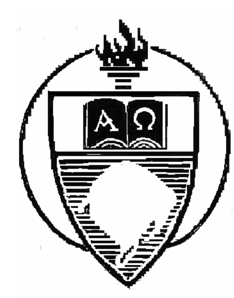

UFRGS-UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

ENVOLVIMENTO DO DANO AO DNA E ESTRESSE OXIDATIVO NO TRANSTORNO BIPOLAR E NO USO DE METILFENIDATO.

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Porto Alegre, 2008

... À minha família Armando, Marlês e Tatiane

pela dedicação, amor e compreensão.

"Não há nenhum mar inavegável, nenhuma terra inabitável."

Robert Thorne, Mercador e Geógrafo, (1527)

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PARTE I

Resumo

Embora há muito tempo se considere que alterações neurobiológicas tenham um papel central no transtorno bipolar (TB), os mecanismos moleculares ligados à sua fisiopatologia permanecem desconhecidos. A hipótese atual sugere que alterações nos circuitos cerebrais associados à regulação do humor e alterações em sistemas de sinalização intracelular associados à plasticidade e sobrevivência neuronal estão envolvidas no TB. Modelos animais são ferramentas úteis que nos permitem testar essas hipóteses e a resposta aos agentes farmacológicos. Um dos mais bem estabelecidos modelos animais de mania é o de hiperatividade induzida por psicoestimulantes. Portanto, o nosso objetivo inicial foi testar no modelo animal de mania, induzido por anfetamina, o envolvimento do dano ao DNA e do estresse oxidativo, bem como verficar o efeito do lítio e do valproato sobre esse modelo. Ainda no modelo animal, testamos se o dano ao DNA estaria envolvido também com o tratamento crônico e agudo de ratos jovens e adultos com metilfenidato. Nos pacientes com TB também avaliamos o dano ao DNA e proteínas, bem como a atividade do sistema antioxidante glutationa. No modelo animal de mania, observamos que a anfetamina é capaz de aumentar a atividade das enzimas antioxidantes, peroxidação lipídica e dano ao DNA. O lítio forneceu proteção ao dano recente ao DNA induzido pela anfetamina e o valproato apenas foi capaz de modular a atividade das enzimas antioxidantes superoxido dismutase (SOD) e catalase. No tratamento com metilfenitado, tanto os ratos jovens como os adultos também apresentaram dano recente ao DNA. Nos resultados em pacientes com TB podemos verificar que o dano permanente ao DNA não está aumentado, porém os pacientes com TB apresentam um aumento da frequência de apoptose. Verificamos ainda que o dano oxidativo a proteínas ocorre por nitração da tirosina, avaliado pelo imunoconteúdo de 3-nitrotirosina, e que este pode ativar a enzima glutationa-S-transferase, aumentada nos pacientes bipolares com maior tempo de doença, indicando um mecanismo compensatório envolvido. Esses resultados apontam para um envolvimento do dano ao DNA na fisiopatologia do TB e que esse pode ser modulado por rotas ativadas pelo estresse oxidativo.

Abstract

Although neurobiological changes have long been considered to have a central role in bipolar disorder (BD), the molecular mechanisms related to the pathophysiology of this condition remain unknown. The current hypothesis suggests that alterations in neurochemistry could be associated with mood regulation and intracellular signaling systems linked to neuronal plasticity and survival are involved in BD. Animal models are useful tools that allow testing hypotheses and response to pharmacological agents. One of the most well established models of mania is psychostimulant-induced hyperactivity. Therefore, our initial objective was assessing DNA damage and oxidative stress in an animal model of amphetamine-induced mania, in addition to verifying the effect of lithium and valproate in this same model. Still in this paradigm we tested if DNA damage is also associated with acute and chronic methylphenidate exposure in young and adult rats. In patients with BD we also assessed DNA and protein damage, as well as activity of the glutathione antioxidant system. In the animal model of mania we observed that amphetamine can increase the activity of antioxidant enzymes, lipid peroxidation and DNA damage. Lithium provided protection against recent amphetamine-induced DNA damage and valproate was only able to modulate the activity of SOD antioxidant enzymes and catalase. Both young and adult rats displayed DNA damage after amphetamine exposure. In patients with BD, we observed that permanent DNA damage is not increased, but they present a higher frequency of apoptosis. It was also verified that oxidative damage to proteins ensues via thyrosine nitration, assessed by 3-nitrotyrosine immunocontent. Possibly this damage activates the GST enzyme, which levels are raised in patients with bipolar disorder with a longer disease evolution, indicating that a compensatory mechanism is implicated. This results point to the importance of DNA damage in the pathophysiology of bipolar disorder, which is possibly mediated by routes activated by oxidative stress.

LISTA DE ABREVIATURAS

ANF = d-anfetamina

BDNF = fator neurotrófico derivado do cérebro (do inglês *brain-derived neurotrophic factor*)

CREB = elemento responsivo ao cAMP (do inglês *cAMP response element binding*)

COMT = catecol-metiltransferase

DA = dopamina

ERO= Espécies Reativas do Oxigênio

GPx = Glutationa peroxidase

GR = glutationa reductase

GSH = glutationa

GSK-3b = glicogênio sintase cinase 3-beta (do inglês *glycogen synthase kinase 3-beta*)

GSSG = glutationa oxidada

GST = glutationa S-transferase

5-HTTR= receptor do transportador de serotonina

Li^+ = lítio

MDA = malondialdeido

MAO = mono-aminotrasferase

NAD = Nicotina adenosina difosfato

NMDA = N-methyl-D-aspartic acid

NGF = nerve growth factor

NO• = oxido nítrico

NOS = oxido nítrico sintases

NOSn= oxido nítrico sintase neuronal

$O_2^{-\bullet}$ = radical superóxido

O_2^1 = oxigênio singlet

$OH^\bullet$ = radical hidroxil

$ONOO^-$ = peroxinitrito

PET = tomografia por emissão de pósitrons

RL = Radicais livres

RO• = radical peroxil

SOD = superóxido dismutase

TDAH = transtorno de déficit de atenção e hiperatividade

TB = transtorno bipolar

TBA = ácido tio-barbitúrico

TBARS = Espécies reativas do ácido tio-barbitúrico (do inglês, *thiobarbituric acid reactive species*)

UB = ubiquinona

VPT = valproato

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1. INTRODUÇÃO

O Transtorno Bipolar (TB) acomete de 1-3% da população em todo o mundo e está associado a um alto índice de suicídio e desemprego (Weissman et al. 1996; Grant et al. 2005; Müller- Oerlinghausen et al. 2002). Segundo dados da Organização Mundial da Saúde, o TB é considerado uma das dez principais causas de incapacitação no mundo (Lopez e Murray 1998). O curso clínico do TB é crônico, usualmente caracterizado por períodos de exacerbação dos sintomas (episódios agudos) intercalados por períodos subsindrômicos e períodos de remissão (eutimia). Um estudo de acompanhamento de pacientes bipolares tipo I por um período médio de 13 anos observou que os pacientes permaneceram metade deste período sintomáticos (Judd et al. 2002), sendo que a persistência de sintomas subsindrômicos estão associados a um maior risco de reagudização da doença (Perlis et al. 2006) e maior índice de incapacitação (Judd et al. 2005).

Do ponto de vista do diagnóstico, os autores pressupõem que a ocorrência de pelo menos um episódio maníaco ou hipomaníaco durante a vida é suficiente para a identificação do TB, na qual a presença de episodio maníaco confere o diagnóstico de TB tipo I, enquanto a presença de episodio hipomaníaco confere o diagnóstico de TB tipo II (Belmaker, 2004). A presença de um episódio maníaco é definida por uma elevação persistente do humor (humor eufórico ou irritável), acompanhado por pelo menos 3 dos seguintes sintomas (4 se humor irritável):

a) aumento da autoconfiança ou grandiosidade;

b) taquilalia ou pressão por falar;

c) diminuição da necessidade do sono;

d) pensamento acelerado ou fuga de idéias;

e) distratibilidade;

f) alteração do comportamento dirigido para atividades prazerosas, freqüentemente imprudentes ou perigosas;

g) ou agitação psicomotora.

O episódio deve ser suficientemente severo para causar prejuízo significativo no âmbito familiar, social ou ocupacional, ou a necessidade de hospitalização ou ter a presença de sintomas psicóticos (American Psychiatric Association 2000). Devido ao curso crônico e à alta recorrência e severidade dos sintomas de humor, o tratamento do TB baseia-se no manejo dos episódios agudos e no tratamento de manutenção como prevenção para ocorrência de novos episódios (Yatham et al. 2005). Entretanto os episódios podem ser incapacitantes, assim, estudos mostram que a demora no diagnóstico e o número maior de crises refletem ou prognosticam uma piora cognitiva e clínica geral do paciente bipolar.

Além disto, estudos ainda indicam que alterações neuroquímicas induzidas pela mania estão associadas a surgimento de efeitos lesionais em células neurais (Post et al., 1982; Friedman et al., 1993; Johnston-Wilson et al., 2000). Ou seja, a conduta para que os pacientes se mantenham eutímicos o maior tempo possível tem um efeito de proteção neuronal, na medida em que durante as fases maníacas e depressivas os mesmos estão mais suscetíveis aos efeitos de danos ao DNA (Andreazza et al., 2006). Entretanto, os índices de recorrência e de resistência aos medicamentos de primeira linha são bastante elevados. Dois estudos que avaliaram indivíduos bipolares tratados em instituições acadêmicas demonstraram que uma alta porcentagem dos pacientes permanece sintomática, mesmo quando “adequadamente tratados” (Post et al., 2003; Dennehy et al., 2005). Embora os medicamentos de última geração possuam um melhor perfil de tolerabilidade e segurança em relação aos tradicionais, muito pouco se agregou, do ponto de vista da eficácia, em relação aos primeiros medicamentos (Castrén, 2005). Possivelmente este pequeno avanço no tratamento do TB se

deve ao pouco conhecimento que ainda se tem acerca dos mecanismos fisiopatológicos envolvidos neste transtorno (Zarate, Singh e Manji 2006). Logo, estudos que se proponham a avaliar os mecanismos fisiopatológicos envolvidos no TB, bem como o mecanismo farmacológico das medicações utilizadas para o TB tornam-se de extrema importância.

1.1 Fisiopatologia do TB

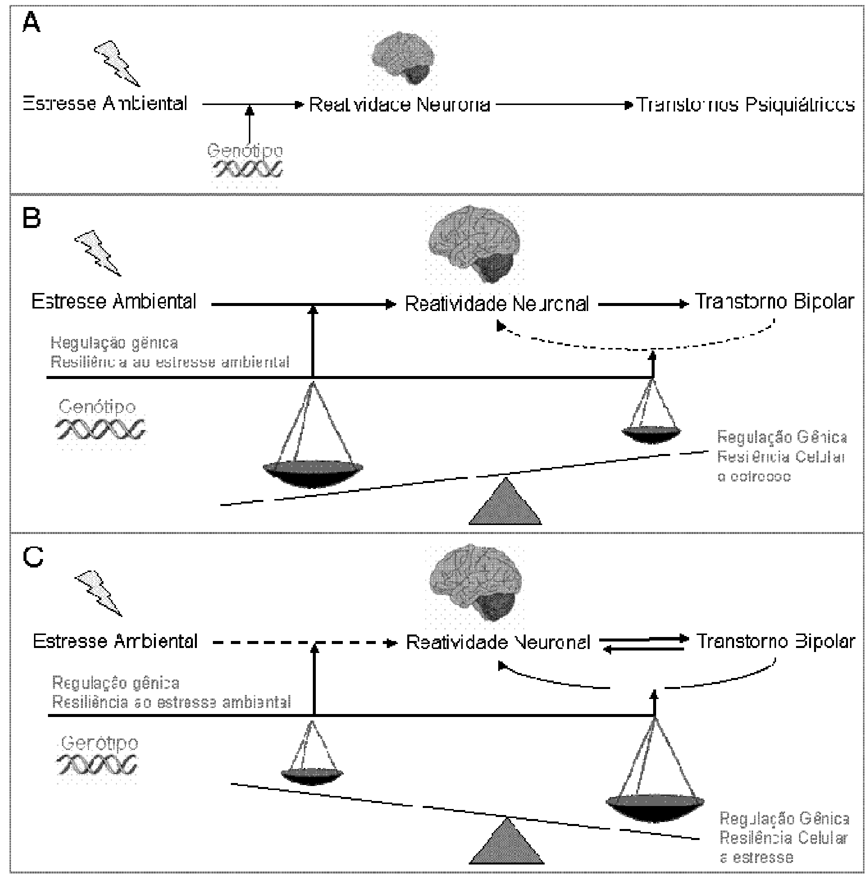
Apesar dos crescentes esforços para o entendimento da neurobiologia do TB, sua exata fisiopatologia permanece indeterminada. As bases biológicas do TB incluem estudos relacionados à genética, às vias neuro-hormonais, à neurotransmissão, às vias de transdução de sinal, à regulação da expressão gênica, ao estresse oxidativo, entre outros (Yatham et al, 2005). As bases biológicas do TB mostram ser um quadro complexo de interação entre os múltiplos genes que causam suscetibilidade, bem como a relação destes com os fatores ambientais e suas conseqüências para o organismo.

1.1.1 Bases genéticas

Dados provenientes de estudos genéticos demonstram que o risco de um indivíduo com familiar em primeiro grau portador de TB de desenvolver a doença é cerca de 10 vezes maior do que a população em geral (Goodwin and Jamison, 1990). Além disso, estudos com gêmeos homozigóticos mostram que o risco de desenvolver TB para um indivíduo cujo gêmeo idêntico possui a doença é de 67% (Goodwin and Jamison, 1990). Embora o TB apresente um elevado padrão de herdabilidade, a busca por genes de suscetibilidade tem demonstrado que a maioria das pesquisas, envolvendo estudos de genes únicos, apresenta resultados negativos. Resultados de meta-análise sugerem, entretanto, que alguns genes funcionais apresentam modesta, mas significativa associação com aumento de suscetibilidade para TB (Craddock e Forty,2006). São eles: o gene do transportador de serotonina (5-HTTR), o gene da enzima que degrada as catecolaminas, catecol-O-metiltransferase (COMT) o gene

da enzima que degrada as monoaminas, monoamine oxidase A (MAOA) e o gene do fator neurotrófico derivado do cérebro (BDNF) (Cradock e Forty, 2006). De fato, esses achados sugerem que a suscetibilidade para TB pode estar associada a genes diretamente envolvidos com os sistemas de neurotransmissão (5-HTTR, COMT e MAOA) e maturação, sobrevivência e plasticidade neuronal (BDNF) (Coyle e Duman, 2002).

Mais recentemente, resultados de estudos de epigenética têm apresentado um meio extremamente promissor na busca de alterações genéticas causadas pelos mais variados estressores (Veldic, et al, 2007). Especificamente, epigenética se refere à regulação adquirida de determinadas funções genéticas através de modificações no DNA e em histonas, incluindo alterações da expressão gênica (Mill et al, 2008). Estudos demonstram que o estresse ambiental pode induzir modificações em histonas, alterações de expressão gênica e, subseqüentemente, desencadear estados de depressão (Mill et al, 2008). Com relevância no TB, tem sido demonstrado que os estabilizadores de humor lítio e valproato aumentam a atividade do promotor IV do gene do BDNF, possivelmente através da inibição da GSK-3b (glicogênio sintase cinase-3b) e da enzima histona desacetilase, respectivamente (Veliedic et al, 2007). Este estudo sugere que o desenvolvimento de medicamentos capazes de inibir a proteína GSK-3b e a enzima histona deacetilase são potenciais candidatos a estabilizadores de humor (figura 1).



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1.1.2 Alterações Estruturais e Neuroquímicas

Estudos neuroanatômicos, baseados nas imagens obtidas por ressonância magnética, demonstram alterações do volume de determinadas regiões cerebrais envolvidas na regulação do humor. Achados que têm sido replicados no TB incluem:

a) diminuição do volume do córtex pré-frontal subgenual (Drevets et al, 2001). O córtex pré-frontal de pacientes bipolares contém menos neurônios não-piramidais, particularmente aqueles com fenótipo GABAérgico (Knable 1999; Beasley et al, 2002).

b) aumento do volume da amígdala e do estriado (Hajek et al, 2005; Strakowski, et al, 2005).

Já os estudos neurofuncionais, baseados em ressonância magnética funcional e tomografia por emissão de pósitrons (PET), apontam para uma diminuição significativa do metabolismo do córtex pré-frontal durante a depressão e subseqüente aumento em algumas regiões do córtex pré-frontal durante a fase maníaca (Malhi et al., 2004; Strakowski, DelBello e Adler, 2005). Além disso, diversos estudos de neuroimagem têm sugerido níveis aumentados de dopamina durante episódios de mania e que os efeitos anti-maníacos estão associados à diminuição de dopamina (para revisão ver Yatham, 2005 e Berk et al, 2007). Um estudo de PET demonstrou que o tratamento com valproato de sódio reduziu a captação de 18F-DOPA no estriado de pacientes bipolares em episódio maníaco, sugerindo uma diminuição da função dopaminérgica pré-sináptica após o uso de valproato de sódio (Yatham et al., 2002). Alterações de receptores dopaminérgicos no TB têm sido demonstradas em dois estudos que observaram um aumento de 25% da expressão do RNAm do receptor dopaminérgico D1 na região CA2 do hipocampo (Pantazopoulos et al., 2004) e uma menor expressão do receptor D3 em linfócitos de indivíduos bipolares (Vogel et al., 2004). Mais recentemente, foi sugerido que variações do gene do transportador de DA podem estar envolvidas na suscetibilidade para o desenvolvimento do TB (Greenwood et al., 2006). Em conjunto, esses estudos indicam que alterações do sistema dopaminérgico podem estar envolvidas nos mecanismos fisiopatológicos do TB. Interessantemente, o aumento dos níveis

de dopamina é importante fonte de estresse oxidativo no cérebro, devido ao metabolismo de oxidação da dopamina (Rees et al, 2007; Chen et al, 2008).

É importante salientar que indivíduos com TB apresentam diminuição da arborização de dendritos apicais, bem como diminuição da densidade de espinhas dendríticas em neurônios piramidais do hipocampo (Rosoklija et al., 2000), bem como diminuição do número de neurônios não-piramidais na região CA2 e também no hipocampo (Benes et al., 1998). Uma avaliação mais detalhada dessas alterações neuropatológicas sugere que estas modificações seguem um padrão de alteração no desenvolvimento neuronal e de neuroplasticidade, e não um padrão de degeneração cerebral como previamente se pensava (Rajkowska, 2003). Nesta mesma linha, estudos conduzidos em tecido post-mortem revelaram que indivíduos bipolares apresentam uma diminuição significativa de células neuronais e gliais no córtex pré-frontal dorsolateral e subgenual (Rajkowska, Halarie e Selemon, 2001; Öngur, Drevets e Price, 1998; Bouras et al., 2001).

Em paralelo, tem sido demonstrado que antidepressivos e estabilizadores de humor atuam modulando diversas cascatas de sinalização celular envolvidas em neuroplasticidade e sobrevivência neuronal (Manji, Drevets e Charney, 2001; Schaltiel, Chen e Manji, 2006). A primeira evidência surgiu dos trabalhos do laboratório do Prof. Ronald Duman, demonstrando que o uso crônico de antidepressivos e de choques eletroconvulsivos aumentam a expressão do RNAm do fator neurotrófico derivado do cérebro (BDNF), do seu receptor TrkB (receptor tirosina-cinase B) e do fator de transcrição nuclear CREB (elemento responsivo ao cAMP) em hipocampo de ratos (Nibuya et al, 1995; Nibuyaet al, 1996). Mais recentemente, estudos pré-clínicos têm demonstrado que o tratamento crônico com Li ou VPT (medicamentos de primeira linha para o tratamento do TB) também são capazes de aumentar a expressão do BDNF no córtex frontal e hipocampo (Einat et al., 2003; Fukumoto et al., 2001).

