processos psicobiológicos responsáveis pela etiologia e fisiopatologia dos sintomas clínicos estejam atuando no modelo.

Muitos modelos que causam a morte neuronal na SN são utilizados como ferramentas para o estudo da DP, dentre esses o MPTP (1-metil-4-phenyl-1,2,3,6-tetrahidropiridina), a 6-OHDA (6-hydroxydopamine) e o LPS (Lipopolisacarídeo), os quais foram escolhidos para a realização desse trabalho.

A injeção estriatal de 6-OHDA leva à degeneração, bem como à ativação astroglial e microglial na via nigroestriatal dopaminérgica, induzindo à geração de ROS que conduzem a degeneração dos neurônios catecolaminérgicos, e podem também danificar a atividade de proteases (Rodrigues et al., 2003; Vercammen et al., 2006). Diferente da toxina MPTP, a 6-OHDA não atravessa a barreira hematoencefálica, e para reproduzir o modelo de parkinsonismo utilizando essa toxina, é necessário que ela seja infundida intracerebral.

Apesar de muito utilizada, a lesão causada pela 6-OHDA difere da verdadeira lesão da DP, e seu mecanismo ainda não está totalmente elucidada (Dauer e Przedborski, 2003).

Segundo Blum et al. (2001), o mecanismo hipotético da toxicidade da 6-OHDA estaria relacionado a morte dos neurônios catecolaminérgicos por três mecanismos principais: geração de ROS por auto-oxidação intra ou extracelular, formação de H_2O_2 pela atividade da MAO ou inibição da cadeia respiratória mitocondrial.

Embora a 6-OHDA seja utilizada como uma neurotoxina seletiva, ela pode causar uma lesão não específica a qualquer tecido cerebral que contenham neurônios catecolaminérgicos (Kondoh et al.,2005).

Animais lesados bilateralmente com 6-OHDA demonstram os sintomas motores parkinsonianos, entretanto, a lesão bilateral não é um modelo comum (Cenci et al., 2002; Ferro et al., 2005; Da Cunha et al., 2008). A 6-OHDA é usualmente injetada unilateralmente, enquanto que o hemisfério intacto funciona como um controle, constituindo um modelo de hemiparkinsonismo, que é caracterizado por um comportamento motor assimétrico após a administração de drogas dopaminérgicas, devido a um desequilíbrio fisiológico entre o lado lesado e não-lesado (Betarbet *et al.*, 2002; Da Cunha et al, 2008).

O MPTP é uma substância lipossolúvel, fato que lhe confere grande capacidade de cruzar a barreira hemato-encefélica, e penetrar nas células gliais e em neurônios serotonérgicos, e sofrer a ação da MAO-B é oxidada em 1-metil-4-fenil-2,3-dihidropiridium (MPDP) e em seguida convertida na forma neurotóxica ativa, o MPP^+ (1-metil-4-fenil-piridinium). O MPP^+ é então seletivamente captado pelos neurônios dopaminérgicos, através dos mesmos

mecanismos de recaptação da dopamina, vindo a produzir degeneração dessas células por interferência com o metabolismo oxidativo (Javitch et al., 1985; Dauer e Przedborski, 2003; Mattson et al., 2008; Kumar et al., 2009). O MPP^+, dentro dos neurônios dopaminérgicos, pode seguir por três vias: (1) Se concentra dentro da mitocôndria, bloqueando o complexo I, e interrompendo a transferência de elétrons para a ubiquinona. Essa perturbação aumenta a produção de ROS e diminui a síntese de ATP (adenosina tri-fosfato), fazendo, então, que ocorra um estresse oxidativo e uma deficiência energética causada pelo MPP^+. (2) Interage com enzimas citosólicas, principalmente com aquelas carregadas com cargas negativas. (3) Liga-se ao transportador vesicular de monoaminas 2 (VMAT-2), que transloca o MPP^+ para dentro das vesículas, expulsando dessa o neurotransmissor dopamina. O acúmulo de dopamina no citosol pode sofrer autooxidação e produzir ainda mais ROS, como radicais superóxidos (Vila e Przedborski, 2003; Dauer e Przedborski, 2003; Schulz e Falkenburger, 2004; Mattson et al., 2008). Segundo Przedborski (2005), o MPTP causa a morte de neurônios dopaminérgicos através do estresse oxidativo e da deficiência energética, sendo que, esses processos não são os principais responsáveis pela morte neuronal, mas atuariam provocando uma injúria inicial que ativaria vias moleculares, essas sim que levariam à morte dos neurônios (Rubio-Osornio et al, 2009; Kumar et al 2009).

Mais recentemente o LPS, uma endotoxina presente na parede celular de bactérias Gram-negativas, têm sido estudada e também usada para desenvolver uma síndrome parkinsoniana. O modelo utilizando LPS se faz devido essa substância ser um potente estimulante de células imunes e a

infusão intranigral dessa toxina ser capaz de induzir a degeneração dopaminérgica em ratos (Asanuma *et al.*, 2004; Reale et al, 2009).

O LPS induz a expressão de citocinas inflamatórias como: IL-1β, IL-6, IL-12, TNF-α e óxido nítrico sintase induzida (iNOS). Além disso, a ativação microglial causada por LPS pode resultar em *upregulation* da enzima ciclooxigenase-2 (COX-2) e desta forma, aumentar a síntese de prostaglandinas que podem ativar diretamente a caspase-3 ou indiretamente liberar glutamato levando a excitotoxicidade (Hald e Lotharius, 2005).

A administração de LPS em cérebros de roedores têm reproduzido determinadas características da DP, como degeneração progressiva de DA na substância negra e desordens de movimento. Mas sua principal importância é no entendimento da participação das células gliais, especialmente da micróglia, no processo neurodegenerativo da DP (Liu, 2006; Sugama et al, 2008).

Pode-se observar que muitas questões ainda devem ser respondidas para o entendimento da DP, e estudos envolvendo modelos animais são necessários para auxiliar na compreensão dos mecanismos fisiopatológicos da doença e na busca de novas estratégias terapêuticas.