Além disso, os estabilizadores do humor possuem outros mecanismos de ação em comum, como inibição da GSK-3b. A proteína GSK-3b está envolvida em uma série de processos que regulam a arquitetura e a função celular. Por exemplo, a GSK-3b regula a atividade de diversas proteínas que se ligam aos microtúbulos, que são complexos protéicos que dão estabilidade estrutural para a célula (Coyle e Manji 2002; Williams et al., 2002). Dessa forma, ao modular a atividade de proteínas ligadas aos microtubulos, a GSK-3b é capaz de alterar a plasticidade neuronal, crescimento dendrítico e, por conseqüência, produzir uma marcada alteração na plasticidade sináptica (Coyle e Manji 2002; Williams et al., 2002). Além disso, também regula fatores de transcrição celular, como o CREB, que estão envolvidos com a regulação da neurogênese e morte/sobrevivência celular (Castrén, 2005; Coyle e Duman, 2003; Nestler et al., 2002; Manji et al, 2001). O envolvimento da GSK-3b no TB é uma importante contribuição do grupo de Housseini Manji. Este autor e seus colaboradores iniciaram com trabalhos mostrando que o lítio é um potente inibidor da GSK-3b tanto *in vitro* como *in vivo* (Manji et al, 2006). Mais tarde, estudos subseqüentes demonstraram que o valproato também é um potente inibidor da GSK-3b, o que propõe um mecanismo de ação em comum entre esses estabilizadores de humor, que possuem estruturas farmacológicas marcadamente distintas (De Sarno e Jope, 2000). Mais recentemente, estudos em modelos animais demonstraram que camundongos geneticamente alterados que possuem um *deficit* marcado na transmissão serotoninérgica apresentam um aumento da atividade da GSK-3b e este aumento é associado a alterações comportamentais compatíveis com quadros depressivos (Beaulieu et al, 2008). Além disso, Gould e Manji (2002) demonstraram que o lítio, quando em concentrações próximas ao limiar de concentração terapêutica (1-2mM), estimula a neurogênese em células progenitoras hipocampais adultas. Tal efeito aparentemente envolve a inibição da GSK-3b e ativação da via intracelular Wnt e B-catenina, mas independe da inibição da via do fosfatidilinositol. Além disso, mais rececentemente

Gould et al (2007) mostraram que camundongos com super-expressão de B-catenina apresentam comportamento semelhante ao observado com administração de lítio. Em conjunto, estes trabalhos sugerem fortemente que a ativação da via Wnt e a inibição da proteína GSK-3b podem estar envolvidas nos efeitos terapêuticos dos estabilizadores de humor.

Atualmente acredita-se que os transtornos de humor estão associados a alterações no sistema de comunicação entre os circuitos cerebrais reguladores do humor, e que antidepressivos e estabilizadores do humor ativam cascatas de sinalização que regulam a plasticidade e sobrevivência celular, com subseqüente melhora gradativa da transmissão da informação nesses circuitos cerebrais (Castrén, 2005; Coyle e Duman, 2003; Nestler et al., 2002; Manji et al, 2001).

1.1.3 Estresse Oxidativo e Disfunção Mitocondrial no TB

O termo radical livre refere-se a um átomo ou molécula altamente reativos que contêm um número ímpar de elétrons em sua última camada eletrônica (Halliwell e Guteridge 1999). Os principais radicais livres e suas principais funções estão relacionados na Tabela 1.

Tabela 1: Principais espécies reativas do oxigênio, nitrogênio e outras. (Tabela adaptada do livro Radicas Livres de Halliwell e Gutteridge 2000).

Principais Espécies Reativas[a]		
Espécies reativas do Oxigênio [b]		
Radical superóxido	$O_2^{\bullet}$	Radical mais abundante nas células, gerado por reações de autoxidação, como a cadeia de transporte de elétrons, algumas enzimas como a xantina oxidase ou pode ser formado por ação das células fagocitárias.
Peróxido de hidrogênio (não-radicalar)	H_2O_2	Gerado principalmente na matriz mitocôndrial durante o processo de redução do O_2 a H_2O. Atua como segundo mensageiro intra e intercelular ativando o fator de transcrição NF-κB em algumas células.
Radical hidroxila	$OH^{\bullet}$	É extremamente reativo, podendo lesar DNA, proteínas, carboidratos e lipídios. Possui uma meia vida de $7x10^{-4}$ s. A capacidade desse radical em lesar as células aumenta visto que o organismo não possui uma defesa especifica para esse radical.
Oxigênio Singlet (não-radicalar)	O_2^1	Esse pode ser gerado por indução luminosa, reações catalisadas por peroxidases ou por células fagocíticas. Pode lesar o DNA diretamente.
Espécies Reativas do Nitrogênio		
Oxido Nítrico (radical)	$NO^{\bullet}$	Possui um importante papel no cérebro na vasoregulação e na neurotransmissão. Não possui alta reatividade, porém quando em excesso pode reagir com o $O_2^{-\bullet}$ e formar o radical $ONOO^-$.
Peroxinitrito (não-radicalar)	$ONOO^-$	Esse radical pode causar a oxidação e a nitração de lipídios, DNA e resíduos de aminoácidos de proteínas.
Outras espécies ou radicais livres		
Radicais do Enxofre	$S^{\bullet}$	Os radicais do enxofre são formados quando um grupo tiol (-SH) reage com vários radicais do oxigênio ou com metais de transição (Fe^{3+}). A homocisteína, por sua vez, é capaz de gerar radicais tióis.
Radicais Lipídicos	$RO^{\bullet}$[c], $ROO^{\bullet}$	Os radicais alcoxil ($RO^{\bullet}$) e peroxil ($ROO^{\bullet}$) possuem reatividade intermediária. O $ROO^{\bullet}$ pode dar início a reações em cadeias de RL, sendo particularmente importante quando ocorrem em lipídios.

[a]As espécies reativas envolvem os radicais livres e os não-radicais.
[b]As ERO são formadas principalmente na cadeia de transporte de elétrons,com exceção do oxigênio singlet.
[c]Utiliza-se o símbolo R para representar de modo geral qualquer grupo alquila.

Nos mamíferos são produzidos radicais livres de carbono, enxofre, nitrogênio e oxigênio, mas os que ganham mais destaque, devido à reatividade e aos danos que podem causar, são os radicais derivados do oxigênio. O termo espécies reativas do oxigênio (ERO) inclui não somente radicais livres, mas também, espécies não radicalares derivadas do oxigênio, como por exemplo, o peróxido de hidrogênio, capaz de levar a formação do radical hidroxila, quando em presença de metais (Halliwell 2000, Halliwell 2001, Shao et al 2002). As ERO e outros radicais livres podem ser provindos de fontes exógenas ou endógenas. Na célula, as ERO são produzidas como conseqüência do metabolismo celular normal (Halliwell 2001). As principais fontes endógenas são a cadeia de transporte de elétrons mitocondrial, a degradação de ácidos graxos nos peroxissomos, os mecanismos de detoxificação mediados pelo complexo enzimático citocromo P-450, o processo de fagocitose e a oxidação de pequenas moléculas como hidroquinonas, ferrodoxinas reduzidas e catecolaminas, entre outras (Salvador et al 2004; Andreazza et al 2004).

O nosso organismo dispõe de um sistema celular de defesa contra as ERO produzidas no organismo (Bonnefoy et al 2002). São as chamadas defesas antioxidantes primárias, que incluem as enzimas superóxido dismutase (SOD), glutationa peroxidase (GPx), catalase (Cat), glutationa- S-transferase (GST) e outras que não participam diretamente do processo, mas fornecem suporte à GPx, como a glutationa redutase (GR) (Halliwell e Gutteridge, 2000; Droge, 2002) (Figura 2). Além das defesas enzimáticas existem ainda antioxidantes não enzimáticos, como a glutationa, vitamina E (α-tocoferol), vitamina C (ácido ascórbico), flavonóides e outras moléculas como β-caroteno e N-acetilcisteína (Borella e Varela 2004). Esses antioxidantes agem principalmente bloqueando a cadeia de peroxidação lipídica, eliminando oxigênio ou quelando íons metálicos (Sies 1993).

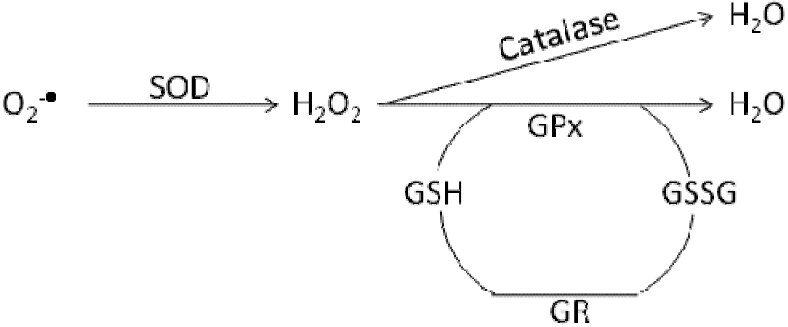

Figura 2. Esquema da atividade das principais enzimas antioxidantes. A enzima SOD é responsável por dismutar o radical superóxido ($O_2^{-•}$) e formar o peróxido de hidrogênio (H_2O_2) que será convertido em água pela ação de duas enzimas, a catalase e a glutationa peroxidase (GPx). Quando a GPx converte o H_2O_2 em água, a glutationa (GSH) é oxidada (GSSG). Assim a enzima glutationa redutase (GR) reduz a glutationa oxidada.

Em condições patológicas onde ocorre um aumento exacerbado da produção de ERO e RL, a qual sobrepõe à capacidade de proteção das enzimas antioxidantes, instaura-se no organismo uma condição denominada estresse oxidativo. Assim, existem evidências de que o estresse oxidativo possui um importante papel na patogênese de diversas doenças, principalmente em doenças neurológicas e transtornos psiquiátricos, tais como transtorno bipolar (Kologlu et al, 2002; Ranjenkar, 2003; Andreazza et al, 2007a) e esquizofrenia (Gama et al, 2006). O sistema nervoso central (SNC) é particularmente sensível ao estresse oxidativo (Mahadik et al, 2001), pois: (1) o SNC possui uma alta taxa de consumo de oxigênio. Em torno de 20% do oxigênio consumido no organismo é metabolizado no SNC, isto conduz a um aumento da atividade da cadeia de transporte de elétrons mitocondrial que por sua vez aumenta a produção de espécies reativas do oxigênio (ERO) (Halliwell 2001); (2) elevados níveis de lipídios poliinsaturados vulneráveis à peroxidação lipídica (Mahadik et al 2001). (3) o glutamato ao se ligar no seu receptor (NMDA) libera Ca^{2+} que estimula a ativação da oxido nítrico sintase neuronal (NOSn), levando à produção de NO^- (Schulz et al 1997; Schulz et al 1996), que pode reagir com o $O_2^{-•}$ levando à formação de $ONOO^-$ que ao regiar com resíduos tirosina nas proteínas forma um composto chamado 3-nitrotirosina (Beal,

2002); (4) auto-oxidação de alguns neurotransmissores, em especial a dopamina, podem levar a formação de ERO (Obata 2002).

O sistema dopaminérgico tem sido de particular interesse no entendimento do TB. Há relatos de um aumento na atividade dopaminérgica em estados de mania aguda. Os antipsicóticos que antagonizam a dopamina são notoriamente antimaníacos (Yatham, 2005). Os sintomas maníacos podem estar relacionados a um aumento na atividade do sistema dopaminérgico cerebral (Yatham, 2005). A dopamina sofre dois tipos de oxidação: enzimática, através das enzimas MAO e COMT, ou não-enzimática, também chamada de auto-oxidação (Obata, 2002; Halliwell, 2001; Asanuma et al, 2004). A auto-oxidação da dopamina ocorre em presença de íons Fe^{2+} livres e H_2O_2, promovendo a hidroxilação da dopamina na posição 6 formando a 6-hidroxidopamina que pode ser convertida, em torno de 20%, a o-quinonas (Baez 1997). O sistema mais efetivo para detoxificação da o-quinona no cérebro é através da enzima GST, a qual utiliza GSH no objetivo de formar um complexo estável com a o-quinona (Obata 2002; Baez 1997). A dopamina, quando oxidada pela enzima MAO, leva à formação do ácido 3,4 diidroxifenilacético e H_2O_2, capaz de gerar OH• através da reação com íons de ferro (Asanuma et al 2004; Obata 2002). Essa via ocorre especialmente nos gânglios da base (putamen, núcleo caudal e substantia nigra) onde existem altas concentrações de dopamina, oxigênio e ferro (Qureshi et al 2004; Obata 2002). Wang et al (2004) encontraram um aumento da atividade da GST em culturas de neurônios tratadas com valproato e/ou lítio indicando uma das vias de proteção utilizada por esses estabilizadores do humor para prevenção do estresse oxidativo, previamente estudado pelo mesmo grupo (Wang et al, 2003). Esses estudos apontam um envolvimento desse sistema na patologia do TB.

Recentemente, McQuillin et al (2006) demonstraram que o transtorno bipolar está associado com o polimorfismo do gene TRPM2 (canais permeáveis a cálcio), o qual estaá

envolvido na manutenção dos níveis intracelulares de cálcio responsivos ao estresse oxidativo. O cálcio é um importante segundo mensageiro envolvido na regulação da plasticidade sináptica em células neuronais (Chinopoulos et al, 2006). A mitocôndria é uma das organelas responsáveis por estocar o cálcio. O influxo de cálcio na mitocôndria e via sistema *uniport* e o efluxo é conduzido por poros permeáveis a cálcio, isso é mediado via potencial de membrana que é gerado durante o gradiente de prótons da cadeia de transporte de elétrons mitocondrial (Chinopoulos et al, 2006). Assim, uma das conseqüências da disfunção mitocondrial é alterar o potencial de membrana e conseqüentemente reduzir a habilidade de captar cálcio (Wang et al, 2007). Na mesma direção, Sun et al (2006) avaliaram o perfil da expressão gênica, através de análise de *microarray*, em córtex pré-frontal de pacientes bipolares. Eles mostraram que muitos desses genes possuem expressão diferenciada do grupo controle, porém a diferença mais expressiva foi encontrada nos genes regulatórios da cadeia de transporte de elétrons, sugerindo fortemente uma disfunção mitocondrial, o que poderia levar a uma produção excessiva de espécies reativas do oxigênio.

Além do papel central na produção de energia, uma das funções principais da mitocôndria é a regulação do estado redox intracelular. A principal fonte geradora de radicais livres é a cadeia transportadora de elétrons, cuja função central é a produção de energia através da fosforilação oxidativa (Adam-Vizi and Chinopoulos 2006) (Figura 3). Em situações patológicas, o excesso de radicais livres pode desencadear reações intracelulares que levam a um aumento na liberação de citocromo c, o que pode ativar a cascata de apoptose (morte programada) celular. Diversos estudos conduzidos em tecido cerebral post-mortem estão de acordo com os estudos de neuroimagem abordados na seção anterior, corroborando com a hipótese de disfunção mitocondrial no TB. Por exemplo, Konradi et al (2004) demonstraram que a expressão de genes mitocondriais está diminuída no hipocampo de pacientes bipolares em relação a voluntários normais, enquanto outros dois estudos

encontraram o mesmo resultado na região do córtex pré-frontal dorsolateral (Iwamoto et al, 2005; Sun et al, 2006). Recente estudo do nosso grupo, ainda não publicado, mostrou que o cortex pré-frontal de pacientes bipolares apresenta uma diminuição de duas vezes na atividade do complexo I da cadeia de transporte de elétrons da mitocôndria (Andreazza et al). Ainda, Benes et al (2006) demonstraram que a expressão de genes que codificam as principais enzimas antioxidantes, SOD e catalase, também está diminuída no hipocampo de indivíduos com TB.

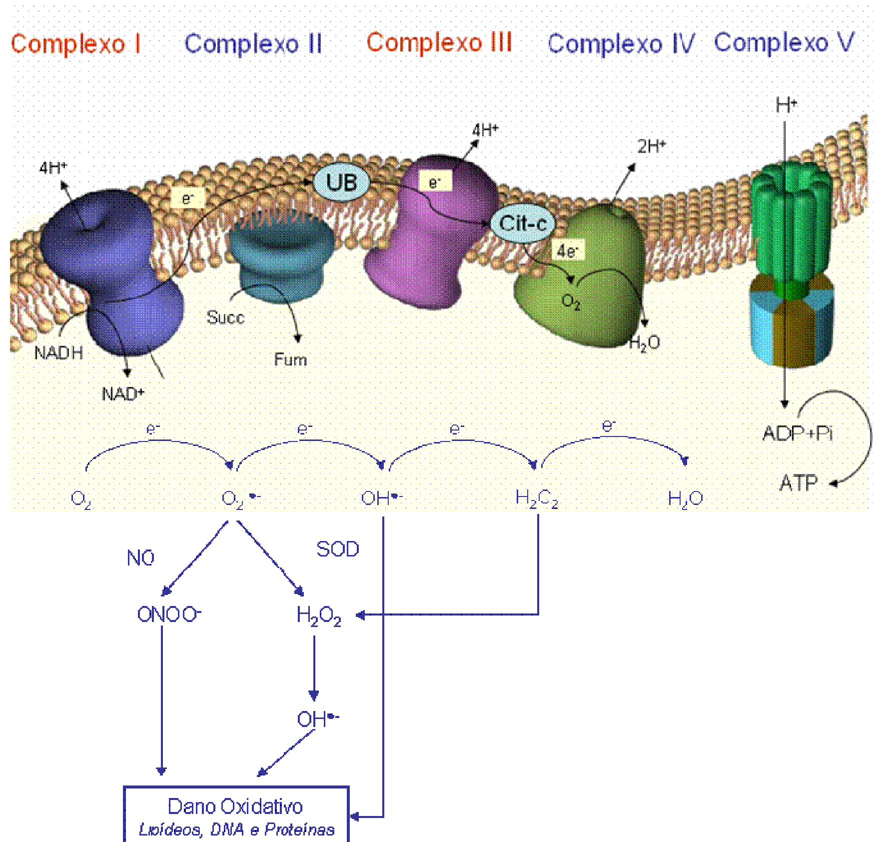

Figura 3. Cadeia de transporte de elétrons mitocondrial e formação de espécies reativas do oxigênio. A formação das espécies reativas do oxigênio (ERO) ocorre durante a transferência de elétrons através dos complexos I-IV da cadeia de transporte de elétrons. Abreviações: dinucletideo de nicotinamida e adenina reduzida (NADH); dinucleotídeo de nicotinamida e adenina oxidada (NAD); Succinato (Succ); fumarato (fum); citocromo-c (Cit-c); ubiquinona (UB); adenosina difosfato (ADP); fosfato inorganico (Pi); adenosina trifosfato (ATP); radical superóxido (O_2^-); radical hidroxil (OH^-) ; peróxido de hidrogênio (H_2O_2); óxido nitrico (NO).

Outros estudos têm demonstrado o envolvimento do estresse oxidativo na patogênese do TB, como por exemplo, pacientes bipolares apresentam alterações em hemácias (Ozcan et al., 2004; Rajenkar et al., 2003; Kuloglu et al., 2002) e no soro (Savas et al., 2006; Andreazza et al, 2007a; Frey et al, 2007) da atividade das enzimas antioxidantes: SOD, catalase e GPx. Recentemente, Gergerlioglu et al (2007) demonstrou que pacientes bipolares apresentam altos níveis de óxido nítrico. Além disso, pacientes bipolares apresentam uma alta taxa de peroxidação lipídica (Andreazza et al, 2007a ; Ozcan et al., 2004; Rajenkar et al., 2003; Kuloglu et al., 2002). É importante ressaltar que o balanço da atividade das enzimas antioxidantes séricas (superoxido dismutase / catalase + glutationa peroxidase), avaliado por Andreazza et al (2007) mostrou ser maior em pacientes deprimidos e maníacos quando comparados a eutímicos e grupo controle, porém a peroxidação lipídica, nesses pacientes, não diferiu entre as diferentes fases da doença, isso alerta para o fato de que a regulação das enzimas antioxidantes está relacionada com a fase da doença, porém o dano é permanente ao organismo estando mais relacionado ao tempo de doença.

1.1.4 Dano ao DNA

O dano ao DNA produzido por oxidação é considerado o mais significante dano oriundo do metabolismo celular. Estima-se que aproximadamente $2x10^4$ lesões oxidativas ao DNA ocorram no genoma humano por dia (Ames & Shigenoga 1992). Acredita-se que desta maneira o reparo dessas lesões possua um papel central na prevenção do aumento de mutações nos organismos vivos (Maluf, 2004). Muitas evidências sugerem que danos cumulativos ao DNA causados pelas ERO contribuam para diversas condições clínicas como câncer (Palyvoda et al 2003; Rajeswari et al 2000), esquizofrenia (Psimadas et al 2004), Alzheimer (Migliore et al 2005). Porém, devemos levar em consideração que o dano ao DNA pode ser induzido por hábitos alimentares e estilo de vida (McCord & Edeas 2005). Alguns estudos têm mostrado que o cigarro pode aumentar o dano ao DNA tanto em jovens como em

adultos (Piperakis et al 2004). Além disso, o dano ao DNA (Singh et al, 1991) e a freqüência de mutações aumentam com o envelhecimento (Betti et al 1994; McCord & Edeas 2005), isso deve-se ao acúmulo de danos e/ou pela diminuição da capacidade de reparo ao DNA.

Assim, é de conhecimento geral que os radicais livres, em especial o radical OH^-, e a espécie reativa do nitrogênio peroxinitro ($ONNO^-$), podem induzir dano ao DNA (Maluf & Erdattman, 2000). Andreazza et al, (2007b) avaliaram o índice de dano ao DNA em 30 pacientes com TB tipo I, não fumantes e que não possuíam comorbidades clínicas associadas. Eles mostraram um aumento de 10 vezes no índice de dano ao DNA desses pacientes. Atualmente ainda não temos dados na literatura que mostrem se esse dano ao DNA é reparado pelo organismo ou se tornará um dano permanente às células, no entanto, esse será um dos objetivos desta Tese. Na mesma linha de pensamento, Simon et al (2006) mostraram que pacientes com TB apresentam uma aceleração no encurtamento do telômero. O telômero é uma estrutura constituída por fileiras repetitivas de DNA que formam as extremidades dos cromossomas, tendo como principal função manter a estabilidade estrutural dos cromossomos, porém a cada vez que a célula se divide, os telômeros são ligeiramente encurtados (Lewin, 1999). O aceleramento do encurtamento dos telômeros está associado ao processo de envelhecimento (Simon et al, 2006). Um dos principais fatores associados ao aceleramento do encurtamento dos telômeros é o aumento da produção de radicais livres no organismo (Passos et al, 2007).

O dano ao DNA pode induzir à ativação do p53 o que promoverá um atraso na progressão do ciclo celular no *checkpoint* da fase G1. Este atraso pode prover tempo para o reparo no dano ao DNA, e para reparo de lesões que seriam perpetuadas como mutações em células entrando na fase S. Ou ainda, a proteína p53 parece iniciar o processo apoptótico celular em resposta a agentes que danificam o DNA (Could and Manji, 2000).

Esses estudos salientados acima apresentam fortes evidências do envolvimento do estresse oxidativo na fisiopatologia do TB. Acreditamos que os próximos estudos necessários nessa área nos auxilem a entender qual ou quais rotas bioquímicas estão associadas ao aumento do estresse oxidativo no TB, com a finalidade de, no futuro, podermos auxiliar na dininução das conseqüências do estresse oxidativo para o organismo de pacientes bipolares.

1.1.5 Modelo Animal de Mania

Embora as evidências sejam mais fortes quando os resultados são em humanos, estudos em modelos animais permitem testar e levantar hipóteses em relação aos mecanismos fisiopatológicos dos diferentes transtornos mentais. Como conseqüência, estudos pré-clínicos são ferramentais fundamentais no desenvolvimento de novos agentes terapêuticos. A escolha de um modelo animal para pesquisa de transtornos mentais baseia-se fundamentalmente no quanto a fisiopatologia do determinado transtorno é conhecida, na especificidade e eficácia dos agentes farmacológicos e na capacidade de reproduzir as alterações comportamentais em animais de laboratório (Einat et al. 2000). Até o presente, não existe um modelo animal ideal para o TB (Einat e Manji 2006; Machado-Vieira et al. 2004), uma vez que o padrão crônico e oscilatório deste transtorno, com variações comportamentais extremas e clinicamente opostas é um verdadeiro desafio a ser modelado. Portanto, a resolução deste problema tem sido o desenvolvimento de modelos animais que reproduzem determinados aspectos do quadro clínico deste transtorno. A validação de um modelo animal de transtorno mental deve seguir os 3 seguintes critérios: validade aparente (*face validity*), validade preditiva (*predictive validity)* e validade interpretativa (*construct validity*) (Willner 1986).

A validade aparente representa o quanto o comportamento do animal consegue mimetizar os sintomas de um determinado transtorno. A validade interpretativa se refere aos aspectos fisiopatológicos que teoricamente explicariam as alterações encontradas no modelo e no correspondente transtorno testado. A validade preditiva, por sua vez, avalia o quanto as

alterações comportamentais induzidas no modelo são revertidas ou prevenidas pelos agentes de primeira linha utilizados no tratamento do transtorno em questão.

Considerando que o TB caracteriza-se fundamentalmente pela presença de episódios maníacos e depressivos, e diversos modelos animais de depressão têm sido desenvolvidos (Nestler et al. 2002), modelos animais de TB têm focalizado no quadro clínico de mania. A dificuldade de desenvolver um modelo adequado de mania inicia na dificuldade de reproduzir o sintoma central, que é o humor elevado ou euforia (aqui cabe lembrar que esta mesma dificuldade existe para reproduzir o "humor depressivo"). Desta forma, modelos animais de mania geralmente avaliam atividade locomotora, agressividade, ou comportamento de risco ou recompensa (Machado-Vieira et al. 2004; Einat et al. 2000). Modelos que possuem relevância quanto ao quadro clínico do TB, mas não têm sido explorados sistematicamente com este propósito incluem comportamento sexual, julgamento e diminuição do padrão de sono.

O modelo de hiperatividade induzido por psicoestimulantes (usualmente anfetamina ou cocaína) é considerado o modelo animal de mania mais bem estabelecido até o presente (Machado-Vieira et al. 2004; Gould et al. 2004; Nestler et al. 2002). Esse modelo é baseado em dois aspectos: um deles é a hiperatividade causada pelo tratamento agudo com psicoestimulantes, que pode ser prevenido com pré-tratamento com lítio (Berggren 1985), enquanto o segundo é o modelo de sensibilização comportamental (behavioral sensitization) induzida pelo tratamento crônico com psicoestimulantes (Robinson e Becker 1986). Após extensa revisão da bibliografia sobre o assunto, nosso grupo decidiu por desenvolver um modelo animal de mania baseado na exposição crônica de d-anfetamina (ANF) em ratos, padronizado durante a tese de doutorado de Benicio Frey, desenvolvida nessa instituição.

A ANF atua principalmente facilitando a liberação de dopamina (DA) pelas vesículas pré-sinápticas, mas também age bloqueando a recaptação de DA pelos transportadores de DA

pré-sinápticos (Sulzer et al. 1995). Desta forma, a ANF aumenta significativamente o conteúdo de DA na fenda sináptica, ativando a transmissão dopaminérgica cerebral. O envolvimento da via dopaminérgica no TB é apoiado por estudos farmacológicos, genéticos, pós-mortem e de neuroimagem como discutido na seção 1.1.2. Além disso, a administração de substâncias dopaminérgicas, como ANF ou L-DOPA, induz sintomas maníacos em indivíduos com TB (Murphy et al, 1971; Gerner et al, 1976).

1.1.6 Metilfenidato

Como a ANF é uma droga com uma forte ação dopaminérgica, o nosso grupo decidiu estudar se as lesões encontradas no modelo animal mania de maina se repetiam com outras drogas estimulantes e com também conhecida ação dopaminérgica. Assim, optamos por testar o efeito da administração crônica e aguda de metilfenidato a ratos jovens e adultos.

Por mais de 50 anos o metilfenidato (Ritalina®) tem sido usado com eficácia no tratamento do transtorno de déficit de atenção e hiperatividade (TDAH). Este fármaco reduz os sintomas de desatenção, hiperatividade e impulsividade em mais de 70% das crianças tratadas (Greenhil et al., 2002; Swanson et al.,1991). No TDAH ocorre a falha na captação do neurotransmissor dopamina pelos neurônios e, assim pesquisas sugerem que o metilfenidato age bloqueando a recaptação da dopamina aumentando o nível de dopamina extracelular no cérebro, assim cortando o ciclo, com isso o neurotransmissor fica mais tempo agindo (Castellanos et al., 1996b; VolKow et al., 1994). Ainda, Volkow et al. (2002) mostraram que o metilfenidato via oral atinge seu pico de concentração no cérebro depois de 60-90 minutos, bloqueando mais de 50% do transportadores de dopamina (DAT) e aumentando significativamente o nível de dopamina extracelular nos gânglios basais. Esses estudos dão suporte, pelo menos em parte, para estudos pré-clínicos que mostram que o metilfenidato atua bloqueando os DAT, tão bem quanto os transportadores de noradrenalina (Dougherty et al.,1999; Krause et al., 2000; Solanto, 1998, Han e Gu, 2006).

A disfunção nos sistemas dopaminérgicos e noradrenérgicos tem uma função auto-reguladora como atenção seletiva (neurônios noradrenérgicos) e motivação (neurônios dopaminérgicos), as quais são implicadas na patogênese do TDAH (Dougherty et al.,1999; Solano,1998). A ação dos estimulantes no córtex pré-frontal está implicada ao desenvolvimento da sensibilização locomotora e a mudanças comportamentais, acreditando-se estar associada a certos aspectos das drogas de abuso (Vanderschurem e Kalinas, 2000). Outro aspecto importante a ser destacado são os estudos sobre os efeitos da administração crônica do metilfenidato na expressão gênica no desenvolvimento do cérebro. Recentes estudos têm mostrado o envolvimento do polimosfimo da COMT (Kereszturi et al, 2008) e da alteração da expressão genênica do DAT-1 (Ouellet-Morin et al, 2007) em pacientes com TDAH.

Pouco se conhece ainda sobre os mecanismos que contribuem para a eficácia dos estimulantes ou sobre a possível conseqüência neuroadaptacional do metilfenidato quando utilizado por um período prolongado (Safer e Allen, 1989; National Institutes Statement, 2000; Greenhill, 2001). Logo, estudos que possam mostrar as possíveis vias de atuação dos neuroestimualntes tornam-se interessantes.

1.2 Objetivos

1.2.1 Objetivo Geral

Avaliar o envolvimento do dano ao DNA, freqüência de apoptose, dano protéico e sistema antioxidante glutationa no transtono bipolar, bem como, comparar os resultados quanto ao dano ao DNA em modelo animal de mania, induzido por anfetamina, com o uso agudo e crônico de metilfenidato.

1.2.2 Objetivos Específicos

1) Caracterizar o dano ao DNA recente, através do ensaio Cometa, e o dano permanente ao DNA, pela freqüência de micronúcleos, no modelo animal de mania, induzido por anfetamina, em linfócitos e no hipocampo.

2) Caracterizar o dano ao DNA recente, através do ensaio Cometa, e o dano permanente ao DNA, pela freqüência de micronúcleos, em ratos jovens e adultos tratados aguda e cronicamente com metilfenidato, em linfócitos, estriado e hipocampo.

3) Caracterizar a freqüência de dano permanente ao DNA, através da freqüência de micronúcleos em linfócitos de pacientes com transtorno bipolar livres de comorbidades clínicas.

4) Caracterizar a freqüência de apoptose, através de conjugados de anexina-V e por coloração com 4',6-diamidino-2-phenylindole (DAPI) em linfócitos de pacientes com transtorno bipolar livres de comorbidades clínicas.

5) Avaliar o envolvimento do dano protéico, através da quantificação de grupos carbonil e da quantificação da 3-nitrotirosina no soro de pacientes bipolares no início da doença em comparação com pacientes bipolares com mais de 10 anos de doença.

6) Avaliar a atividade das enzimas antioxidantes, glutationa peroxidase, glutationa redutase e glutationa S-transferase no soro de pacientes bipolares no início da doença em comparação com pacientes bipolares com mais de 10 anos de doença.

PARTE II

2. RESULTADOS

Os objetivos dessa Tese foram compilados em quatro capítulos:

Capitulo I: Effects of mood stabilizers on DNA damage in an animal model of mania. Ana C. Andreazza, Marcia Kauer-Sant'Anna, Benicio N. Frey, Laura Stertz, Caroline Zanotto, Leticia Ribeiro, Karine Giasson, Samira S. Valvassori. Gislaine Z. Réus, Mirian Salvador, João Quevedo, Carlos A. Gonçalves, Flavio Kapczinski.
Artigo aceito no Journal of Psychiatry and Neuroscience

Referente ao objetivo 1.

Capitulo II: DNA damage in rats after treatment with methylphenidate. Ana Cristina Andreazza, Benício N. Frey, Samira S. Valvassori, Caroline Zanotto, Karin M. Gomes, Clarissa M. Comim, Carina Cassini, Laura Stertz, Letícia C. Ribeiro, João Quevedo, Flavio Kapczinski, Michael Berk, Carlos Alberto Gonçalves
Artigo publicado na Progress in Neuro-Psychopharmacology & Biological Psychiatry

Referente ao objetivo 2.

Capitulo III: DNA damage and Apoptosis in Bipolar patients. Ana Cristina Andreazza, Benicio N. Frey, Julio Wals, Laura Stertz, Gabriel Fries, Fernanda Weyne, Leonardo Evangelista da Silveira, Fabiano Gomes, Keila Ceresér, Alexandre Escargueil, Mirian Salvador, Carmen Gottfried, Carlos Alberto Goncalves, Flavio Kapczinski.
Artigo em preparação.

Referentes aos objetivos 3 e 4.

Capitulo IV: 3-Nitrotyrosine and Glutathione System in Bipolar Patients in early and late course of illness. Ana Cristina Andreazza, Marcia Kauer-Sant'Anna, David J Bond, Flavio Kapczinki, Carlos Alberto Golcalves, Lakshmi Yatham, Trevor Young.
Artigo submetido ao Journal Psychiatry and Neuroscience.

Referentes aos Objetivos 5 e 6.

CAPITULO I

EFFECTS OF MOOD STABILIZERS ON DNA DAMAGE IN AN ANIMAL MODEL OF MANIA

Ana C. Andreazza, Marcia Kauer-Sant'Anna, Benicio N. Frey, Laura Stertz, Caroline Zanotto, Leticia Ribeiro, Karine Giasson, Samira S. Valvassori, Gislaine Z. Réus, Mirian Salvador, João Quevedo, Carlos A. Gonçalves, Flavio Kapczinski

Artigo aceito para publicação no Journal Psychiatry and Neuroscience

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03-Mar-2008

Dear Dr. Andreazza:

It is a pleasure to accept your revised manuscript entitled "Effects of mood stabilizers on DNA damage in an animal model of mania" in its current form for publication in the Journal of Psychiatry and Neuroscience.

We ask that you complete the attached Copyright Transfer Agreement and, if you have not already done so, the Contributors' Information Form and Disclosure of Competing Interests Form and return them by fax to 613 565-5471. These must be received before copy editing and layout can begin.

Thank you for your fine contribution. On behalf of the Editors of the Journal of Psychiatry and Neuroscience, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Simon Young
Editor in Chief, Journal of Psychiatry and Neuroscience
simon.young@mcgill.ca

Effects of mood stabilizers on DNA damage in an animal model of mania

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Abstract

Objectives: Recent studies have suggested that oxidative stress and DNA damage may play a role in the pathophysiology of bipolar disorder (BD). In the present study, we investigated the effects of the mood stabilizers lithium (Li) and valproate (VPA) on amphetamine-induced DNA damage in an animal model of mania and its correlation with oxidative stress markers. ***Methods:*** In the first experiment (reversal model), adult male Wistar rats were treated with d-amphetamine (AMPH) or saline for 14 days; between the 8th and 14th days, rats also received Li, VPA or saline. In the second experiment (prevention model), rats received either Li, VPA or saline for 14 days and between the 8th and 14th days, AMPH or saline was added. DNA damage was evaluated using single cell gel electrophoresis (Comet assay) and the mutagenic potential was assessed using the Micronucleus test. Oxidative stress levels were assessed by lipid peroxidation levels (TBARS) and antioxidant enzymes activities (superoxide dismutase and catalase). DNA damage and oxidative stress markers were assessed in blood/plasma and hippocampal samples. Mutagenesis was evaluated in fresh lymphocytes. ***Results:*** In both models, we found that AMPH increased peripheral and hippocampal DNA damage. The index of DNA damage correlated positively with lipid peroxidation, whereas Li and VPA were able to modulate the oxidative balance and prevent recent damage to the DNA. However, Li and VPA were not able to prevent micronucleus formation. ***Conclusions:*** These results support the notion that Li and VPA exert central and peripheral antioxidant-like properties. In addition, the protection to the integrity of DNA conferred by Li seems to be limited to transient DNA damage and does not alter micronuclei formation.

Key words: animal model, bipolar disorder, lithium, DNA damage, dopamine, oxidative stress.

Introduction

Bipolar disorder (BD) is a prevalent, chronic and highly disabling psychiatric disorder (1). BD is considered one of the leading causes of disability amongst all medical and psychiatric conditions (2), while untreated BD has been associated with increased morbidity and mortality due to general medical conditions, such as vascular disorders and cancer (3). In addition, evidence from post-mortem studies suggest that the pathophysiology of BD may involve apoptotic cell death and neuronal and glial cell loss (4), which may in part explain the high rates of morbidity and the persistent inter-episode cognitive impairment in bipolar patients (4). It has been suggested that increased oxidative stress may be a common mechanism underlying neurobiological basis to these conditions (5). Reactive Oxygen Species (ROS) are known to play an important role in the pathogenesis of several general medical diseases (6) and in neurodegenerative and psychiatric disorders (8), including Alzheimer's disease (8), schizophrenia (6, 7) and BD (9, 10).

The central nervous system is extremely vulnerable to peroxidative damage. The human brain has a high concentration of oxidizable substrates and high oxygen tension because it metabolizes 20% of total body oxygen (11). In addition, the human brain contains a large amount of polysaturated fatty acids, which are very susceptible to lipid peroxidation. Thus, under pathological conditions levels of ROS can easily exceed antioxidant capacity, which is relatively low in the central nervous system (6). Such excess of ROS may cause direct damage to cellular proteins, DNA, and lipids thereby affecting cellular functioning (12, 13). The amount of DNA damage and the integrity of the repair system will determine whether any damage occurred to DNA strands would be transient (DNA single and double-strand breaks) or permanent (micronucleus formation). The single cell gel electrophoresis technique (Comet assay) is used to detect transient DNA single and double strand breaks

whereas the DNA damage that persists after the action of different repair systems can be assessed using the Micronucleus test (14, 15).

The understanding of the transient and permanent consequences of oxidative stress to the DNA integrity as well as the protective potential of mood stabilizers have turned into central issues in field of BD. We have recently shown that BD patients have increased oxidative stress (10) and DNA damage (10, 16) in peripheral blood. Moreover, it seems that increased oxidative stress occurs in portion with DNA damage in such patients (10). A recent study showed that telomere shortening, which is thought to occur as a consequence of increased oxidative stress, is increased among patients with BD (17). As the main source of free radicals is the respiratory chain in the mitochondria, attention should be drawn to the fact that abnormalities in respiratory complex activity (18) and mitochondrial dysfunction (19, 20) were also demonstrated in BD. Using an animal model of mania, we have shown that chronic treatment with d-amphetamine (AMPH) increased superoxide dismutase (SOD) and catalase activity (21) as well as lipid peroxidation and protein carbonyl formation (22). Chronic AMPH exposure induced a 3- to 6-fold increase of thiobarbituric acid reactive substances (TBARS) and a 1.5- to 2-fold increase in superoxide production in submitochondrial particles of prefrontal cortex and hippocampus (23). Further, we showed that lithium and valproate exert protective effects against AMPH-induced oxidative stress (24). However, the deleterious effects of oxidative stress on DNA integrity and whether such effects can be attenuated by mood stabilizers have not been examined.