2. OBJETIVOS

2.1 Objetivo geral:

O objetivo desse trabalho foi estudar as alterações comportamentais e neuroquímicas em diferentes modelos animais de parkinsonismo 1, 3 e 7 dias após infusão das toxinas: MPTP, 6-OHDA e LPS.

2.2 Objetivos específicos:

Avaliar comportamento motor de ratos após a infusão de MPTP, 6-OHDA e LPS na SN no primeiro, terceiro e sétimo dia após a cirurgia.

Analisar os níveis de dopamina e seus metabólitos no estriado nos dias 1, 3 e 7 após lesão na SN com as diferentes toxinas.

Verificar a concentração de glutationa na SN de ratos lesados com MPTP, 6-OHDA e LPS, 1, 3 e 7 dias após cirurgia estereotáxica.

3.

MPTP, 6-OHDA OR LPS: WHAT IS THE BEST MODEL OF PARKINSON'S DISEASE?

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ABSTRACT

Parkinson´s disease (PD) is the second most common neurodegenerative disease. The current study investigated the behavior, dopamine (DA) and glutathione (GSH) level changes in three different PD models. Rats were infused bilaterally in the Substantia nigra (SN) with MPTP, 6-OHDA or LPS. The behavior of the animals was observed in an open field 1, 3, and 7 days after the surgery. The striatal levels of DA and its metabolites were measured by HPLC with electrochemical detection. The levels of GSH in SN were also measured. The MPTP and 6-OHDA rats presented a decrease in locomotion and rearing frequencies 1 day after surgery. In addition, the 6-OHDA rats presented a reduction in locomotion and rearing frequencies 1, 3, and 7 days after surgery. The behavior of the LPS rats in the open field did not differ from the control group. Reduced levels of striatal DA was observed 3 and 7 days after surgery in the MPTP rats, 1, 3, and 7 days after surgery in the 6-OHDA rats, and 7 days after surgery in the LPS rats. The nigral levels of GSH were reduced on day 3 after surgery for the MPTP rats, and on days 1, 3 and 7 after surgery for the LPS rats. These data suggest that MPTP and 6-OHDA, but not LPS, were able to cause motor impairment as expected for a good animal model of PD. DA turnover was reduced in the three models studied. GSH nigral levels were reduced in both MPTP and LPS models.

Key words: Parkinson’s disease, MPTP, 6-OHDA, LPS, Dopamine, Glutathione, rats

INTRODUCTION

Research into the pathogenesis of Parkinson`s disease (PD) has rapidly advanced with the development of animal models that allow the investigation of new treatments. Animal models are important tools in experimental medical science because they permit the study of pathogenetic mechanisms and help to understand the therapeutic principles of treating the functional disturbances (symptoms) of human diseases (Gerlach and Riederer, 1996). Some of these models are achieved by the use of neurotoxins, in particular 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), and recently lipopolysaccharide (LPS) (Castano et al., 1998; Iravani et al., 2002; Lima et al., 2006).

According to Willner (1986), the validity of animal models is evaluated by three sets of criteria: predictive validity (capacity of the model to predict some event of the clinical condition), face validity (phenomenological similarities between the model and the clinical condition) and construct validity (some similarities between the mechanisms underlying animal behavior and psycho- or neurobiological mechanisms underlying the clinical condition).The criterion for face validity is that the model should simulate the etiology, biochemistry, symptomatology and treatment of the clinical condition being modeled.

Motor symptoms (including slowness of motor activity and rigidity) are the major symptoms of PD (Birkmayer and Riederer, 1985). In most of the studies of PD models, a decrease in locomotion and/or rearing was observed following MPTP administration (Sedelis et al., 2001; Perry et al., 2004, 2005; Lima et al., 2006, Reksidler et al. 2007, 2008). 6-OHDA is an animal model of PD that is

used for screening new therapeutic drugs for PD through their capacity to induce turning behaviour (Da Cunha et al., 2008). However, LPS-lesioned rats do not show this feature (Lima et al., 2006). Moreover, impairment in cognitive function and depression were observed both in PD patients and in animal models (Da Cunha et al., 2001; Gervaerd et al., 2001; Skalisz et al., 2002; Perry et al., 2005; Reksidler et al., 2007). The administration of LPS in the brains of rodents have shown certain features of PD, including the progressive degeneration of the SN and disorders of movement, but its main importance is to unravel the role of glial cells, especially of microglia in neurodegenerative disease (Liu, 2006).

Neurochemical parameters in these PD models indicated that MPTP (Da Cunha et al., 2001; Gevaerd et al., 2001; Perry et al., 2004), 6-OHDA (Lindner et al., 1999; Da Cunha et al., 2001; Ferro et al., 2005) and LPS (Castãno et al., 1998; Herrera et al., 2000; Iravani et al., 2002) caused reduction in DA striatal levels besides the reduction of nigral tyrosine-hydroxylase (TH)-positive neurons. In this line, we demonstrated in a comparative study that intranigral injection of LPS, MPTP or 6-OHDA elicited an up-regulation of COX-2 protein in a time dependent manner (1, 3 and 7 days) after the neurotoxin insult. In addition, we observed approximately the same reduction of TH protein content in the SN on these models.

From another point of view, GSH, an essential tripeptide, is an endogenous antioxidant found in all animal cells which reacts with free radicals to protect cells from singlet oxygen, hydroxylradical and superoxide radical damage. GSH depletion is the first indicator of oxidative stress in the progression of PD (Kumar et al. 2008). Nevertheless, depletion of GSH may

render cells more sensitive to toxic effects of oxidative stress and potentiate the toxic effects of reactive microglia (Ibi et al., 1999; Chen et al., 2001; Lee Mosley et al., 2006).

Although each model recapitulates significant neuropathological features of PD representing parts of the same puzzle, combinatorial study of multiple models are warranted to provide a more comprehensive view of PD pathogenesis (Dawson et al., 2002). Unfortunately there is still no model that alone can mimic all the signs and symptoms of the disease, however, there are some features of models that allow the elucidation of the etiology of the disease and also new features in attempt to therapeutic treatment. The objective of the present study was to evaluate a comparative study between MPTP, 6-OHDA and LPS PD models. The comparison mainly focuses the LPS model because it was recently proposed by some authors, while the others are considered well established models. This comparison was based on behavioral effects, striatal DA levels and GSH depletion 1, 3 and 7 days after lesion induced by LPS, MPTP or 6-OHDA.