Thus, the purpose of the present study is to investigate the effects of lithium and valproate on transient and permanent DNA damage (as assessed with Comet and Micronucleus assays, respectively) in peripheral blood and in the hippocampus using an animal model of mania (repeated exposure to AMPH). Moreover, parameters of oxidative

stress, such as lipid peroxidation and antioxidant enzymes activities, were evaluated in order to test the correlation between DNA damage and oxidative stress markers.

Material and Methods

Chemicals. d-Amphetamine (Sigma, St Louis, USA), low and normal melting point agarose (Gibco, USA), ethylene-diamine-tetracetic acid (EDTA) (Labsynth Produtos de Laboratório Ltda, SP, Brazil), tris (Labsynth Produtos de Laboratório Ltda, SP, Brazil), triton X-100 (Labsynth Produtos de Laboratório Ltda, SP, Brazil), dimethyl sulfoxide (DMSO) (Labsynth Produtos de Laboratório Ltda, SP, Brazil), ethidium bromide (Sigma, USA), RMPI 1640 medium (Nutricell, Campinas- SP, Brazil). Cytochalasin – B, valproic acid sodium salt and lithium carbonate (Sigma, USA) and giemsa (Merck).

Animals. The experiments were performed in male Wistar rats (age: 3-4 months; weight: 220-310 g), obtained from our breeding colony. Rats were housed five to a cage, on a 12-h light/dark cycle (lights on between 7:00 a.m. – 7 p.m.), with food and water available *ad libitum*. All experimental procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. This research project was approved by the local ethics committee (Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil).

Reversal model. The first model was designed in order to reproduce the management of an acute manic episode. Animals received one daily intraperitoneal (i.p.) injection of either AMPH 2 mg/kg or saline for 14 days (total number of animals = 48). Between the 8th and the 14th day, the animals that received saline or AMPH were divided in six experimental groups (6-8 animals per group): saline+saline (SAL), saline+lithium (Li), saline+valproate (VPA),

amphetamine+saline (AMPH), amphetamine+lithium (AMPH+Li) and amphetamine+valproate (AMPH+VPA). Li-treated animals received Li 47.5mg/kg i.p. twice a day, and VPA-treated animals received VPA 200mg/kg i.p. twice a day. Locomotor activity was measured 2h after the last injection of AMPH or saline, and the rats were submitted to intra-cardiac punction immediately after the open field task and sacrificed by decapitation after the intra-cardiac punction. The hippocampus was rapidly dissected and a slice was immediately used for the analyses of DNA damage; the remaining hippocampal tissue was rapidly frozen and stored at –80°C until assayed for oxidative stress markers.

Prevention model. The second model was designed to mimic the maintenance phase of BD treatment. Animals received either Li 47.5 mg/kg i.p. twice a day, VPA 200 mg/kg i.p. twice a day or saline for 14 days (total number of animals = 48). Between the 8th and the 14th day, the animals that received Li, VPA or saline were divided in six experimental groups (6-8 animals per group): saline+saline (SAL), saline+lithium (Li), saline+valproate (VPA), saline+amphetamine (AMPH), lithium+amphetamine (Li+AMPH) and valproate+amphetamine (VPA+AMPH). The AMPH-treated group received one daily i.p. injection of either AMPH 2 mg/kg or saline. Locomotor activity was measured 2h after the last injection AMPH or saline, and the rats were submitted to an intra-cardiac punction and sacrificed by decapitation after the open field task. The hippocampus was rapidly dissected and a slice was immediately used for DNA damage, while the remaining hippocampal tissue was stored at –80°C until assayed for oxidative stress markers.

Locomotor activity. Locomotor activity was assessed in the open-field task. The task was performed in a 40 x 60 cm open field surrounded by 50 cm high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal

rectangles by black lines. The animals were gently placed on the left rear quadrant, in order to freely explore the arena for 5 minutes. Number of crossings of the black lines and rearings were considered as a measure of horizontal and vertical locomotor activity, respectively.

Sample preparation. Blood was extracted by intra-cardiac punction with heparin. Total blood was used for micronucleus test and comet assay. For enzyme assays, we used plasma that was obtained by centrifugation at 1800g for 10 minutes. Hippocampus was homogenized in cold PBS for comet assay and antioxidant enzymes assay, which were carried out immediately.

Single cell gel electrophoresis - Comet assay. A standard protocol for comet assay preparation and analysis was adopted (25). The comet assay was performed under alkaline conditions (pH = 12.6), which detects double- and single-strand breaks and alkali-labile sites (25, 26). The slides were prepared by mixing 5 µl of whole blood, or 20 ul of hippocampus homogenate (homogenized in 20 vol (wt/vol) of STM buffer [sucrose 250mmol/L, Tris-HCl 50 mmol/L, pH 7.4, MgSO4 5 mmol/L, phenylmethylsulfonyl fluoride 0.5 mmol/L]), with 95 µl (for blood samples) or 80 µl (for hippocampus samples) of low melting point agarose (0.75%). The mixture (cells/agarose) was added to a fully frosted microscope slide coated with a layer of 500 µl of normal melting agarose (1%). After solidification, the cover slip was gently removed and the slides were placed in lyses solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0 – 10.5, with freshly added 1% Triton X-100 and 10% DMSO) for 1 day. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 10 min. The DNA was electrophoresed during 20 min at 25 V (0.90 V/cm) and 300 mA at alkaline condition (pH=12.6). After that, the slides were neutralized with 0.4 M Tris (pH 7.5). Finally, the DNA was stained with ethidium bromide. Negative and positive controls were used for each electrophoresis assay in order to ensure the

reliability of the procedure. Images of 100 randomly selected cells (50 cells from of two replicated slides) were analyzed from each treatment. Cells were also scored visually according to size of the tails into five classes: no tails (marked as 0), to maximally (marked as 4) resulting in a single DNA damage score for each cell and, consequently, for each study group. The index of DNA damage was calculated by multiplying the number of cells by your respectively index score and then summed up. Therefore, a group damage index could range from 0 (all cells no tail, 100 cells x 0) to 400 (all cells with maximally long tails, 100 cells x 4) (26).

Cytokinesis-blocked lymphocyte MN (Micronucleus Test). For each blood sample, duplicate blood cultures were set up in culture flasks by adding 0.3 ml of whole blood to 5 ml of RPMI 1640 medium containing 20% fetal calf serum and 1% (v/v) phytohemaglutinin. The flasks were incubated at 37 ◦C for 44 h before adding 5 μg/ml of cytochalasinB (27) and continuing incubation until the total incubation time reached 72 h. After incubation, the lymphocytes were harvested by centrifugation at 800 revs/min for 8 min, recentrifuged, fixed in 3:1 (v/v) methanol/acetic acid, placed onto a clean microscope slide and stained with 5% (v/v) Giemsa. For each blood sample, 1000 binucleated lymphocytes (i.e. 500 from each of the two slides prepared from the duplicate lymphocytes cultures) were scored for micronuclei presence, using bright-field optical microscopy at a magnification of 200–1000 ×. All slides were coded blinded.

Lipid peroxidation. The plasma levels of lipid peroxidation were measured using the thiobarbituric acid reactive substances (TBARS) method described by Wills (28). Data are expressed as nmol/mL.

Superoxide Dismutase activity. Superoxide dismutase activity was determined spectrophotometrically in peripheral plasma and hippocampal homogenates by measuring the inhibition of the ratio of autocatalytic adrenochrome formation at 480 nm in a reaction medium containing 1 mM adrenaline and 50 mM glycine (pH 10.2). This reaction was conducted at a constant temperature of 30 ºC during 3 minutes. Enzyme activity is expressed as superoxide dismutase units per g of protein. One unit is defined as the amount of enzyme that inhibits the ratio of adrenochrome formation to 50% (29).

Catalase activity. Catalase activity was assayed by the method of Aebi (30). Briefly, first 2 mL quartz cuvette contained in 1.980 mL phosphate buffer (pH=7.4, 50 mM) and 20 µL of peripheral plasma or hippocampal homogenates were added and the spectrophotometer was adjusted to auto zero. Then, 2 µL of H_2O_2 (3 mM freshly diluted) was added. Values are expressed as µmol of H_2O_2 consumed per minute per mg of protein.

Statistical analysis. All data are presented as mean ± S.D. Differences in oxidative stress parameters, index of DNA damage and micronuclei frequency between experimental groups were determined by one-way ANOVA, followed by Tukey post-hoc test when ANOVA was significant. Spearman Coefficient was used to calculate the correlation between lipid peroxidation and DNA damage. In all experiments, p values less than 0.05 were considered to indicate statistical significance.

Results

Locomotor activity. In the first experiment (reversal treatment), we replicated our previous findings (24) in order to confirm that DNA damage was measured in a proper model of mania: AMPH increased crossings ($F_{(5,39)}$=12.89; p<0,001) and rearings ($F_{(5,39)}$=22.91;

$p<0.001$) in saline-treated rats, while Li and VPA reverted AMPH-related hyperactive behavior (Fig. 1A). The administration of Li or VPA in saline-treated animals did not change behavioral measures, indicating that the effects of mood stabilizers in AMPH-treated rats were not associated with sedation. Behavioral measures of the second experiment (prevention treatment) demonstrated that both Li and VPA pretreatment were also able to prevent AMPH-related hyperactivity (crossings $F_{(5,40)}=21.44$; $p<0.001$) and rearings ($F_{(5,40)}=31.56$; $p<0.001$). Saline administration in mood stabilizer-pretreated animals did not affect locomotor behavior (Fig. 1B).

Transient and Permanent DNA damage. The results of transient DNA damage (Comet assay) are showed as an index of DNA damage (Table 1), while permanent DNA damage are showed by frequency of micronuclei (Figure 2). AMPH treatment increased transient DNA damage in central (hippocampus) samples in both prevention ($F_{(5,40)}=10.14$; $p<0.001$) and reversal ($F_{(5,39)}=13.57$; $p<0.001$) models (Table 1). The same results was observed in blood samples ($F_{(5,40)}=21.25$; $p<0.001$; prevention $F_{(5,39)}=19.01$; $p<0.001$; reversal). Li treatment was able to reverse and prevent the transient DNA damage in peripheral ($p=0.233$ reversal model; $p=0.235$, prevention model; Tukey test p values) and hippocampal samples in both reversal ($p=0.345$; Tukey test p value) and prevention ($p=0.641$; Tukey test p value) models, while VPA did not have any effect. In prevention ($F_{(5,40)}=10.01$; $p<0.001$) and reversal models ($F_{(5,39)}=9.55$; $p<0.001$), animals treated with AMPH showed an enhanced micronucleus frequency. In the prevention model, administration of Li and VPA diminished the frequency of micronuclei, albeit such decrease was not statistically significant (Figure 2A). In reversal treatment, neither Li nor VPA were able to reverse such damage (Figure 2B).

Oxidative Stress. Tables 2 and 3 show the results of oxidative stress markers in reversal and prevention model, respectively. AMPH increased plasma (prevention $F_{(5,40)}=18.91$; $p<0.001$ and reversal $F_{(5,39)}=11.99$ $p<0,001$ models) and hippocampal (prevention $F_{(5,40)}= 11.11$ $p<0.001$ and reversal $F_{(5,39)}=9.81$ $p<0.001$; models) lipid peroxidation, as indicated by increased levels of TBARS, in both treatment models. In addition, Li reversed and prevented hippocampal and peripheral AMPH-induced lipid peroxidation, whereas VPA prevented hippocampal lipid peroxidation, and reverted and prevented peripheral AMPH-induced lipid peroxidation. AMPH lead to about 3-fold increase of hippocampal (prevention $F_{(5,40)}=10.98$; $p<0.001$ and reversal $F_{(5,39)}=18.45$ $p<0.001$; models) and 5-fold increase of plasma (prevention $F_{(5,40)}=18.99$; $p<0.001$ and reversal $F_{(5,39)}=21.45$ $p<0.001$; models) SOD activity in both models. In the reversal model, Li decreased SOD activity after AMPH exposure in plasma and hippocampal samples, while in the prevention model Li and VPA prevented the increase of SOD activity in both samples. AMPH alone increased catalase activity in hippocampus (prevention $F_{(5,40)}=12.48$; $p<0.001$ and reversal $F_{(5,39)}=19.49$; $p<0.001$; models) and plasma (prevention $F_{(5,40)}=15.61$; $p<0.001$ and reversal $F_{(5,39)}=11.95$; $p<0.001$; models). VPA alone increased catalase and SOD activity in hippocampus of reversal model. Li and VPA when administered before AMPH increased catalase activity in hippocampus, but only VPA had the same effect in plasma samples. Li and VPA were able to reverse AMPH-induced increase in catalase activity.

Correlation between DNA damage and oxidative stress markers. We found a positive correlation between TBARS and transient DNA damage in plasma ($r_s=0.62$; number of pairs= 29; $p=0.003$) and hippocampus ($r_s=0.4$; number of pairs= 29; $p=0.005$) in the AMPH group + saline group, which indicates that AMPH-induced oxidative stress is associated with increased DNA damage. In addition, the frequency of type-IV DNA damage (highest degree

of damage) was positively correlated with frequency of micronuclei in rats treated only with AMPH (r_s=0.45; number of pairs=16; p=0.034). No significant correlation between TBARS levels and micronucleus frequency was observed.

Discussion

The present results suggest that our earlier report of increased oxidative stress in an animal model of mania (24), which was replicated here, is associated with transient and permanent DNA damage. We also found that Li and VPA were able to prevent and/or reverse DNA damage, lipid peroxidation and antioxidant enzymes changes, along with the increased locomotor activity induced by AMPH. These results are consistent with recent reports indicating that the neuroprotective effects of Li and VPA may be related to their antioxidant properties (24, 31, 32). However, neither Li nor VPA were able to alter permanent DNA damage (as indicated by the micronuclei frequency), suggesting that these mood stabilizers may protect against transient, but not permanent, DNA damage. It is conceivable that the transient DNA damage reported herein may be a consequence of increased oxidative stress, as there was a positive correlation between the lipid peroxidation markers and the index of transient DNA damage.

Our findings are in line with recent evidence showing that mood stabilizers may regulate numerous pathways that are involved in the oxidative stress response (33, 34). In our experiment, Li and VPA were able to modulate the antioxidants enzymes and Li, in particular, prevented oxidative DNA damage. De Vasconcelos et al (13) showed that Li decreased free-radical formation and increased the total antioxidant capacity in hippocampus. Moreover, in primary culture of rat cerebral cells, chronic Li and VPA treatment prevented glutamate-induced oxidative stress and DNA fragmentation (31) and increased glutathione-S-transferase mRNA and protein levels (34). Compared to our previous findings, we found

higher activity of catalase and superoxide dismutase, possibly because in the present work the method was changed to allow the comet assay and micronucleus technique to be performed in the same sample as the oxidative stress markers.

The effects of chronic Li and VPA treatments seem to take place through multiple pathways, including the induction of the survival signaling systems such as: activation of PI3K/Akt and MEK/ ERK cascades, which up-regulate BDNF expression (35, 36); increase of neuroprotective and anti-apoptotic protein bcl-2 levels in rat frontal cortex, hippocampus, and striatum (37); inhibition of glycogen synthase kinase-3β activity by phosphorilation in Serine-9, an apoptotic promoter involved in neurodegenerative and affective diseases (38); and prevention of lipid and protein peroxidation (24, 25, 34). Moreover, VPA increases the expression of heat shock protein 70 (Hsp70) and the binding of Hsp70 to apoptosis-proteases-activating factor 1 (39), which prevents this factor to activate procaspase 9 (38).

Our results showed that VPA did not prevent transient DNA damage, which is in line with previous reports (40, 41). For instance, VPA has been reported to function as a histone deacetylase inhibitor, leading to the acetylation of histone tails (41, 42). Histone acetylation results in attenuation of the electrostatic charge interactions between histones and DNA and has been associated with chromatin decondensation (42). VPA-induced chromatin decondensation may lead to enhanced sensitivity of DNA to nucleases and increased DNA binding by intercalating agents (40), increasing the access of macromolecules to DNA. In addition, it has been suggested that H_2O_2 can increase histone acetylation and decrease deacetylation (40). Thus, an increase in ROS concentration and VPA intake may increase the susceptibility of the cell to DNA damage. Our data corroborates the notion that Li treatment may be more protective against oxidative stress and DNA damage than VPA. However, it is conceivable that VPA and Li share part of their protective mechanisms. For instance, both Li and VPA decreased AMPH-induced ROS generation, an effect that may be partly due to a

decrease in dopamine levels (37, 43). AMPH increases dopamine release, which may suffer auto-oxidation, thereby leading to the formation of highly reactive quinones (44). It has been demonstrated that reactive quinones may cause a direct inhibition of mitochondrial electron transport chain complexes (45) and increase glutamate release (46), which may alter the redox status.

Along with the effects of mood stabilizers, the neurochemical effects of AMPH itself should be considered. Repeated exposure to AMPH is known to be associated with neurotoxicity (47, 48), and our results showed increased lipid peroxidation, DNA damage and SOD activity in AMPH-treated rats. Importantly, SOD accelerates the dismutation of $O_2^{\bullet-}$ to H_2O_2 and, thus, diminishes $ONOO^-$ formation from $O_2^{\bullet-}$ as well as direct damage caused by $O_2^{\bullet-}$ (6). Therefore, it seems that the increment of SOD activity after AMPH treatment, as found in the present study, was a response against AMPH-induced oxidative DNA damage.

Some limitations should be considered in the interpretation of the present results. The animal model of mania using AMPH is restricted to mimic one aspect of BD, which is a far more complex disorder. However, the unique hallmark of bipolar illness is the presence of mania (1). Manic symptoms can be reproduced in normal volunteers after administration of AMPH and a number of case reports have suggested that the euphoric effects induced by AMPH in humans can be attenuated by lithium administration (49, 50). AMPH administration increases heart rate, blood pressure and cortisol levels similar to the changes associated with mania (49). Indeed, the magnitude of ventral striatal dopamine release induced by AMPH correlated positively with the hedonic response in human subjects (51). Another limitation of the present study is that apoptosis and the efficiency of the repair system were not assessed. However, the purpose of this study was to assess the correlation between oxidative stress and DNA damage and further investigation of the fate of cells after DNA damage was beyond the scope of the present communication.

In conclusion, the present study suggests that increased oxidative stress in an animal model of mania may be associated with both transient and permanent DNA damage. Further studies are needed to clarify whether this is a consequence of a dysfunctional repair system and whether this damage will ultimately result in neuronal apoptosis. More importantly, data from the treatment models indicate that Li and (to a lesser extent) VPA exert antioxidant properties, which are likely to be associated with protective effects to DNA. However, the extent of the protection conferred by these drugs seems limited to transient DNA damage (Comet assay) – once the cell has undergone permanent DNA damage (micronuclei formation), neither Li nor VPA were able to prevent or reverse this type of DNA damage. Further investigation is needed to ascertain if these antioxidant/protective properties are correlated with the ability of these drugs to control the symptoms as well as negative outcomes in BD. We are tempted to speculate that new treatments targeted to lessen the oxidative stress burden in BD have the potential to move the field beyond the control of symptoms, getting closer to the preventive model already consolidated in other medical areas.