RESULTS

The open-field data indicated that these models have different patterns of motor changes. Table 1 illustrates the effect of MPTP, 6-OHDA and LPS infusion in the immobility time of the rats. Figures 1 and 2 demonstrate the locomotion and rearing frequencies 1, 3 and 7 days after the stereotaxic surgery. The MPTP group had a decrease in locomotion [$F(14.92)= 5.11$;

P<0.01] and rearing frequencies [F(5.81)= 3.81; P<0.05] 1 day after surgery in comparison to the control group (Table 1). The 6-OHDA group showed a reduction in all motor parameters: locomotion 1 [F(14.92)=9.85; P<0.001] and 3 days after surgery[F(4.18)= 5.42; P<0.01]; rearing 1 [F=(5.81)=6.61; P<0.001] and 3 days [F(2.61)= 4.09;P<0.05] after surgery as shown in Table 1. Moreover, the rats of this group also exhibited an increase in the immobility time 1[F(8.34)= 7.32;P<0.001] and 3 days after surgery[F(3.95)= 4.83; P<0.01] compared to the control group (Table 1). And the LPS group showed no difference in motor parameters when compared to the control group.

In the second experiment, the DA turnover changes induced by these models was evaluated. The neurochemical analysis indicated that DA levels in the MPTP group presented a reduction 3 [F(6.91)= 3.64; P<0,05] and 7 days [F(17.1)= 6.14; P<0,001] (Figure 1B and 1C) after lesion. In the 6-OHDA lesioned rats (Figure 1A) a significant increase on striatal DA levels on day 1 [F(6.02)= 5.56; p<0.01] was observed. However, a reduction in DA content on days 3 [F(6.91)= 5.95; P<0.01] and 7 after surgery [F(17.1)= 11.0; P<0.001] (Figure 1B and 1C) was observed. There was a decrease in DA levels on the LPS group 7 days after the toxin [F(17.1)= 5,11; P<0.01], in comparison to the control group (Figure 1C).

As shown in Table 2, 3,4-dihydroxyphenylacetic acid (DOPAC) striatal levels of the MPTP group did not differ from control rats. The lesion with 6-OHDA caused a decrease of this metabolite on days 3 [F(4.46)= 5.08; P<0.01] and 7[F(8.76)= 7.89; P<0.001] after surgery. The LPS group exhibited a decrease in DOPAC levels on day 7 [F(8.76)= 4,23; P<0.05]. In addition, there was a significant increase in striatal homovanillic acid (HVA) levels 1 day after

MPTP [F(11.0)= 3.84; P<0.05], 6-OHDA [F(11.0)= 4.98; P<0.01] and LPS [F(11.0)= 8.17; P<0.001] infusion. A reduction in HVA levels 3[F(5.62)=4.48 ; P<0.01] and 7 days [F(5.52)=6.22; P<0.001] after surgery was also observed in the 6-OHDA group.

GSH levels in SN were found to be significantly reduced in the MPTP lesioned rats 3 days after lesion [F(5,12)=6,12, P<0,01], compared to the non-operated group (Figure 2B). In the 6-OHDA lesioned rats there wasn`t significant difference in GSH levels when compared to the control group (Figure 2). A significant decrease in GSH levels on days 1 [F(3,59)=4,73, P<0,05], 3 [F(5,12)=4,06, P<0,05] and 7 [F(5,42)=2,10, P<0,01] was observed after lesion with LPS (Figure 2).

DISCUSSION

The most important finding in this paper is that the GSH striatal levels were reduced in both MPTP and LPS PD models. Surprisingly, the rats of the 6-OHDA group did not show changes in GSH levels.

Our behavioral results showed that the MPTP group exhibited a motor impairment mainly 24 h after surgery. These data corroborate many other studies of our group (Perry et al., 2004; Lima et al., 2006; Reksidler et al., 2007, 2008; Capitelli et al., 2008). However, this feature was more persistent in the 6-OHDA lesioned rats. LPS did not produce motor signs that are representative of a parkinsonian syndrome. Striatal DA turnover indicated that DA levels decreased 3 (MPTP and 6-OHDA) and 7 (MPTP, 6-OHDA and LPS) days after surgery, while striatal DOPAC levels were reduced 3 and 7 days after surgery in the 6-OHDA lesioned rats. In the LPS group there was a significant reduction of DOPAC in the 7^{th} day after lesion. HVA levels increased 1 day after lesion in all models and decreased 3 days after surgery in the 6-OHDA group.

Numerous neurochemistry studies have shown that MPTP administration damages the nigrostriatal pathway, causing cell loss in Substantia nigra pars compacta (SNpc) (Heikkila et al, 1984; Sundström et al, 1987; German et al, 1996; Gevaerd et al, 2001) and DA depletion in the neostriatum (Sonsalla and Heikkila, 1986; Sundström et al, 1987; Da Cunha et al, 2001; Perry et al, 2004). Such effects observed in this animal model mimic the neurochemical alterations observed in PD. Moreover, when MPTP was given in a chronic fashion, it induced the formation of Lewy body-like inclusions in surviving SNpc neurons (Fornai et al., 2005).

In a similar study, we demonstrated that 24 h after infusion MPTP caused a reduction of TH content by 37% in the SN and an increase in ciclooxigenase 2 (COX-2) expression observed in the SN, suggesting damage in the nigroestriatal pathway (Lima et al., 2006). In this line, COX-2 expression is induced specifically within SNpc dopaminergic neurons in human postmortem PD specimens and in the MPTP mouse model of PD during the destruction of the nigrostriatal pathway (Teismann et al., 2003).