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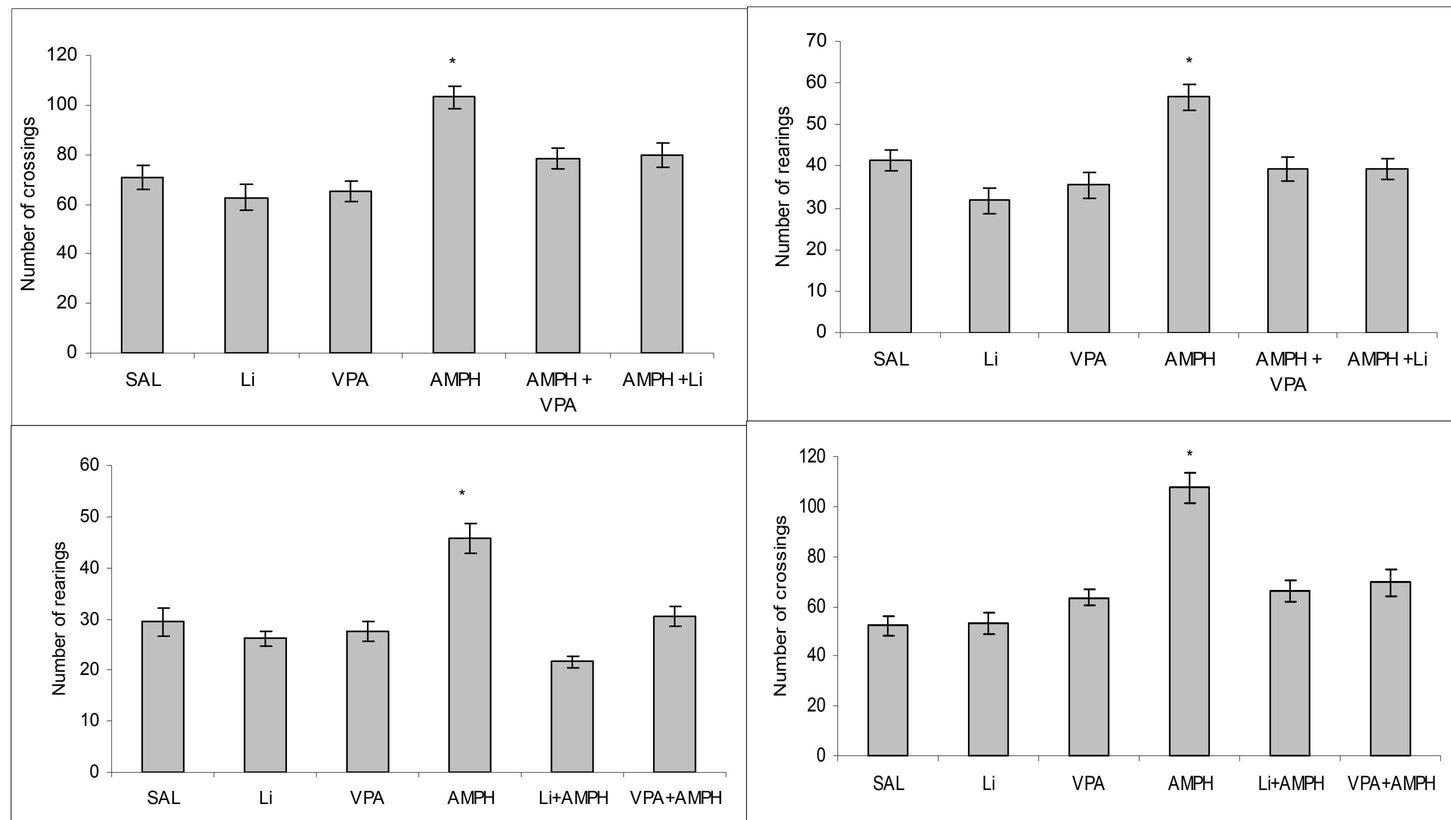

Figure 1. Effects of d-Amphetamine (AMPH), lithium (LI) and valproate (VPA) on horizontal (crossings) and vertical (rearings) behavior in reversal (A) and prevention (B) treatments. Values are expressed as mean ± standard error. *Statistically different from control group (SAL) *One-Way ANOVA Test follow by Duncan Test ($p<0.05$).

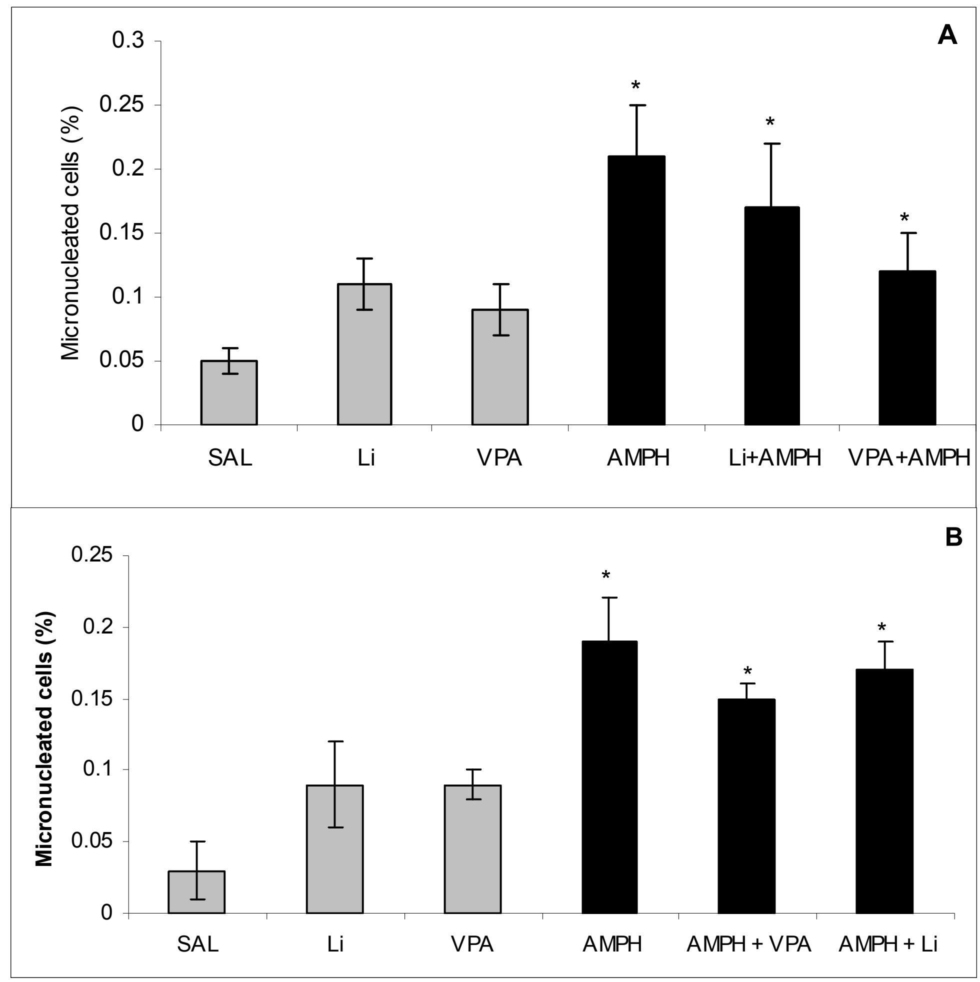

Figure 2. Frequency of micronucleus in prevention (A) and reversal (B) treatments. The micronucleus analysis was carried out in lymphoblastic cells. Values are expressed as mean ± standard error. *One-Way ANOVA test follow by Tukey post-hoc test; ($p<0.01$) differences in relation to control group (SAL). Treatments with amphetamine + mood stabilizers did not differ to amphetamine + saline in any of the models. Abbreviations: SAL= saline; Li=lithium; VPA= valproate; AMPH= amphetamine.

Table 1. Effects of d-amphetamine and mood stabilizers (lithium and valproate) on transient DNA damage in reversal and prevention treatments. The comet assay was evaluated in lymphoblastic cells and was carried out under alkaline conditions. That permit verify the single and double-strand-breaks and alkali-labile sites.

		Index of DNA Damage[#]							
		Reversal model				*Prevention model*			
	Treatment	N	Mean	(SD)	***P****	N	Mean	(SD)	***P****
Hippocampus	SAL	6	21.3	(1.30)		7	21.3	(1.30)	
	Li	8	19.45	(3.10)	0.231	8	19.45	(3.10)	0.455
	VPA	7	16.4	(6.10)	0.098	8	16.4	(6.10)	0.314
	AMPH	8	*32,5*	(3.20)	0.001	8	*28,5*	(3.20)	0.012
	Li + AMPH	8	25.1	(3.56)	0.345	8	23.4	(3.56)	0.641
	VPA + AMPH	8	*33,1*	(3.20)	0.012	8	*32,1*	(3.20)	0.001
Plasma samples	SAL	6	13.71	(1.70)		7	14.71	(3.55)	
	Li	8	20.57	(7.09)	0.235	8	23.88	(10.33)	0.005
	VPA	7	19.33	(9.07)	0.311	8	24.2	(4.89)	0.125
	AMPH	8	*36,2*	(10.18)	0.008	8	*38,29*	(5.94)	<0.001
	Li+AMPH	8	21.33	(3.67)	0.233	8	22.33	(2.78)	0.235
	VPA+AMPH	8	*36,8*	(4.76)	<0.001	8	*27,71*	(1.63)	0.011

* $p<0.05$; One- Way ANOVA test followed by Duncan Test. Differences in relation to control group (saline+saline). [#] Index of DNA damage was calculated by multiplying the number of cells by your respectively index score and than summed up. Therefore, a group damage index could range from 0 (all cells no tail, 100 cells x 0) to 400 (all cells with maximally long tails, 100 cells x 4). Abbreviations: SAL= saline; Li=lithium; VPA= valproate; AMPH= amphetamine.

Table 2: Antioxidants enzymes activities and level of lipid peroxidation in reversal model. The analysis of antioxidant activity was carried out in hippocampal cells and in plasma samples.

	Treatment		Sod (U/mg protein)			Catalase (U/mg protein)			TBARS (nmol/ml)		
		N	**Mean**	**(SD)**	***P****	**Mean**	**(SD)**	***P****	**Mean**	**(SD)**	***p****
Hippocampus	SAL	6	35.45	(5.73)		12.66	(1.09)		2.19	(0.47)	
	Li	8	*66,59*	(21.09)	0.001	*45,02*	(10.63)	<0.001	3.61	(1.00)	0.879
	VPA	7	40.61	(0.51)	0.213	13.32	(1.40)	0.788	4.71	(1.85)	0.456
	AMPH	8	*95,83*	(0.96)	<0.001	*28,66*	(4.00)	<0.001	*9,29*	(0.91)	<0.001
	AMPH + VPA	8	*93,56*	(35.51)	<0.001	9.34	(0.76)	0.566	*7,03*	(0.56)	<0.001
	AMPH +Li	8	41.68	(0.51)	0.189	8.04	(2.40)	0.231	4.41	(1.85)	0.233
Plasma samples	SAL	6	7.12	(1.18)		15.82	(1.63)		1.87	(0.47)	
	Li	8	12.33	(1.00)	0.089	*113,67*	(14.04)	<0.001	2.05	(0.98)	0.544
	VPA	7	9.56	(1.51)	0.269	*85,62*	(1.51)	<0.001	3.51	(0.51)	0.356
	AMPH	8	*22,34*	(1.34)	<0.001	*32,38*	(1.34)	0.001	*9,99*	(1.12)	0.012
	AMPH + VPA	8	*15,55*	(1.00)	<0.001	44.09	(1.82)	0.566	2.28	(0.34)	0.233
	AMPH + Li	8	4.66	(1.40)	0.456	24.72	(1.40)	0.621	1.96	(1.85)	0.725

* One-Way ANOVA test follow by Duncan Test ($p<0.05$). Differences in relation to control group (SAL). Abbreviations: TBARS= thiobarbituric acid reaction substances; SOD= Superoxide dismutase; SAL= saline; Li=lithium; VPA= valproate; AMPH= amphetamine.

Table 3. Antioxidant enzymes activities and level of lipid peroxidation in prevention model. The analysis of antioxidant activity was carried out in hippocampal cells and in plasma samples.

	Treatment		**Sod (U/mg protein)**			**Catalase (U/mg protein)**			**TBARS (nmol/ml)**		
			Mean	**(SD)**	***P****	**Mean**	**(SD)**	***P****	**Mean**	**(SD)**	***P****
Hippocampus	SAL	7	15.76	(5.73)		6.78	(2.00)		1.64	(2.11)	
	Li	8	13.3	(1.14)	0.369	6.88	(1.14)	0.899	5.29	(1.14)	0.322
	VPA	8	11.72	(1.14)	0.411	6.09	(1.14)	0.745	4.68	(1.14)	0.415
	AMPH	8	*59,97	(2.11)	<0.001	*19,37	(2.11)	<0.001	*9,06*	(2.11)	0.001
	Li+AMPH	8	13.06	(1.23)	0.740	*15,91	(1.23)	0.002	5.48	(1.23)	0.678
	VPA+AMPH	8	11.11	(1.22)	0.658	*17,52	(1.22)	0.001	3.20	(1.22)	0.450
Plasma samples	SAL	7	5.33	(1.40)		15.90	(5.00)		2.32	(2.11)	
	Li	8	8.88	(2.30)	0.569	35.71	(9.44)	0.121	3.32	(2.00)	0.556
	VPA	8	9.22	(1.90)	0.623	33.75	(9.90)	0.089	2.93	(2.33)	0.756
	AMPH	8	*34,99	(2.11)	0.005	*93,25	(6.70)	<0.001	*12,76	(1.98)	0.001
	Li+AMPH	8	13.44	(2.98)	0.234	37.03	(7.44)	0.101	3.26	(1.55)	0.899
	VPA+AMPH	8	11.00	(2.22)	0.366	*56,25	(7.99)	0.002	3.50	(2.11)	0.786

*One-Way ANOVA test followed by Duncan Test (p<0.05). Differences in relation to control group (SAL). Abbreviations: TBARS= thiobarbituric acid reaction substances; SOD= Superoxide dismutase; SAL= saline; Li=lithium; VPA= valproate; AMPH= amphetamine.

CAPITULO II

DNA DAMAGE IN RATS AFTER TREATMENT WITH METHYLPHENIDATE.

Andreazza AC, Frey BN, Valvassori SS, Zanotto C, Gomes KM, Comim CM, Cassini C, Stertz L, Ribeiro LC, Quevedo J, Kapczinski F, Berk M, Gonçalves CA.

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CAPITLO III

DNA DAMAGE AND APOPTOSIS IN BIPOLAR PATIENTS.

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Artigo em preparação.

DNA DAMAGE AND APOPTOSIS IN BIPOLAR PATIENTS.

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Abstract

Objectives: Recent studies have suggested that oxidative stress and DNA damage may play a role in the pathophysiology of bipolar disorder (BD). In the present study, we investigated the involvement of permanent DNA damage and frequency of apoptosis in BD patients in comparison with healthy subjects. ***Methods:*** Forty-five BD outpatients without clinical comorbidities (diabetes, cardiovascular diseases and cancer), diagnosed according to DSM-IV criteria, were matched to 45 healthy volunteers. Clinical outcome was measured using the Hamilton Depression Rating Scale (HAM-D) and Young Mania Rating Scale (YMRS). Permanent DNA damage was evaluated using the Micronucleus test. Apoptosis was assessed by annexin V Cy3 staining and confirmed by 4′6-Diamidino-2-phenylindole, dihydrochloride *(*DAPI) staining. ***Results:*** The present study showed that BD outpatients present an increased frequency of apoptosis relative to controls; however the frequency of micronucleus was not increased. C***onclusions:*** The present findings may be used to augment strategies tailored to prevent apoptosis in BD patients.

Key words: Bipolar disorder; DNA damage; Apoptosis; Micronucleus; Oxidative stress.

Introduction

Bipolar disorder (BD) is a major affective disorder with an estimated prevalence of about 1–3% of the population worldwide (Belmaker, 2004; Merikangas et al, 2007). It has been increasingly recognized that individuals with BD are at higher risk of suffering from chronic general medical conditions, such as cardiovascular disease, obesity and diabetes mellitus (Kupfer, 2005; Mclntyre et al., 2006), which is directly associated with increased morbidity and mortality observed in this disorder. Notably, recent studies have found impairment in a number of cognitive functions in BD patients that are present even after remission of symptoms (Martinez-Aran et al., 2004; Robinson et al., 2006; Torres et al., 2007), suggesting that BD may be associated with persistent cognitive dysfunction. The exact neurochemical mechanisms underlying the pathophysiology of BD are not completely understood. Several hypotheses have been postulated including a role for monoamines, gamma-amino butyric acid (GABA), glutamate, and second messenger signaling pathways. More recently, oxidative stress has been implicated and there is accumulating evidence to support its' role (Andreazza et al., 2007; Gergerlioglu et al., 2007; Machado-Vieira et al., 2007; Selek et al., 2007; Savas et al., 2006; Ozcan et al., 2004; Ranjekar et al., 2003; Kuloglu et al., 2002; Abdalla et al., 1986).

Several postmortem studies have reported reduced neuronal and glial density in discrete regions of the prefrontal cortex (Ongur et al 1998; Rajkowska et al 2001; Uranova et al 2001; Vostrikov et al 2007), whereas in amygdala it has been found decreased number and density of neurons, as well as volume loss (Ongur et al, 1998). In addition, Davis et al (2004) found a reduction in cortical gray matter and cerebral white matter volumes in male patients with familial bipolar I disorder. The shrinkage and neuronal loss can result not only from necrotic process, but also from apoptosis. Apoptosis and necrosis can be distinguished from

one another histopathologically based on their energy requirements, histological profiles, plasma membrane changes, polymerase enzymes, structural DNA phagocytic mechanisms, and the presence or absence of inflammatory reaction and patterns of DNA breakdown (Hetes et al, 1998). An important difference is that damage to DNA occurs in apoptosis, but not in necrosis, and it is believed to involve the activation of a DNA fragmentation factor by a caspase enzyme (Evan and Littelwood, 1998; Ansari et al, 1993) If a cell is unable to express poly(adenosine diphosphate-ribosyl) polymerase, an enzyme that repairs DNA breaks in the chromatin strands, such damage may persist indefinitely in a surviving neuron (Bouchard et al, 2003; Yun et al, 2003). On the other hand, if damage to DNA continues to accrue within a population of neurons subjected to oxidative stress, then these cells may progress towards apoptotic cell death. Studies investigating the damage to DNA, the sources of DNA damage and pathways involved, may be particularly important to BD, because it has the potential to affect expression of a wide variety of genes. Furthermore, it can stimulate cell cycle-related intrinsic DNA-repair mechanisms that may be either reparative or deleterious (Halliwell and Gutteridge, 1999).

The cells are continuously exposed to exogenous and endogenous agents that damage its DNA (Barzilai and Yamamoto, 2004; Neeley and Essigmann, 2006). One of most important agents are free radicals, especially the radical hydroxyl (OH^{-}) (Cooke et al., 2006; Culmsee and Mattson, 2005). In this way, our group has recently showed that BD patients have increased oxidative stress (Andreazza et al, 2007a) and DNA damage (Andreazza et al, 2007b) in peripheral blood. Moreover, it seems that increased oxidative stress occurs in tandem with DNA damage in such patients (Frey et al, 2007). A recent study showed that telomere shortening, which is thought to occur as a consequence of increased oxidative stress, is increased among patients with BD (Simon et al, 2006). As the main source of free radicals is the respiratory chain in the mitochondria, attention should be drawn to the fact that

abnormalities in respiratory complex activity (18) and mitochondrial dysfunction (19, 20) have been also demonstrated in BD. For instance, studies conducted in postmortem brain tissue demonstrated that mitochondrial genes are downregulated in hippocampus (Konradi et al, 2004) and dorsolateral prefrontal cortex (Iwamoto et al, 2005 Sun et al, 2006), as well as the expression the major antioxidant enzymes, SOD and catalase, and that apoptotic genes are upregulated in hippocampus of individuals with BD (Benes et al, 2006). Similarly, recent studies found increased DNA fragmentation in the anterior cingulate cortex (Buttner et al, 2007) and in lymphocytes (Andreazza et al, 2007; Naydenov et al, 2007) of BD patients. Notably, Simon et al (65) showed that the amount of telomere shortening (an indicator of cumulative oxidative stress) of individuals with chronic bipolar and unipolar disorder represent about 10 years of accelerated aging.

The amount of DNA damage and the integrity of the repair system will determine whether any damage occurred to DNA strands would be transient (DNA single and double-strand breaks) or permanent (micronucleus formation). DNA damage that persists after the action of different repair systems can be assessed using the Micronucleus (MN) test (Fenech, 2006). MN originates from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division (Fenech 2000). The MN index in human cells has become one of the standard cytogenetic tests for genetic toxicology testing (Fenech 2006). Other types of cell that have been studied with this method are the nucleoplasmic bridges (NPB) and apoptotic cells, as displayed in figure 1. NPB are assumed to occur when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. It is rarely possible to observe dicentric anaphase bridges before the nuclear membrane is formed, because cells proceed through anaphase and telophase rapidly, completing cytokinesis and ultimately breakage of the NPB when daughter cells separate.

Considering the results from cell-counting studies, it is conceivable that oxidative stress influence DNA damage in BD. The understanding of the transient and permanent consequences of oxidative stress to the DNA integrity as well as the frequency of apoptosis have turned into central issues in field of BD. Thus, the aim of this study is to verify the frequency of permanent DNA damage using the micronucleus test, and the frequency of apoptosis using annexin V/propidium iodide staining, confirmed by DAPI staining in bipolar disorder patients in comparison to health controls.

Material and Methods

Subjects

This was a case-control study of 45 patients with BD, 18 years of age or older (mean age ± S.D.= 43.28±12.72), consecutively assessed from October 2006 to June 2007. All patients were recruited from the Bipolar Disorders Program- Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Patients were non-smokers, were free of active medical conditions (diabetes, chronic inflammation and cancer) and were not on any medication, other than those necessary for the management of their psychiatric illness. The diagnosis of BD was confirmed using the Structured Clinical Interview for DSM-IV – Axis I (SCID-I) (First et al., 1998). Manic and depressive symptoms were assessed using the Young Mania Rating Scale (YMRS) (Young et al., 1978) and the Hamilton Depression Rating Scale (HDRS) (Hamilton, 1960), respectively. Healthy volunteers (n=45) had no personal or family history in first degree relatives of major psychiatric disorders, dementia, mental retardation, cancer or tumor, and were matched to patients for age, gender and education. They were nonsmokers and were not taking medication. This study was approved by the Clinical Hospital of Porto Alegre Ethics Board (Brazil). All patients and healthy volunteers provided written informed consent prior to taking part in any study-specific activities.

Procedures

Collection and processing of blood samples

Each patient and healthy volunteer provided 5 ml blood samples which were collected by venipuncture with heparine anticoagulants. The lymphocytes were extracted using Ficoll-Hypaque gradient (Histopaque 1077; Sigma, St. Louis, MO), by mix equal part of ficoll and blood and centrifuge for 40 minutes at 1000g and wash 2 times with RPMI 1640 medium. The lymphocytes were used immediately to measure the frequency of micronucleus and apoptosis.

Cytokinesis-blocked lymphocyte MN (Micronucleus Test).

For each blood sample, duplicate blood cultures were set up in culture flasks by adding 0.3 ml of whole blood to 5 ml of RPMI 1640 medium containing 20% fetal calf serum and 1% (v/v) phytohemaglutinin. The flasks were incubated at 37 ◦C for 44 h before adding 5 μg/ml of cytochalasinB (27) and continuing incubation until the total incubation time reached 72 h. After incubation, the lymphocytes were harvested by centrifugation at 800 revs/min for 8 min, recentrifuged, fixed in 3:1 (v/v) methanol/acetic acid, placed onto a clean microscope slide and stained with 5% (v/v) Giemsa. For each blood sample, 1000 binucleated lymphocytes (i.e. 500 from each of the two slides prepared from the duplicate lymphocytes cultures) were scored for micronuclei presence, using bright-field optical microscopy at a magnification of 200–1000 ×. All slides were coded blinded. We also scored the frequency of apoptotic cells and nucleoplasmic bridges (Fenech et al 2006).

Annexin V- Cy3 staining

The Annexin V-Cy3 was measured using the Apoptosis Detection Kit, from Sigma (Saint Louis, Missouri, USA). The suspension of lymphocytes was washed two times with binding

buffer (10 mM HEPES, pH 7.5, containing 140 mM NaCl and 2.5 mM CaCl2), then the cellular suspension was mixed with 50ul of double label staining solution (AnnCy3 + 6-Carboxyfluorescein diacetate) and incubated for 10 minutes. After staining, the results was observed using a fluorescence microscope, the live cells will only stain with 6-Carboxyfluorescein diacetate in green fluorescence and cells starting the apoptotic process will stain both with Annexin-Cy3 (red fluorescence). We counted 100 cells in the same visual optical to calculate the frequency of apoptosis (figure 2A).

4'6-Diamidino-2-phenylindole, dihydrochloride (DAPI) staining

In order to morphologically confirm the finding of enhanced lymphocytes cell apoptosis, DAPI staining was performed. Briefly, the lymphocytes were washed with phosphate-buffered saline (PBS), fixed with 70% ethanol for 20 min at room temperature and washed again with PBS. Cells were then treated with DAPI (1μg/ml; Sigma, Saint Louis, Missouri, USA) at a 1:1000 dilution, incubated for 10 min and washed again with PBS for 5 min. Stained nuclei were visualized under a fluorescence microscope. Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and chromatin condensation or fragmentation. The percentage of apoptotic cells was calculated as a proportion of 100 total cells visualized in 10 different fields of each slide/experiment (figure 2B).

Statistical Analysis

Data were expressed as mean ± S.D. Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) Version 15.0. The Kolmogorov-Smirnov Test was used to compare the observed cumulative distribution function to a theoretical cumulative distribution. Our data had parametric distribution. The outcome measures for the groups were

compared using Independent T Test. Categorical variables (ethnicity and gender) were analysed used Chi-square test.

Results

Subjects

A total of 45 BD patients and 45 healthy volunteers were enrolled. Demographic variables are displayed in Table 1. No significant differences in age, gender, and education were detected between patients and matched volunteers. BD patients present 28.16(3.94) of age of onset and 15.80(13.32) length of illness. Patients are in euthymic phase with 9.25(7.15) of HAM-D scores and 4.98(5.67) of YMRS scores. All patients were on psychiatric medications: mood stabilizers (95.2%), antipsychotics (71.4%) and/or antidepressants (23.8%).

Frequency of Micronucleus, nucleoplasmic bridges and apoptosis

There was no difference in MN frequency between BD patients and HC subjects ($t= -1.34$; $df=70.42$; $p=0.184$). However, BD patients had higher frequency of NPB ($t= -7.49$; $df=57.43$; $p<0.001$) and apoptotic cells, as assessed by micronucleus test ($t= -7.92$; $df=49.42$; $p<0.001$) or annexin V-Cy3 test ($t= -5.85$; $df=47.67$; $p<0.001$) (Figure 3). We found a positive correlation between frequency of apoptotic cell measured by micronucleus test and annexinV-Cy3 ($r=0.452$; $p=0.01$) (figure 4).

Discussion

The present results suggest that BD patients have increased frequency of cell apoptosis, but do not have increased micronucleus formation. Different mechanisms can be involved in the generation of DNA damage, and the repair of such DNA damage may be effective or not.

The human genome is composed of three billion base-pairs divided into 23 DNA molecules that make up chromosomes, and 30,000–50,000 genes (Ewing and Green, 2000; Venter et al., 2001) encoded in only 3% of the DNA sequence. The function of 40% of the genes and the remaining non-gene encoding genome is unknown. Some portion of the genome is needed to maintain chromosome structure and regulate gene expression (Sadock and Sadock, 2000). Cells are continuously exposed to exogenous and endogenous agents that may damage its DNA (Barzilai and Yamamoto, 2004; Neeley and Essigmann, 2006). The vast majority of DNA damage affects the primary structure of the double helix; that is, the bases themselves are often chemically modified (Ames et al, 1993; Neeley and Essigmann, 2006; Markesbery and Lovell, 2007). These modifications can in turn disrupt the molecules' regular helical structure by introducing non-native chemical bonds or bulky adducts that do not fit in the standard double helix. Unlike proteins and RNA, DNA usually lacks tertiary structure and therefore damage or disturbance does not occur at that level. DNA is, however, supercoiled and wound around "packaging" proteins called histones, and both superstructures are vulnerable to the effects of DNA damage (Braig and Smith, 2006; David at al, 2007).

The endogenous reactions that are likely to contribute to ongoing DNA damage are depurination, deamination, methylation, and oxidation (Wiseman and Halliwell, 1996; David at al, 2007). Typically, the DNA damage caused by environmental factors (radiation, mutagenic chemical or chemotherapy) and by endogenous reactions inside the cell occur at a rate of 2x104 molecular lesions per cell per day (Lodish, et al 2004; Ames et al, 1993). DNA damage produced by oxidation from reactive oxygen/nitrogen species appears to be the most significant endogenous damage (Ames et al, 1993; Barzilai and Yamoto 2004; David at al, 2007). As early as 1952, Conger and Fairchild demonstrated that increased pressure could lead to accumulation of chromosomal aberrations. Reactive oxygen species (ROS) are constantly produced in the living cells (Halliwell and Guteridge, 1999; Halliwell, 2001).

Major endogenous sources are metabolic process, primarily oxidative metabolism in the mitochondrial electron transport chain, and pathological processes such as inflammation (Brazilai and Yamamoto, 2004). The different types of DNA lesions that result from endogenous insults are rapidly detected, with subsequent activation of an intricate web of signaling pathways known as the DNA damage response (Shou and Elledge, 2000; Barzilai and Yamamoto, 2004). This response culminates in activation of cell-cycle checkpoints and the appropriate DNA repair pathways, or, in certain conditions, initiation of apoptotic programming (Iliakis et al, 2003).

In the past few years, there has been an increasing body of evidence suggesting the involvement of oxidative stress, DNA damage and mitochondria dysfunction in the patophysiology of BD. Several studies reported that patients with BD have significant alterations in antioxidant enzymes (Andreazza et al., 2007; Gergerlioglu et al., 2007; Machado-Vieira et al., 2007; Selek et al., 2007; Savas et al., 2006; Ozcan et al., 2004; Ranjekar et al., 2003; Kuloglu et al., 2002; Abdalla et al., 1986), increased lipid peroxidation (Andreazza et al., 2007; Gergerlioglu et al., 2007; Savas et al., 2006; Ozcan et al., 2004; Ranjekar et al., 2003; Kuloglu et al., 2002) and increased nitric oxide levels (Gergerlioglu et al., 2007; Selek et al., 2007; Savas et al., 2006; Savas et al., 2005; Yanik et al., 2004; Savas et al., 2002). In addition, the expression of the major antioxidant enzymes, SOD and catalase, and apoptotic genes are upregulated in hippocampus of individuals with BD (Benes et al, 2006). Notably, recent findings have demonstrated that involvement of DNA damage is remarkably increased in individuals with BD. Andreazza et al (2007) showed that lymphocytes of BD patients have increased transient DNA damage, a type of DNA damage that is detected before the action of repair systems. This is consisten with Butter et al (2007) who showed that non-gabaergic neurons from anterior cingulated of BD patients present

increased DNA fragmentation. Moreover, it seems that increased oxidative stress occurs in tandem with DNA damage in BD patients (Frey et al, 2007).

A recent study showed that telomere shortening, which is thought to occur as a consequence of increased oxidative stress, is increased in individuals with chronic bipolar and unipolar disorder, which represent about 10 years of accelerated aging (Simon et al, 2006). As the main source of free radicals is the respiratory chain in the mitochondria, attention should be drawn to the fact that abnormalities in respiratory complex activity (Adam-Vizi et al, 2005) and mitochondrial dysfunction (Kato et al, 2006) have also been demonstrated in BD. Kato et al in 1997 first reported that deletion of mitochondrial DNA was significantly higher in postmortem cerebral cortex of bipolar disorder subjects than in age-matched controls. Subsequent studies have shown that the polymorphisms of mitochondrial DNA coding complex I genes are significantly associated with bipolar disorder (Kato et al 2000; Kato et al 2001). Studies conducted in postmortem brain tissue demonstrated that mitochondrial genes are downregulated in hippocampus (Konradi et al, 2004) and dorsolateral prefrontal cortex (Iwamoto et al, 2005 Sun et al, 2006).

Our results showed that BD patients do not present increased of frequency of MN, but present higher frequency of NPB and rate of cell in apoptotic process. MN reflects the chromosome breakage or loss, whereas NPB occur when the centromeres of dicentric chromosomes or chromatids are pulled to the opposite poles of the cell at anaphase (Fenech et al, 2003). The formation of anaphase and NPBs has been observed in models of rodent and human intestinal cancer *in vivo* and shown to correlate positively with telomere length indicating that nucleoplasmic bridge formation can also be used as a surrogate measure of critically short telomeres (Rudolph et al, 2001). Thus, previous findings in BD showing accelerate telomere shortening (Simon et al, 2006) are consistent with the present results of increased NPB in BD. Accordingly, the most important factor that accelerate telomere

shortening is oxidative stress (Passos et al, 2007), which has also been related to BD patophysiology.

Apoptosis is a programmed and highly controlled form of cell death. Apoptosis can be triggered by a wide variety of stimuli, all of which lead to the same cell death machinery (Rowe and Chuang, 2004). Apoptosis is regulated by two general methods: (1) the extrinsic pathway is activated by death receptors, such as Fas and tumour necrosis factor receptor, which have a cytosolic death domain (Lawen 2003). (2) The intrinsic, or mitochondrial, pathway is activated by a wide variety of pro-apoptotic stimuli, which cause the release of cytochrome c from mitochondria, generation of reactive oxygen species, and subsequent activation of caspases (Lawen 2003). Key regulators of this pathway are the Bcl-2 family members, which determine the integrity of the mitochondria (Rowe and Chuang, 2004). Bax is a pro-apoptotic Bcl-2 family member that binds to the anti-apoptotic Bcl-2 protein, and can antagonize its function to block apoptosis (Oltvai et al., 1993). Induction of Bax also promotes cytochrome c release from the mitochondria, which is inhibited by anti-apoptotic Bcl-2 family members (Rossé et al., 1998). The bax gene is known to be transcriptionally regulated by p53 during induction of apoptosis (Buckbinder et al., 1995; Miyashita and Reed, 1995). p53 is activated by cell stress and DNA damage and, depending upon the severity of the stress and the particular cell type, may aid in adaptive responses to the stress or may trigger cell cycle arrest or apoptosis (Joers et al, 2003). In many types of post-mitotic neurons, p53 may mediate apoptosis induced by a range of insults including DNA damage, hypoxia, withdrawal of trophic support, hypoglycemia, oxidative stress, and viral infections (Morrison et al, 2003).

In summary, our results give support to the involvement of apoptotic processes in BD. One of the potential underlying mechanisms may be the increased DNA damage, which can activate p53 and initiate the apoptotic process. However, future studies are necessary to look

together if the DNA damage could altered the expression or phosphorilation of p53. In the same way, future studies should assess the molecular mechanisms implicated in DNA damage induced by oxidative-stress.

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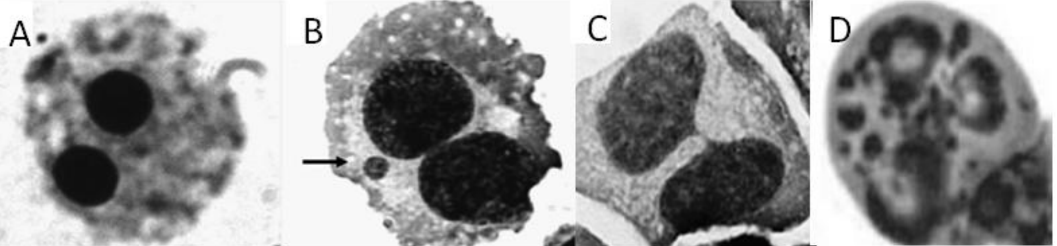

Figure 1. (A) Binucleated cell, (B) micronucleus, (C) nucleoplasmic bridges and (D) apoptotic cell detect by cytokinesis-block micronucleus assay. Micronucleus originates from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division. The nucleoplamic bridges are assumed to occur when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. It is rarely possible to observe dicentric anaphase bridges before the nuclear membrane is formed, because cells proceed through anaphase and telophase rapidly, completing cytokinesis and ultimately breakage of the NPB when the daughter cells separate. Apoptosis is a programmed and highly controlled form of cell death. In apoptosis, the cellular contents are packaged into apoptotic bodies.

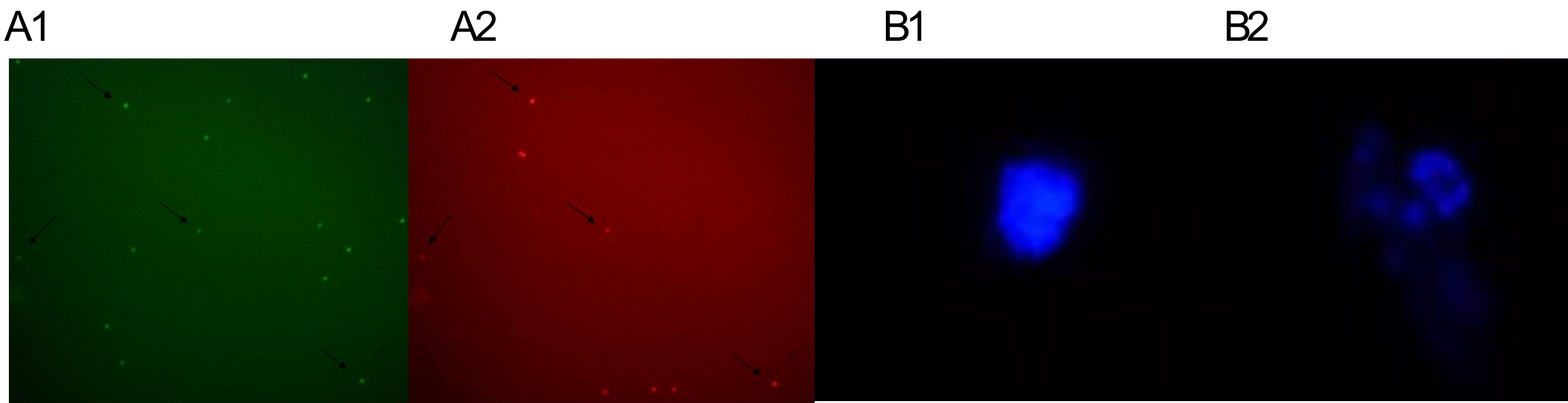

Figure 2. Annexin V-Cy3 test , the live cells will only stain with 6-Carboxyfluorescein diacetate in green fluorescence (A1) and cells starting the apoptotic process will stain both with Annexin-Cy3 (red fluorescence) (A2). Apoptotic cells, by DAPI stain were defined morphologically by cytoplasmic and nuclear shrinkage and chromatin condensation or fragmentation (B2). The normal nucleus by DAPI stain were showed in B1 picture. The annexins are a group of homologous proteins that bind phospholipids in the presence of calcium. The cellular changes involved in the apopototic process include loss of cell membrane phospholipid asymmetry during early stages of apoptosis. In living cells the phosphatidylserine is transported to the inner plasma membrane leaflet by the enzyme Mg-ATP dependent aminophospho-lipid translocase.3 However, during the onset of apoptosis, the PS is transported to the external leaflet of the plasma membrane. The PS is then available for binding to annexin V and any of its conjugates in the presence of Ca^{2+} ions.