Neurochemical data indicated that the MPTP-lesioned rats exhibited a decrease in striatal DA levels 3 and 7 days after surgery. DOPAC levels were not changed by this neurotoxin in this time-course analysis. HVA had an increase 24 h after the lesion. These data are in accordance with the literature (Da Cunha et al., 2001; Ferro et al., 2005; Perry et al., 2005).

An important factor for the appearance of motor parkinsonian signs is the extent of striatal DA depletion once it reaches a loss of 80% (Heikkila and Sonsalla, 1992). On the other hand, Vingerhoets, et al. (1994) pointed out that clinical features begin to emerge when there is a 40-60% reduction of nigral neurons and striatal DA. Differences in rat strain, experimental conditions, and MPTP dose might account for the discrepancy. In fact, a chronic low dose of MPTP in monkeys resulted in a decrease of approximately 90% in striatal DA and in its metabolites DOPAC and HVA (Schneider, 1990). Moreover, in C57BL/6 mice that received four injections of MPTP (10 mg/kg) intraperitoneally, the striatal DA concentrations were markedly decreased in 82% three days after the toxin injection as compared with the saline-treated group (Araki et al., 2001). One possible explanation for the discrete reduction observed in the present study may be related to the interval between MPTP

administration and DA detection. Perhaps, this had the influence of a compensatory increase in DA turnover in the remaining dopaminergic neurons. In this sense, of particular interest are the studies of Tanji et al. (1999), who investigated the chronological changes in DA receptors and uptake sites in the striatum and SN of mouse brain. They demonstrated that DA uptake sites had slightly recovered by 21 days. This finding possibly indicates that dopaminergic neurons recover or regenerate in the chronic phase.

The other explanation for the mild DA depletion verified is associated with the incomplete lesion produced by MPTP. In this way, many authors have indicated that, despite the high depletion of DA, the lesion of dopaminergic neurons is partial. In previous studies we demonstrated that bilateral intranigral MPTP produced a 40% reduction in striatal DA compared to control (Gevaerd et al., 2001; Perry et al., 2004) and a 57% decrease in the number of TH immunoreactive neurons in the SN. In the present paper the reduction of DA content was approximately 40% on the third day and 44,88% on the seventh day.

Current data showed that MPTP caused GSH depletion 3 days after surgery indicating an increase in oxidative stress and are in accordance with others from literature (Riederer et al., 1987; Kumar et al., 2008). The GSH deficit in the nigra in PD has been known for at least 20 years. This observation has been independently verified in several groups of research (Jenner et al., 1992; Riederer et al., 1989; Pearce et al., 1997) providing confidence in its validity. Decreased levels of total glutathione (GSH plus GSSG) in autopsied brains from PD patients were first observed by Perry and colleagues (Perry et al., 1982; 1986; Zeevalk et al., 2008). The abundant evidence for oxidative damage

in the SN of PD also attests to an ongoing oxidative stress and it is a reasonable assumption that the low level of GSH in the region contributes to this phenomenon (Zeevalk et al., 2008).

This suggests that the MPTP rat model showed good face and predictive validity. On the other hand, nowadays, this model is sometimes considered inadequate because of the discrepancies caused by differences in strain, administration schedule and regimen of administration. This is considered its principal drawback, mainly when compared to the primate and mouse model of PD. Thus, the MPTP-lesioned rat seems to be a good model of early stage PD since the neurotransmitter depletion was specific for DA and spared the mesohippocampal neurons and also because the SNpc lesion and the consequent DA depletion barely reached the low striatal levels of about 40-60% observed in early stage PD patients, when the first motor symptoms begin to emerge (Obeso et al., 2000; Da Cunha et al., 2001).

The 6-OHDA is another neurotoxin able to induce nigrostriatal regional degeneration when infused in rodents, in a topographic standard very similar to idiopathic PD, but the injury differs from the real injury of the PD, and its mechanism is unclear (Rodriguez et a., 2001; Dauer and Przedborski, 2003). 6-OHDA is an animal model of PD that is used to screen new therapeutic drugs for PD through their capacity to induce rotational behaviour. These results indicate that the infusion of 6-OHDA caused severe damage, indicated by the decrease in the frequencies of locomotion and rearing.

It was verified in this study, that 6-OHDA rats presented an increase in the immobility time 1 and 3 days after surgery. Moreover, the locomotion and rearing frequencies were also reduced in the rats of this group 1 and 3 days

after lesion. Taken together, these results indicate a motor impairment that is directly correlated to the hypokinesia observed in PD patients. Thus, these data are in accordance with a previous study conducted by Lima et al. (2006).

In the brain, 6-OHDA is associated with loss of functions and behaviors related to catecholaminergic activity (Andén et al., 1964; Breese and Taylor, 1970; Ungerstedt, 1971). Administered unilaterally into the nigrostriatal pathway, the lesion leads to restricted ipsilateral damage and the animals do not present deficits in ingestive behaviors (Ungerstedt, 1971).

The lesion of the nigrostriatal pathway by 6-OHDA causes a motor asymmetry that results in robust and dose-dependent contralateral turning behavior when these animals are challenged with DA receptor agonists, like apomporphine (Ungerstedt, 1968; Ungerstedt and Arbuthnott; 1970; Hefti et al., 1980; Przedborski et al., 1995; Deumens et al., 2002). The ipsilateral rotation occurs when indirect DA agonists produce a DA imbalance through activation of the intact nigrostriatal terminal. Contralateral turning behavior induced by direct DA agonists is due to a stimulation of the supersensitive DA receptors localized in the lesioned nigrostriatal terminals when most dopaminergic SNpc neurons have been destroyed (Hefti et al., 1980; Deumens et al., 2002).

Neurochemical results showed that DA and HVA striatal levels increased 24 h after surgery and after that they were reduced 3 and 7 days after the 6-OHDA infusion. In this line, DOPAC levels were reduced 3 and 7 days after lesion. These data corroborate others from the literature (Da Cunha et al., 2001; Ferro et al., 2005). Lindner et al. (1999) demonstrated that young (2 months old) and middle-aged rats (12 months old) that received bilateral striatal infusions of 6-OHDA exhibited a DA depletion of 53 and 77%, respectively.