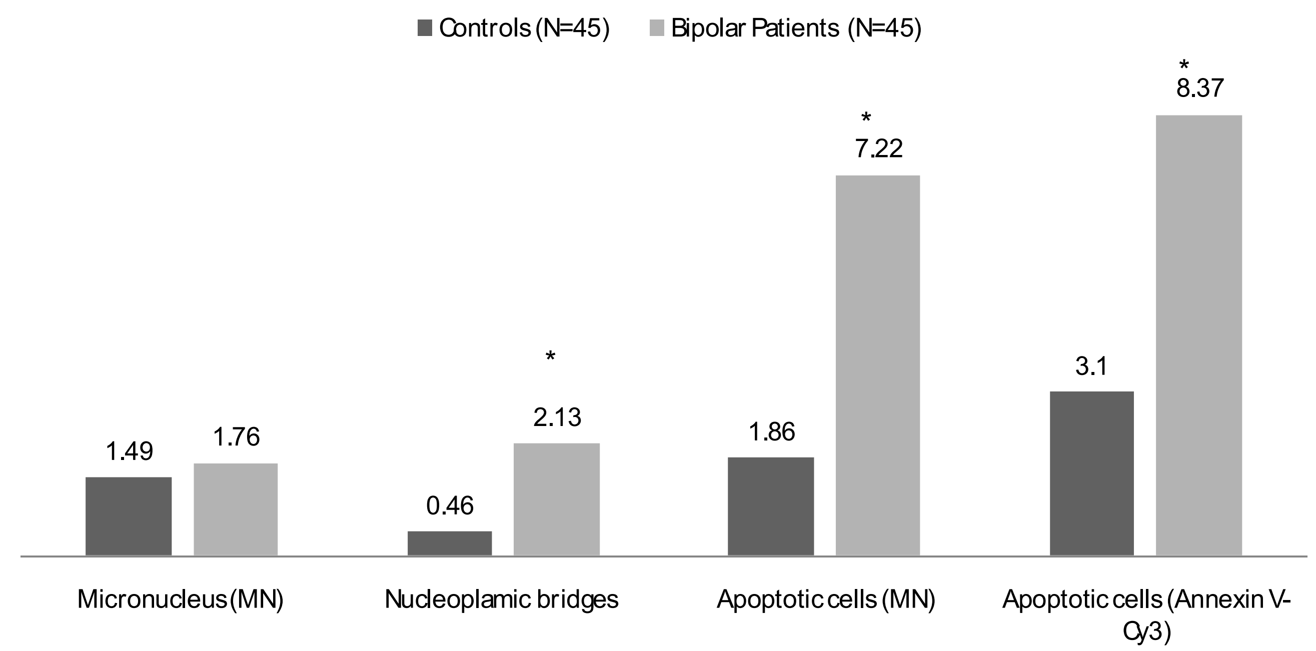

Figure 3. Frequency of micronucleus, nucleoplamic bridges, apoptotic cells assessed by MN test and annexin V-Cy3.

*$p<0.001$; in relation to control group (Independent T test)

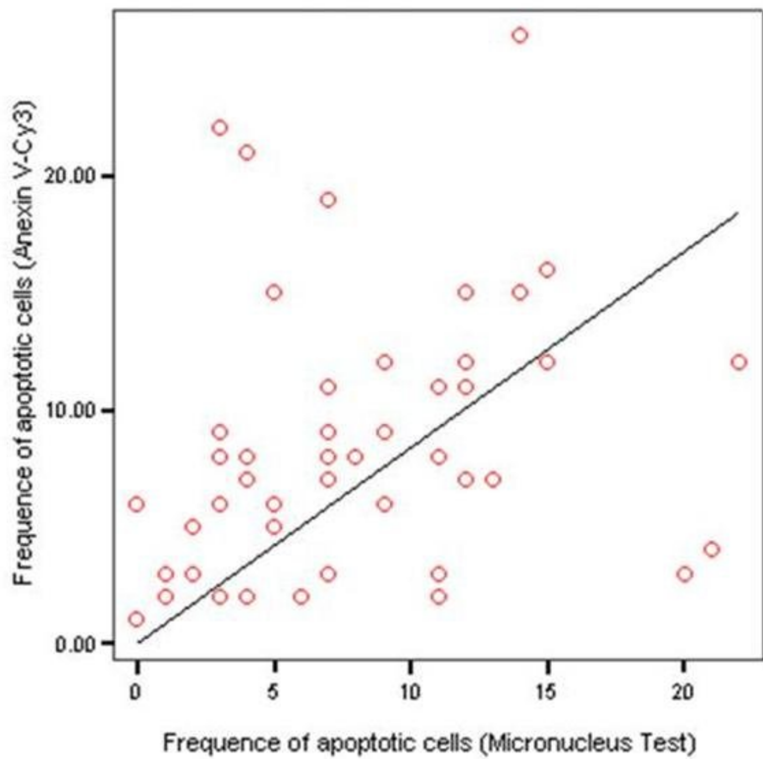

Figure 4. Correlation between frequency of apoptotic cells assessed by micronucleus test and annexin V-Cy3 test.

CAPITULO IV

3-NITROTYROSINE AND GLUTATHIONE SYSTEM IN BIPOLAR PATIENTS IN EARLY AND LATE COURSE OF ILLNESS.

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3-Nitrotyrosine and Glutathione Antioxidant System in Bipolar Patients in early and late stage of illness

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Abstract

Background and aims: There has been an increasing interest in the role of oxidative stress in the pathophysiology of bipolar disorder (BD). To explore this further, we evaluated the activity of glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST), as well as 3-nitrotyrosine level and carbonyl content in BD patients with early (within 3 years of illness onset) and late (a minimum of 10 years of illness) stages of illness. *Method:* 30 patients in the early stage and 30 patients in the late stage of BD, diagnosed according to DSM-IV criteria, were matched to 30 healthy volunteers for each group of patients. Symptomatic status was measured using the Hamilton Depression Rating Scale (HAM-D) and Young Mania Rating Scale (YMRS). *Results:* We found a significant increase in 3-nitrotyrosine levels in early ($p<0.01$) and late stage BD patients ($p<0.01$). GR and GST activity were increased in late stage patients only. GPx activity and carbonyl content were not different between the groups. *Conclusion*: Our data indicates a possible nitration-induced damage in BD patients that is present from the early stage of illness. Our data also indicate that late stage BD patients demonstrate enhanced activity of GR and GST, which could suggest the involvement of compensatory system in BD

Key Words: bipolar disorder; glutathione peroxidase; glutathione reductase; glutathione S-nitrotyrosine; oxidative stress; protein carbonyl.

Introduction

Bipolar disorder (BD) is a chronic psychiatric illness characterized by recurrent episodes of mania, hypomania, mixed states, and depression. BD is a highly disabling condition which is estimated to affect 1-3% of the population worldwide (1-4). It has been increasingly recognized that individuals with BD are at higher risk of suffering from chronic general medical conditions, such as cardiovascular disease, obesity and diabetes mellitus (3, 5), which are directly associated with the increased morbidity and mortality observed in this disorder. In addition, there is an emerging body of evidence correlating chronic and severe medical conditions to oxidative stress (6).

The exact neurochemical mechanisms underlying the pathophysiology of BD are not completely understood. Recently, the number of studies investigating biochemical aspects of BD has increased. Notably, recent studies have demonstrated alterations in diverse oxidative stress parameters in the course of BD. For example, studies conducted with peripheral blood cells have demonstrated that BD is associated with alterations in antioxidant enzymes (7-10), increased lipid peroxidation (8, 9), increased levels of nitric oxide (11-13) and increased DNA fragmentation (14-16).

Sies (17) has defined oxidative stress as *a disturbance in the pro-oxidant–antioxidant balance in favor of the former, leading to potential damage.* Oxidative stress can result from diminished levels of antioxidants and/or increased production of reactive species from oxygen (ROS) or nitric oxide (RNS). Under physiological conditions, mitochondria are the major sources of reactive oxygen species (ROS), which are quenched by the antioxidant defense system. However, in situations where there is an imbalance between pro-oxidant/antioxidant states, ROS may accumulate and oxidative damage can occur. The antioxidant system is composed of three key enzymes: superoxide dismutase (SOD) which converts the superoxide radical ($O_2^{-\bullet}$) into hydrogen peroxide (H_2O_2), and catalase and

glutathione peroxidase (Gpx) both of which metabolize H_2O_2 into H_2O + O_2 (18). Other important free radicals are the nitric oxide (NO), a reactive nitrogen species (RNS). NO reacts rapidly with O2-• to form peroxynitrite (ONOO–) which is a very reactive species (18). The central nervous system is extremely vulnerable to peroxidative damage, since it is rich in oxidizable substrates, has a high oxygen tension (it metabolizes 20% of total body oxygen) and a relatively low antioxidant capacity (18, 19). Reduced glutathione (GSH) plays a key role as an essential cellular antioxidant in the defense of brain cells against oxidative damage (20). GSH is a tripeptide, composed of glutamate, cysteine and glycine and it is used as an electron donor for GPx action, leading to the production of oxidized glutathione (GSSG). This latter process is assisted by glutathione reductase (GR) to recycle GSSG to GSH. In addition, glutathione is also involved in the conjugation of foreign molecules, and these reactions are catalyzed by glutathione S-transferases (GST) (20). An important role of GST in the brain is the detoxification of quinones, formed during the oxidation of dopamine and other catecholamines (6, 21-24).

If ROS or RNS are not effectively eliminated, they can cause oxidative damage to DNA, lipids (cell and organelle membranes) and proteins (receptors and enzymes) (18). The oxidative modification of proteins is critical in many biological processes; however, this damage may also lead to the deleterious functional inactivation of proteins and enzymes (25). ROS can cause oxidation of the side chains of lysine, proline, arginine, and threonine residues by binding Fe^{+2} and Cu^{+2}, and further attack by H_2O_2 or $O_2^{-\bullet}$ may lead to the formation of protein carbonyl groups (25, 26). Peroxynitrite ($ONOO^-$), a RNS, can cause the nitration of tyrosine residues generating 3-nitrotyrosine (25). In addition, nitration of the tyrosine residues of GST can result in increased enzyme activity (27).

As several studies have demonstrated elevated levels of NO in bipolar patients (12-13, 28-29), and given the importance of the glutathione system for antioxidant capacity in the

brain, our aim in this study was to determine if there is evidence for involvement of glutathione system (GPX, GR and GST) and protein damage by ROS and RNS (Carbonylation and 3-nitrotyrosine) in bipolar patients in early (0-3 years of illness) and late (10-20 years of illness) stages of illness, with objective to evaluate if there are cumulative effect of oxidative stress in bipolar disorder.

Material and Methods

Subjects

This was a double case-control study of 60 patients with BD (30 patients within the first 3 years of illness [early stage]; and 30 patients with a minimum of 10 years of illness [late stage]), with each group of patients matched to 30 healthy volunteers. All patients and healthy volunteers were 18 years of age or older. Early stage patients were recruited from the Mood Disorder Centre at the University of British Columbia, Vancouver, Canada. Late stage patients were recruited from the Bipolar Disorders Program, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Patients were non-smokers, were free of active medical conditions, and were not prescribed medications, other than those necessary for the management of their psychiatric illness. The diagnosis of BD was confirmed using the Structured Clinical Interview for DSM-IV – Axis I (SCID-I) (30). Manic and depressive symptoms were assessed using the Young Mania Rating Scale (YMRS) (31) and the Hamilton Depression Rating Scale (HDRS) (32), respectively.

Healthy volunteers (n=60) had no personal or family history in first degree relatives of major psychiatric disorders, dementia, mental retardation, cancer or tumor, and were matched to patients for age, gender and education. They were nonsmokers and were not taking medication.

This study was approved by the University of British Columbia Clinical Research Ethics Board and the Clinical Hospital of Porto Alegre Ethics Board (Brazil). All patients and healthy volunteers provided written informed consent prior to taking part in any study-specific activities.

Procedure

Collection and processing of blood samples

Each patient and healthy volunteer provided 5 ml blood samples which were collected by venipuncture without anticoagulants. Serum was obtained by centrifugation at 3,000xg for 5 minutes and kept frozen at -70°C for up to 6 months until the biochemical assays were performed.

Glutathione peroxidase activity

GPx activity was measured using the Cayman Chemical Glutathione Peroxidase Assay kit (USA, lot number 185936). This kit utilizes an indirect measure of GPx activity. Oxidized glutathione (GSSG) is produced via reduction of hydrogen peroxide by GPX, and is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by decreased absorbance of light at 340nm. One unit of GPx is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 25°C. The results were recorded as units per mg of protein.

Glutathione reductase activity

GR activity was measured using the Cayman Chemical Glutathione Reductase Assay kit (USA, lot number 183406). This kit assays GR activity by measuring the rate of oxidation

of NADPH to $NADP^+$, which is accompanied by a decrease in absorbance at 340nm. One unit of GR is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 25°C. The results were recorded as units per mg of protein.

Glutathione S-transferase activity

GST activity was measured using the Cayman Chemical Glutathione S-Transferase Assay kit (USA, lot number 184236). This kit measures total GST activity (cytosolic and microsomal) by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. This conjugation is accompanied by an increase in absorbance at 340nm. One unit of enzyme will conjugate 1.0 nmol of CDNB with reduced glutathione per minute per mg of protein at 25°C. The results were recorded as units per mg of protein.

Protein Carbonyl Assay

Protein carbonyl levels were measured using the Cayman Chemical Protein Carbonyl Assay kit (USA, lot number 184236). This kit utilizes the dinitrophenylhydrazine (DNPH) reaction to measure the protein carbonyl content in the serum. The amount of protein-hydrozone produced is quantified spectrophotometrically by absorbance at 360nm.

3-Nitrotyrosine

Plasma 3-nitrotyrosine content was determined by enzyme linked immunosorbent assay (ELISA) with a polyclonal anti-3-nitrotyrosine antibody (Abcam, Cambridge, UK) and nitrated bovine serum albumin (BSA) as a standard, as previously described (33, 34). Briefly, a nitrated protein solution was prepared for use as a standard by incubating BSA (10 mg/ml in PBS [pH 7.4]) with 100 µM tetranitromethane in 50 mM KH_2PO_4 (pH 8) for 30 min at 37°C. After the pH was adjusted to 10 with 3M NaOH, the amount of 3-nitrotyrosine present

in this solution was measured by absorbance at 430 nm and expressed as nanomoles of 3-nitrotyrosine per milligram of BSA (using a εM of 4,400 M^{-1} cm^{-1}). 3-nitrotyrosine in the proteins of human plasma was determined by competitive ELISA as described in Davis et al. (34), with some modifications. The assay was performed in 96-well plates at 37°C coated overnight with 4 µg/ml (100µl per well) of nitro-BSA which had been blocked with nonfat dry milk for 2h at 37°C to prevent non-specific binding. After blocking and washing with PBS buffer containing 0.6% Tween 20, serial diluted standard (200µg/ml-0.049µg/ml) and diluted serum (1:10 in PBS, pH 7.4) were bound to 96-well plate and incubated for 30 min on an orbital shaker, followed by incubation with anti-3-nitrotyrosine (1:1000; Abcam, Cambrige, U.K.) for 2h at 37°C. Sequential incubations were then performed with peroxidase-conjugated anti-rabbit immunoglobulin G (Abcam, Cambrige, U.K.). After further washing, color development was initiated by the addition of substrate and was allowed to develop for up to 30 min at room temperature and terminated by the addition of 4 M sulphuric acid. Antibody binding was determined from the absorbance at 450 nm. The concentrations of nitrated proteins that inhibit anti-nitrotyrosine antibody binding were estimated from the standard curve and are expressed as nitro-BSA equivalents.

Statistical Analysis

Data were expressed as mean ± S.D. Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) Version 15.0. The Kolmogorov-Smirnov Test was used to compare the observed cumulative distribution function to a theoretical cumulative distribution. Our data had parametric distribution. Each group of patients (early stage and late stage) was assigned a separate control group as age and sex differed between the patient groups. The outcome measures for the four groups were compared using ANOVA and Tukey post-hoc tests. In order to adequately adjust for confounders, an ANCOVA was used, having

each biochemical marker as the outcome. Covariates included sex, age, education and mood symptoms (HAM-D and YMRS) for patients' analyses. Other variables, such as use of mood stabilizers, antidepressants or antipsychotics, number of suicide attempts, and number of hospitalizations, were not associated with biochemical markers, and therefore were not included as covariates.

Results

A total of 60 BD patients and 60 healthy volunteers were enrolled. Demographic variables are presented in Table 1. No statistically significant differences in age, gender, and education were detected between patients in the early stage of BD (N=30) and matched volunteers (N=30) and BD patients in late stage of BD (late stage; N=30) and matched volunteers (N=30). As expected, late stage BD patients had higher YMRS (3.6 ± 4.1) and HAM-D scores (9.2 ± 6.0) in comparison to early stage BD patients (1.5 ± 2.8, YMRS and 3.8 ± 7.1 HAM-D). GAF scores were similar between the 2 groups of BD patients. Medications prescribed for each group of BD patients are listed in Table 2. All patients were prescribed psychotropic medications (mood stabilizers, antipsychotics and/or antidepressants).

Our first aim in this study was to quantify the involvement of glutathione enzymes in bipolar patients in early and late stages of illness. Fig. 1 shows a significant increase in GST activity in late stage BD patients ($F_{(3;116)}=33.11$; $p<0.001$) but not in those with early stage BD. Similarly, GR activity was also increased in late stage BD patients only ($F_{(3;116)}=17.60$; $p<0.001$). Suggesting that GSH are using for detoxify toxic products by GST and need recycling by GR. No changes were observed in GPx activity in either group of patients ($F_{(3;116)}=1.54$; $p = 0.210$).

Fig. 2 shows that protein carbonyl levels did not differ significantly between either group of BD patients and their matched group of healthy volunteers ($F_{(3;116)}$=2.66; p = 0.060). However, the content of 3-nitrotyrosine was increased in bipolar patients in both the early stage ($F_{(3;116)}$=23.55; p<0.001) and the late stage of illness ($F_{(3;116)}$=23.55; p<0.001) compared to controls which suggests that nitration of tyrosine residues is more involved with BD than oxidation of lysine, proline, arginine, and threonine amino-residues by ROS.

We investigated correlations between biochemical markers and certain clinical variables (age of onset; time of illness; HAM-D; YMRS, GAF; number of previous mania, depression and hipomaina episodes). There was a positive correlation between length of illness and both GR (r=0.492; p<0.001) and GST levels (r=0.333; p=0.010) (figure 3). The associations between biochemical markers and the use of mood stabilizers, antipsychotics, and antidepressants are presented in Table 3. GR and GST activity and carbonyl content did not differ between patients taking vs not taking any of these medications. GPx activity was increased in BD patients taking any antipsychotic or any antidepressant or any mood stabilizer at the time of measurement; however GPx activity was not different from the control groups.

Discussion

This is the first study to evaluate the activity of glutathione enzymes and the levels of protein oxidation and nitration in BD patients at different stages of the disorder. Our results indicated that BD patients in the late stage of illness have increased activity of GR and GST and increased levels of 3-nitrotyrosine relative to healthy volunteers with no personal or family history of psychiatric illness, while early stage patients demonstrate only increased 3-nitrotyrosine levels. Protein oxidation, verified by carbonyl levels, and GPx activity did not differ between early or late stage patients and matched controls. These results suggest that

nitration of tyrosine residues occurs early in the course of the BD, and that with increased duration of illness, BD patients up-regulate antioxidant enzymes (GR and GST) suggesting the involvement of compensatory systems during illness progression in BD.