As reported previously (Da Cunha et al., 2008; Tanaka et al., 1999; Schwarting and Huston, 1996; Ungerstedt, 1968), the infusion of 6-OHDA into the rat medial forebrain bundle, caused an almost complete depletion of DA and its metabolites in the ipsilateral striatum. However, differences in the experimental schedule of dose, route and place of 6-OHDA infusion may contribute to small or moderate lesions (20-40%) (Przedborski et al.,1995; Kirik et al.,1998; Blandini et al., 2007).

Cytotoxicity of 6-OHDA is due to its pro-oxidant activity; the toxin undergoes rapid auto-oxidation in the extracellular space, thus promoting a high rate of reactive oxygen species formation (Hanrott et al., 2006; Blandini et al., 2007). The 6-OHDA leads to the generation of reactive oxygen species (ROS) leading to degeneration of catecholaminergic neurons, but can also damage the activity of protease (Vercammen et al., 2006). The elevation of ROS production by 6-OHDA was less than that by H2O2. Although the precise mechanism of 6-OHDA-mediated cell death has not been clarified, our previous study demonstrated that its cytotoxicity is mediated by its oxidized product, *p*-quinone in SH-SY5Y cells (Izumi, et al., 2005). Interestingly, the current data indicated that nigral GSH from 6-OHDA-lesioned rats did not differ from control groups.

Thus, this model is valuable as a model of an end stage of PD (Deumens et al., 2002) and also for testing new drugs and therapeutic strategies to minimize this neurodegenerative syndrome; therefore this model also presents a good face and predictive validity.

For the last years, neuroinflammation is believed to be involved in the pathogenesis of PD (Gebicke-Haerter, 2001). Epidemiological studies suggest

that inflammation increases the risk of the development of a neurodegenerative condition such as Alzheimer's disease and PD (Wyss-Coray and Mucke, 2000).

LPS, a highly conserved cell wall component in gram-negative bacteria, can initiate a signaling cascade that induces the expression of inflammatory genes, including those that encode cytokines such as interleukin (IL)-1b, IL-6, and TNF-α (Anisman et al, 2008). It has been reported that several enzymes, including COX and nitric oxide synthase, which are induced during LPS-induced inflammation, contribute to tissue injuries and cell death induced by LPS (Togbe et al, 2007; Giulian et al., 1996). In this line, the damage to the dopaminergic neurons in the SN was permanent, as observed 1 year after LPS injection (Herrera et al., 2000).

In the current study, LPS-lesioned rats did not exhibit motor impairment when they were observed in an open–field. On the other hand, DA and DOPAC striatal levels were reduced 7 days after surgery. HVA striatal levels increased 24 h after the toxin infusion. Otherwise, in the present data, LPS caused an increase in DA levels 3 days after surgery, which could be viewed as a compensatory reaction of dopaminergic neurons that survived the lesion. Thus, Lima et al., (2006) demonstrated an LPS induced reduction of TH protein content in the SN, but not in the striatum, which can indicate an impairment of DA production and, consequently, their transmission.

Several studies have shown that nigral LPS injection results in the reduction of DA and its metabolites besides the reduction of nigral TH-positive neurons (Castãno et al., 1998; Herrera et al., 2000; Iravani et al., 2002). DA neurons were sensitive to LPS *in vivo* and this sensitivity occurs exclusively when LPS is injected into the SN (Castãno et al., 1998).

Microglial activation can be triggered by pathogenically-modified activation of central nervous system proteins, antigens from infection agents such as LPS, prion proteins, or by a complex combination of molecules including ATP, cAMP, IL-1β, IL-6, and IL-10 (Hanisch, 2002; Nakamura, 2002). There is an increase in reactive microglia in the striatum and SN of patients with idiopathic PD (McGeer et al., 1988; Mirza et al., 2000). In postmortem investigations of humans exposed to MPTP, activated microglia have been detected 16 years after the last drug exposure (Langston et al., 1999). Activated microglia and dopaminergic cell loss were also found in the SN of primates years after they were treated with MPTP (McGeer et al., 2003). Moreover, a transient microglial reaction was verified from the 1st until the 14th day in the SN and striatum of mice that had received intraperitoneal MPTP injection (Kohutnicka et al., 1998). These findings suggest that microglia plays an active role in the pathology of PD and it may indeed perpetuate the degeneration of dopaminergic neurons once activated (Hald and Lotharius, 2005). Thus, the brain area that encompasses the SN has the highest density of microglia (Kim et al., 2000).

From another point of view, the decrease in GSH levels may be a particularly important component of the cascade of events leading to cell death because it occurs in the presymptomatic stage of PD and may directly induce nigral cell degeneration or render neurons susceptible to the actions of toxins (Jenner and Olanow, 1998).

PD patients show a marked depletion of an approximately 40% nigral GSH depletion (Perry et al., 1982). The reduction in nigral GSH content in PD reflects impairment of a major defense system against oxidative stress. It

emphasizes the potential importance of GSH in the pathogenesis of PD (Pearce et al., 1997). Our data presented in Figure 2 indicates that nigral GSH depletion was observed in LPS-lesioned rats 1, 3 and 7 days after surgery. This result corroborates others from literature. In this way, Tyagi et al. (2008) showed that LPS produced an increase in proinflammatory cytokines, malondialdehyde and a decrease in GSH level 24 h in rat brain.

The mechanism responsible for GSH depletion in the nigra is not known (Zeevalk et al., 2008). Since animals prenatally exposed to LPS had higher levels of TNF-α, increased numbers of activated microglia, and a lower ratio of GSH/GSSG relative to controls at baseline, it is possible that this pre-existing neuroinflammation altered the second inflammatory response in such a way that it produced progressive DA neuron loss (Ling et al., 2006).

Taken together, the data suggesting that the infusion of MPTP into the SNpc can be considered an adequate animal model in the investigation of the impairment of motor activity, DA turnover reduction and GSH depletion associated with PD and may mimic the early stage of PD patients. According to the literature data, 6-OHDA infusion is valuable as a model of an end stage of PD and also for testing new drugs. The current paper demonstrated that this toxin also reproduced some motor signs and reduction of DA levels as expected for a PD model. Surprisingly, 6-OHDA did not change striatal GSH levels. Finally, LPS infusion did not cause motor impairment in rats but reduced DA striatal and GSH nigral levels.

In conclusion, the available animal models of PD have contributed to our better comprehension about the pathophysiology and new therapeutic strategies. These data suggest that all models are able to mimic features of PD,

but each with its merits and limitations. However, the current data indicated that MPTP model was the best animal model because it mimics all the behavioral and neurochemical parameters as expected from a good animal model of PD.

Experimental procedures

Animals

Male Wistar rats from our breeding colony weighing 280–320 g at the beginning of the experiments were used. The animals were randomly housed in groups of six in polypropylene cages with wood shavings as bedding and maintained in a temperature-controlled room (22±2 °C) on a 12-h light-dark cycle (lights on at 7:00 a.m.). The animals had free access to water and food throughout the experiment. The studies were carried out in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals, United States National Institutes of Health. In addition, the protocol complies with the recommendations of Universidade Federal do Paraná and was approved by the University Ethics Committee (Number 0145).

Drugs

The animals were anesthetized with equitesin (0.3 ml/kg). MPTP–HCl (100 µg in 1 µL of saline; Sigma, St. Louis, MO, USA), 6-OHDA (6 µg in 2 µL of artificial cerebrospinal fluid, a CSF, supplemented with 0.2% ascorbic acid; Sigma) and LPS (2 µg in 1 µL of saline; from Escherichia coli, serotype 0111:B4; Sigma) were bilaterally administered.

Experimental protocols

In the first study, to evaluate motor behavior in the open field test, 48 animals were used, divided in 5 groups (n=8-11/group). Control or non operated animals, sham operated animals, MPTP-injected group, 6-OHDA-injected

group, and LPS-injected group. Locomotion frequency, latency to start the movement, rearing frequency, and immobility were evaluated. This procedure was performed 1, 3 and 7 days after the stereotaxic surgery.

The neurochemical investigation by HPLC-ED evaluated levels of striatal DA and its metabolites. Nigral GSH levels were also measured: Wistar rats (n= 6-9/group), control or non operated animals, sham operated animals, MPTP-injected group, 6-OHDA-injected group, and LPS-injected group. The striatum and the SN of the two hemispheres were dissected from the anterior part and mid brain of the same brains.

Stereotaxic surgery

The animals were anesthetized (intraperitoneal - i.p.) and were bilaterally administered through a 30-gauge stainless steel needle at a rate of 0.33 µL/min, for 3 min, according to the following coordinates adapted from the atlas of Paxinos and Watson (1986): anteroposterior (AP): −5.0 mm from the bregma; mediolateral (ML): ±2.1 mm from the midline; dorsoventral (DV): 8.0 mm from the skull. Animals from the sham groups were submitted to the same general procedure but did not receive MPTP, 6-OHDA, or LPS infusion, and control animals were not operated upon. After surgery, the animals were left in a temperature-controlled chamber until they recovered from anesthesia.

Open-field

To determine behavioral alterations within these three models of Parkinson’s disease, we resorted to the open-field as a test of general activity. The apparatus consisted of a rectangular box (40×50×63 cm) whose floor was

divided into 20 (10×10) small rectangles. The animals were gently placed in the right corner of the open field and were allowed to freely explore the area for 5 min. The frequency of locomotion (number of crossings from one rectangle to the other) and rearing (number of times the animals stood on their hind paws) were determined. Hand-operated counters were used to score immobility time (number of seconds of lack of movement during testing). The open field was washed with a 5% water–alcohol solution before behavioral testing to eliminate possible bias due to odors left by previous rats.

Determination of dopamine and metabolites by HPLC-ED.

The endogenous levels of DA and its non-conjugated metabolites DOPAC and HVA were assayed by reverse-phase HPLC with electrochemical detection (ED). The system consisted of a Synergi Fusion-RP C-18 reverse-phase column (150 x 4.6 mm i.d., 4 μm particle size) fitted with a 4 x 3.0 mm pre-column (SecurityGuard Cartridges Fusion-RP); an electrochemical detector (ESA Coulochem III Electrochemical Detector) equipped with a guard cell (ESA 5020) with the electrode set at 350 mV and a dual electrode analytical cell (ESA 5011A); a LC-20AT pump (Shimadzu) equipped with a mannual Rheodyne 7725 injector with a 20μl loop. The column was maintained inside a temperature-controlled oven (25ºC - Shimadzu). The cell contains two chambers in series: each chamber includes a porous graphite coulometric electrode, a double counter electrode and a double reference electrode. Oxidizing potentials were set at 100 mV for the first electrode and at 450 mV for the second electrode. DA and metabolites were detected at the second electrode. The tissue samples were homogenized with an ultrasonic cell

disrupter (Sonics) in 0.1 M perchloric acid containing sodium metabisulfite 0.02% and internal standard. After centrifugation at 10.000 G for 30 min, 4ºC, 20 μl of the supernatant was injected into the chromatograph. The mobile phase, used at a flow rate of 1 ml/min, had the following composition: 20 g citric acid monohydrated (Merck), 200 mg octane-1-sulfonic acid sodium salt (Merck), 40 mg ethylenediaminetetraacetic acid (EDTA) (Sigma), 900 ml HPLC-grade water. The pH of the buffer running solution was adjusted to 4.0 then filtered through a 0.45 μm filter. Methanol (Merck) was added to give a final composition of 10% methanol (v/v). The peak areas of the external standards were used to quantify the sample peaks.

Glutathione levels

Briefly , a 1:6 dilution of tissue with phosphate buffer 0,1M (pH 6,5) was made and 0.5 ml of the homogenate was mixed with 12,5% trichloroacetic acid (0.5 ml) and kept in ice for 30 min for precipitation of protein. The supernatant was separated by centrifugation at 10,000rpm for 10min at 4 ◦C. Then, 50μl of the clear supernatant was mixed with 250μl of 0,1M phosphate buffer (pH 8,0) and 5μl of 5,5`- dithiobis-(2-nitrobenzoic acid) in Methanol. The reaction mixture was then incubated for 10min at 37 ◦C followed by measurement of absorbance at 412 nm in microplate reader using reduced GSH as an external standard.

Statistical analysis

Differences between groups were analyzed by one-way analysis of variance (ANOVA) with repeated measures (trials), followed by the Newman-Keuls test, with the level of significance set at $P < 0.05$.

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Table legend

Table 1: Locomotion, rearing frequencies, latency to start movement and immobility time of MPTP, 6-OHDA and LPS lesioned rats. The animals were treated with MPTP 100mg/mL, 6-OHDA 6 mg/mL and LPS 2 mg/mL and observed in open-field test 1, 3 and 7 days after the stereotaxic surgery. The values are expressed as mean ± SEM (*n* = 8-11/group). **P < 0.01 and ***P<0,001 compared to the non-operated group. Results were analyzed by ANOVA followed by the Newman- Keuls test.

Table 2: Striatal DOPAC and HVA levels after MPTP (100mg/mL), 6-OHDA (6mg/mL) or LPS (2mg/mL) analyzed 1, 3 and 7 days after de lesion. Results were analyzed by ANOVA followed by the Newman- Keuls test. Values are expressed as mean ± SEM (*n*=6-9/group). *P<0,05, **P<0,01, ***P< 0,001 compared to the non-operated group.

Figure Legends

Figure 1: Striatal dopamine levels 1 day (A), 3 days (B) and 7 days (C) after MPTP (100mg/mL), 6-OHDA (6mg/mL) or LPS (2mg/mL). Values are expressed as mean ± SEM (*n*=6-9/group). Results were analyzed by ANOVA followed by the Newman- Keuls test.**P<0,01, **P< 0,001 and ***P< 0,001 compared to the non-operated group.

Figure 2: Glutathione levels in SN of MPTP (100mg/mL), 6-OHDA (6mg/mL) or LPS (2mg/mL) lesioned rat levels 1 (A), 3 (B) and 7 days (C) after surgery. Results were analyzed by ANOVA followed by the Newman- Keuls test. Values are expressed as mean ± SEM (*n*=6-9/group). *P<0,05, **P<0,01, ***P< 0,001 compared to the non-operated group.

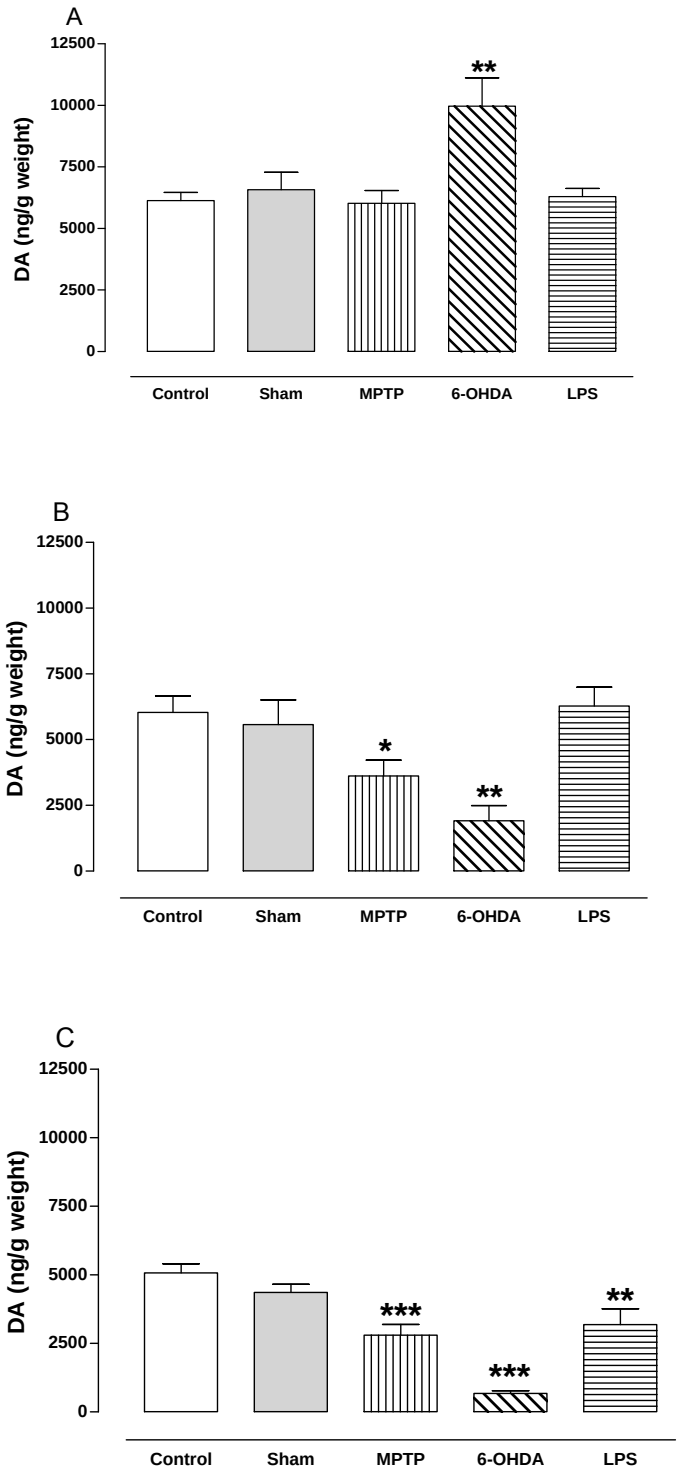
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Figure 1

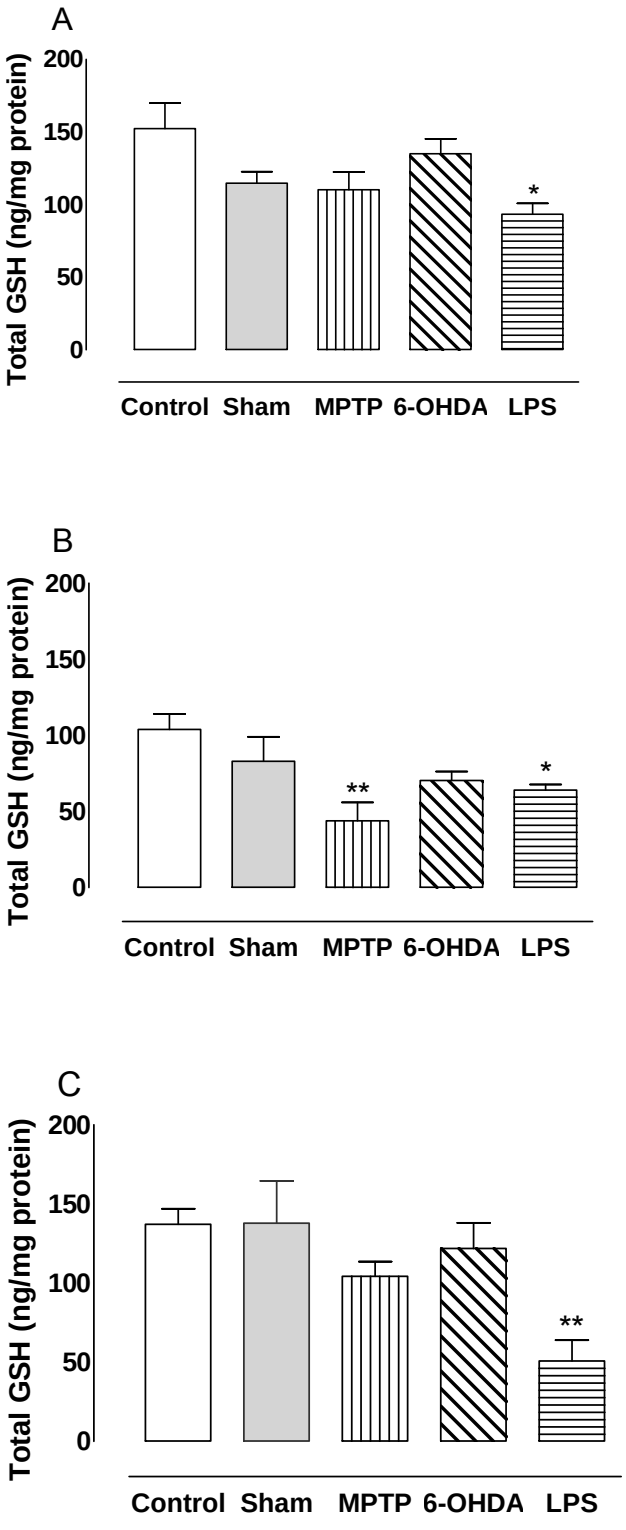
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Figure 2

Table 1

		Day 1	Day 3	Day 7
	Control	85.33±5.17	78.05±6.37	61.05±7.23
Locomotion	Sham	78.50±8.86	74.70±9.45	51.00±9.05
Frequency	MPTP	52.18±8.57**	64.54±7.28	58.63±7.38
	6-OHDA	17.22±6.63***	35.66±9.61**	36.55±11.19
	LPS	81.60±6.54	57.90±8.04	62.70±8.03
	Control	13.72±3.90	31.83±6.52	55.17±14.28
Immobility time (s)	Sham	56.90±29.57	47.90±17.58	45.50±10.80
	MPTP	26.09±11.43	28.91±8.57	39.82±10.28
	6-OHDA	131.22±30.13***	96.33±22.99**	90.33±25.34
	LPS	7.30±2.15	36.90±12.95	37.80±9.95
	Control	28.00±2.28	20.94±1.67	19.88±2.90
Rearing Frequency	Sham	21.20±3.77	19.40±4.13	12.50±2.04
	MPTP	17.90±2.37*	19.90±2.80	15.72±2.64
	6-OHDA	9.33±3.23***	10.55±2.32*	11.55±2.67
	LPS	22.00±2.99	14.90±2.22	16.50±2.15

Table 2

	Days	Control	Sham	MPTP	6-OHDA	LPS
HVA						
	1	696.87±30.81	873.82±126.65	1347.94±238.21*	1572.55±193.88**	2082.07±153.41***
	3	688.67±48.26	706.91±96.12	581.64±46.27	364.30±83.49**	841.02±89.33
	7	766.36±51.96	727.11±70.15	597.79±73.49	351.61±44.39***	577.06±80.92
DOPAC						
	1	1630.84±110.68	1827.42±374.93	2188.69±393.08	2940.19±361.64	2256.88±248.46
	3	1582.34±158.39	1514.56±194.91	1219.40±120.62	739.98±166.66**	1592.55±187.20
	7	1722.90±128.46	1565.17±147.02	1277.78±193.42	576.20±56.87***	1151.28±134.93*

4. CONCLUSÕES

Concluímos através do presente trabalho, que todos os modelos são capazes de mimetizar características da DP, mas cada um com suas particularidades e limitações.

A toxina MPTP foi capaz de provocar alterações motoras como diminuição da freqüência de locomoção e levantar 1 dia após a infusão, mimetizando características motoras iniciais da DP. Além disso, foi capaz de promover diminuição nos níveis de DA 3 e 7 dias após infusão, aumento do HVA no primeiro dia, e diminuição da GSH 3 dias após infusão.

A infusão da 6-OHDA causou severo prejuízo motor com diminuição da freqüência de locomoção e levantar, além do aumento da imobilidade nos dias 1 e 3 após a cirurgia. Os níveis de DA e seu metabólito HVA mostraram-se aumentados no primeiro dia e ao terceiro e sétimo dia a DA e seus metabólitos, HVA e DOPAC, estavam diminuídos. Surpreendentemente os níveis de GSH não se alteraram em nenhum dos tempos testados.

Não ocorreram alterações na atividade motora dos animais após infusão de LPS. Houve aumento do metabólito HVA no primeiro dia após a lesão e diminuição de DA e DOPAC no sétimo dia. Os níveis de GSH mostraram-se diminuídos em todos os dias testados.

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