If oxidative stress is present in BD, what might be responsible for this in BD? There is evidence that increased dopamine levels are associated with the symptoms of mania and that a reduction in dopamine transmission through reduction in dopamine synthesis or blockade of dopamine D2 receptors may be associated with antimanic effects (see 35 and 36 for reviews). Interestingly, increased dopamine levels are an important source of oxidative stress in the brain, due to oxidative metabolism of dopamine (37, 38). Dopamine may be metabolized via monoamine oxidase with production of H_2O_2 and dihydroxyphenylacetic acid (39, 40) or can go through nonenzymatic hydroxylation in the presence of Fe^{2+} and H_2O_2 leading to the formation of 6-hydroxydopamine (6-OHDA) (21, 41). 6-OHDA is toxic to the nervous systems, and the mechanisms involved in this toxicity include: endoplasmic reticulum stress (ER-stress), activation of GSK-3-beta by phosphorylation at tyrosine 216, and inhibition of protein kinase AKT by phosphorylation at Ser473 (24). *In vivo* electrochemical measurements have shown that within a few minutes after injection into rat brain, about 20% of 6-OHDA is converted to *p-quinone* (24, 25), which can also be conjugated with GSH by GST (6, 22). This reaction is similarly thought to combat degenerative processes in the dopaminergic system in human brain (42). Moreover, Tirmenstein et al. (43), using human neuroblastoma cells, showed that after 4 h of treatment, 6-OHDA significantly depleted cellular ATP and GSH concentrations, while after 24 hours it induced a concentration-dependent increase in GSH and total glutathione concentrations, suggesting that 6-OHDA induces oxidative stress in SH-SY5Y cells resulting in an adaptive increase in cellular GSH concentrations. Our results demonstrate that only BD patients in the late stage of illness have increased levels of GR and GST (figure 1). In addition, GR and GST levels show positive

correlations with length of illness (figure 3). The increase in antioxidant enzymes in late stage BD patients may be a consequence of cumulative effect of oxidative stress with progression of BD. It is important to not that GR and GST levels did not appear to be related to the use of psychiatric medications in our patients (Table 3).

Neuroimaging studies have shown increased levels of glutamate+glutamine and lactate levels in discrete sub-regions of the prefrontal cortex in adult bipolar patients (see 44 to review). NMDA receptors are ionotropic glutamate receptors that, when stimulated by glutamate, allow the passage of calcium into the cell, promoting activation of CAMKIV; which may activate nitric oxide synthases (NOS1 and NOS3), thereby leading to increased nitric oxide (NO) production (36). Supporting this scenario, increased NO levels have been demonstrated in bipolar patients (11, 12, 45). As well, increased intracellular calcium levels are a consistent finding in studies of bipolar disorder (see 46 for review).

NO can react with superoxide leading to the formation of peroxynitrite ($ONOO^-$) which has the ability to nitrosylate free tyrosine and tyrosine residues in proteins, forming 3-nitrotyrosine (47). Our results indicate that BD patients in early and late stages of illness have increased levels of 3-nitrotyrosine (figure 2).The modification of critical cellular proteins by $ONOO^-$ -induced tyrosine nitration has been proposed as an early event in the process of dopamine neuronal damage (48). In addition, Ji and Bennet (27) showed that microsomal GST is activated on exposure to $ONOO^-$ by nitration of tyrosine residues. The activation of GST by $ONOO^-$ may play an important role in limiting the extent of oxidative tissue injury when other cellular antioxidant enzymes, such as SOD (49) and xantine oxidase (50), are compromised under pathophysiological conditions of excessive $ONOO^-$ formation. Given that 3-nitrotyrosine is commonly measured as a biomarker of reactive nitrogen species generation (51) our results suggest that nitration process may play an important role in pathophysiology of BD.

Recent findings have suggested that mitochondrial dysfunction may be associated with the pathophysiology of BD (see 52 for review). Studies conducted in post-mortem brain tissue have demonstrated that mitochondrial genes are down-regulated in the hippocampus (53) and dorsolateral prefrontal cortex of BD patients (54, 55), whereas SOD and catalase expression is decreased in the hippocampus of individuals with BD (56). Interestingly, 3-nitrotyrosine (57) and 6-OHDA (27) may promote inhibition of the complex I of the mitochondrial electron transport chain. In addition Ferger et al. (58) suggests that 6-OHDA can increase levels of NT. In our view, reactive nitrogen species may be a link between dopamine related oxidative stress and mitochondrial dysfunction. Future studies will need to investigate the levels of 6-OHDA and the relation of this with increase of reactive nitrogen species in BD.

The limitations of our study should be considered in interpreting our results. All of our patients were taking psychoactive medications. However, we analyzed the levels of biochemical markers in patients taking vs not taking antipsychotics, antidepressants and mood stabilizers, including lithium (Table 3). We did not find any difference in the activity of GR, GST and carbonyl depending on medication use. While GPx showed increased activity in patients with current use of any medication, we did not find a difference between patients and healthy volunteers in the GPx activity. 3-nitrotyrosine levels were increased in patients taking lithium. Lithium is believed to have neuroprotective effects; for example lithium has been associated with dose-dependent induction of glutathione levels in humam neuroblastima cells (59) and increased GST mRNA (60, 61).

In BD, there is strong evidence for involvement of mitochondrial dysfunction and oxidative injury. The reason for this vulnerability is still unclear. One possibility is that it is related to the dopamine content of these cells and the oxidative potential of this catecholamine. In a recent report, our group has proposed the involvement of allostatic load

in the pathophysiology of BD. The term ‘allostatic load’ was introduced to refer to a cumulative, multisystemic view of the physiologic toll that is required for adaptation (see 62 for review). The capacity to achieve stability through change is called allostasis (63). Using this knowledge, in this study, we separated the patients in early and late stage of illness with the objective of verifying the involvement of cumulative damage and/or adptative/compensatory process involved in BD. Our data indicate a possible nitrosylation-induced damage in BD patients that is present early in the course of illness. Our data also indicate that bipolar patients in late stage of the disorder demonstrate enhanced activity of GR and GST, which could suggest the involvement of compensatory system in BD. Thus, our results provide some support to the theory of involvement of allostatic process in BD.

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Table 1. Sociodemographic variables among patients and controls.

Variable	Early Course (N)		P value	Late Course (N)		P value
	Patients with Bipolar Disorder (30)	Controls (30)		Patients with Bipolar Disorder (30)	Controls (30)	
Sex Male	43.3% (13)	33.3% (8)	0.596[b]	30% (9)	36.7% (11)	0.785[b]
Age (years) Mean (SD)	22.4(3.9)	22.1(3.6)	0.734[a]	41.4(8.4)	43.2(6.4)	0.376[a]
Education (years) Mean (SD)	13.5(2.0)	12.9(2.8)	0.331[a]	9.3(3.8)	10.6(1.6)	0.086[a]
Ethnicity Caucasian	73.3% (22)	83.3% (25)	0.532[b]	90% (27)	93.3%(28)	1.0[b]

[a] T-test
[b] χ^2 test

Table 2. Clinical features of BD patients with early course illness (N=30) and late course illness (N=30).

	Early Course (30)	Late Course (30)	
Age onset illness Mean (SD)	20.2(4.2)	27.2(7.4)	<0.001[a]
Length of illness Mean (SD)	2.1(2.9)	13.9(5.1)	<0.001[a]
Number previous Depression - Mean (SD)	1.1(1.5)	6.2(8.1)	
Number previous Hypomania - Mean (SD)	0.8(2.1)	0.05(0.2)	0.046[a]
Number previous Mania - Mean (SD)	1.0(0.0)	7.1(9.1)	0.004[a]
YMRS Mean (SD)	1.53(2.8)	3.6(4.1)	0.026[a]
HAM-D Mean (SD)	3.8(7.1)	9.2(6.0)	0.002[a]
GAF Mean (SD)	63.3(12.5)	61.4(17.5)	0.643[a]
Medication			
Mood Stabilizer	83.3% (25)	86.7% (26)	1.0[b]
Antipsychotic	76.7% (23)	60.0% (18)	0.267[b]
Antidepressant	10% (3)	20% (6)	0.472[b]

[a] T-test
[b] χ^2 test

Table 3. Biochemical marker in bipolar patients with taken or not different medication.

	GPx nmol/min/ml			GR nmol/min/ml			GST nmol/min/ml			Carbonyl Content (nmol/mg)			3-Nytrotyrosine levels		
Any Antipsychotic															
	Mean	**SD**	**P***	**Mean**	**SD**	**P***	**Mean**	**SD**	**P***	**Mean**	**SD**	**P***	**Mean**	**SD**	**P***
No	33.27	8.21	**0.02**	13.65	8.88	0.99	16.76	9.12	0.32	110.81	16.92	0.3	66.01	46.47	0.22
yes	38.84	8.83		13.66	6.68		14.34	8.56		103.74	27.12		80.29	38.60	
Any Antidepressant															
No	36.101	8.2065	**0.04**	13.523	7.383	0.74	15.515	9.0482	0.39	105.49	24.254	0.71	77.089	42.044	0.56
yes	42.582	11.418		14.417	7.6745		12.772	6.6174		108.77	26.733		68.284	38.957	
Any Mood Stabilizers															
No	36.23	10.69	**0.08**	12.15	7.67	0.51	14.63	6.64	0.86	103.34	16.36	0.73	65.58	31.03	0.43
yes	41.87	8.45		13.92	7.36		15.19	9.11		106.45	25.69		77.57	42.97	
Lithium															
No	37.283	9.0161	0.86	12.124	7.4086	0.12	13.707	7.3729	0.23	102.2	18.48	0.25	60.592	33.794	**0.01**
yes	36.877	9.0425		15.091	7.1509		16.411	9.7805		109.52	28.779		89.966	43.288	

* T-test

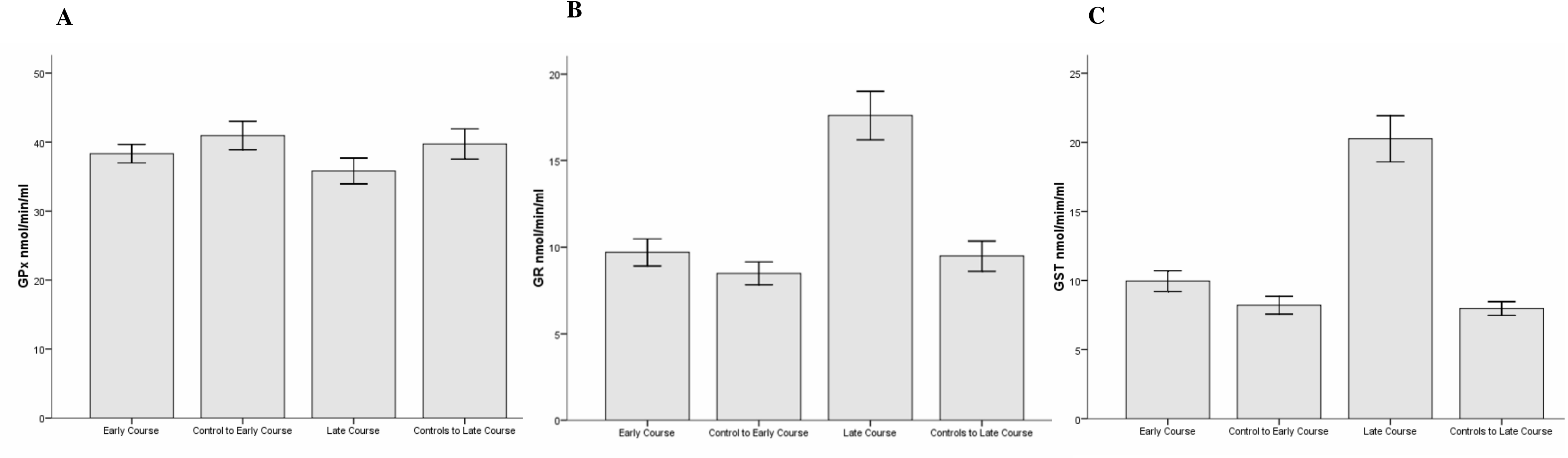

Figure 1. Glutathione peroxidase (A), glutathione reductase (B) and glutathione S-transferase (C) activity in serum samples from bipolar disorder patients with 0-3 years of illness (early course) and patients with 10-20 years of illness (late course). Results are the mean ± S.D. * indicates $p<0.01$ between two comparison groups determined by one-way ANOVA followed with the post-hoc analysis.

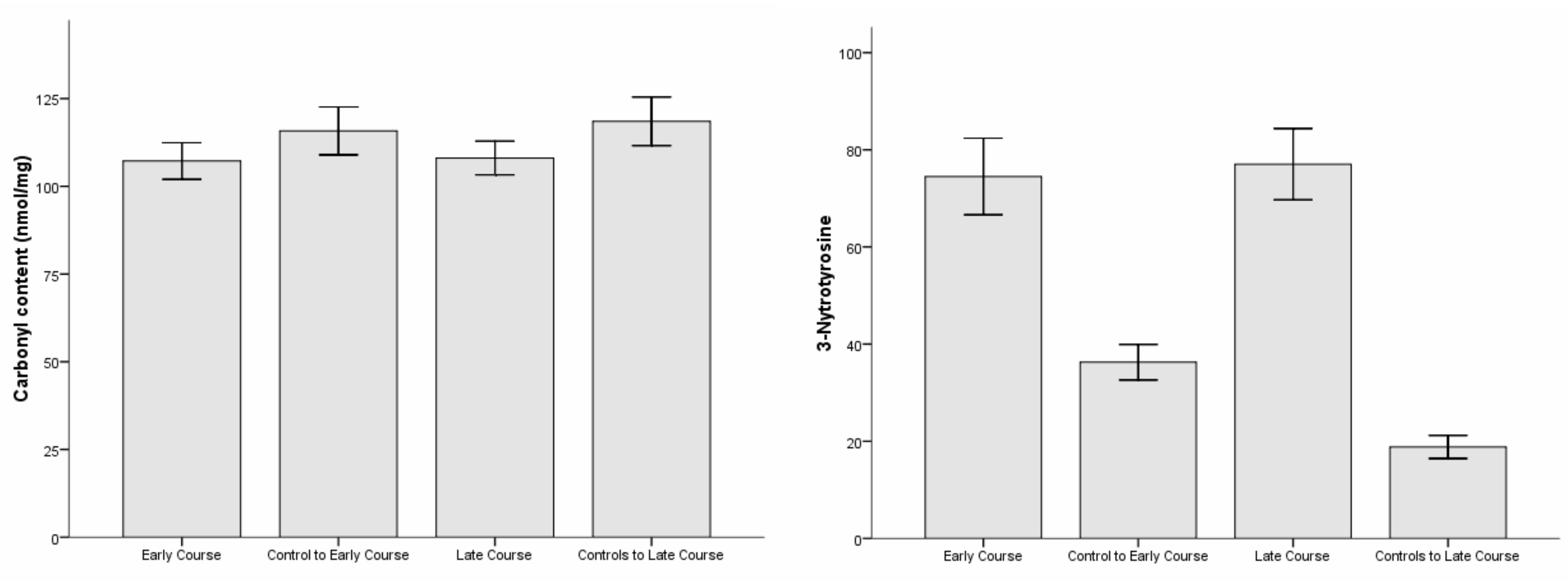

Figure 2. Levels of protein oxidation (Carbonyl levels) and nitration (3-nitrotyrosine) in serum samples from bipolar disorder patients with 0-3 years of illness (early course) and patients with 10-20 years of illness (late course). Results are the mean ± S.D. * indicates $p<0.01$ between two comparison groups determined by one-way ANOVA followed with the post-hoc analysis.

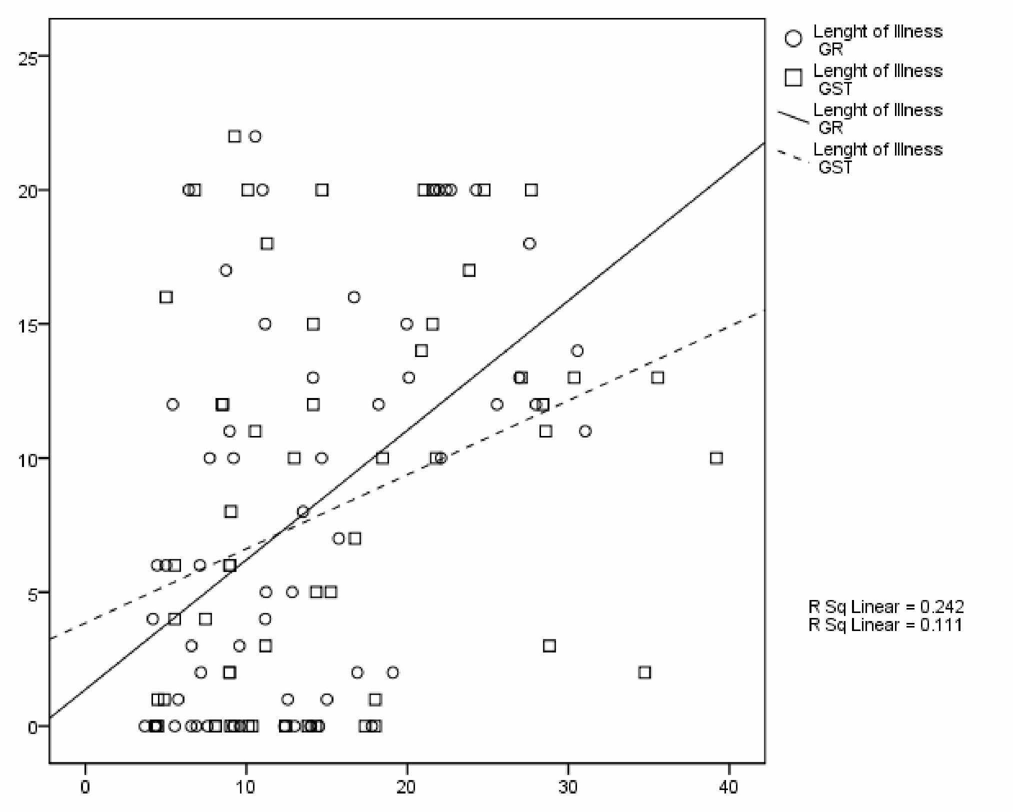

Figure 3. Correlation between glutathione reductase or glutathione S-transferase levels with length of illness in all bipolar patients (N=60). * Pearson Correlation Coefficient.

PARTE III

3. SUMÁRIO DOS RESULTADOS

3.1 Resultados em modelo animal.

a. *Dano recente e permanente ao DNA e marcadores de estresse oxidativo no modelo animal de mania induzida por administração intraperitoneal de anfetamina.* A administração de anfetamina induziu o aumento do dano ao DNA, tanto recente como permanente em linfócitos e hipocampo, bem como alterou a atividade sérica e hipocampal das enzimas antioxidantes, além de aumentar a peroxidação lipídica no soro e no hipocampo. A administração de lítio e valproato foram capazes de modular a alteração na atividade das enzimas antioxidantes, porém somente o lítio foi capaz de prevenir a formação de dano recente ao DNA. Nem o lítio nem o valproato mostraram capacidade de prevenir ou reverter aformação do dano permanente ao DNA.

b. *Dano recente e permanente ao DNA em ratos jovens e adultos tratados com metilfenidato*. Nossos resultados mostraram que o metilfenidato aumentou o dano recente ao DNA, sendo esse mais pronunciado com o tratamento crônico de metilfenidato e no estriado e linfócitos do que no hipocampo tanto de ratos jovens como adultos. Nem o tratamento agudo nem o crônico com metilfenidato foram capazes de induzir a formação de micronúcleus.

3.2 Resultados em humanos:

a. *Dano ao DNA e Apoptose.* Os pacientes bipolares livres de comorbidades clínicas (diabetes, câncer, asma, reumatite) apresentaram uma elevação na freqüência de apoptose, porém não apresentaram um aumento no dano permanente ao DNA.

b. *Dano protéico e sistema glutationa.* Pacientes bipolares no inicio da doença apresentaram uma elevação do conteúdo de 3-nitrotirosina, enquanto pacientes bipolares com mais de 10 anos de tempo de doença apresentaram uma elevação tanto no conteúdo

da 3-nitrotirosina como na atividade da enzima glutationa redutase e glutationa S-transferase.

4. DISCUSSÃO

De acordo com a Organização Mundial de Saúde o TB é a sexta causa de incapacidade entre as condições médicas e psiquiátricas. Além disso, o TB está associado ao aumento da morbidade e mortalidade por diversas condições médicas gerais, como doenças cardiovasculares, obesidade e diabetes mellitus (Kupfer et al, 2005). Essas doenças, bem como o câncer, estão associadas ao estresse oxidativo (Takuma et al, 2004) e ao aumento do dano ao DNA (Faust et al, 2004). Além disso, evidências atuais têm mostrado o envolvimento tanto do estresse oxidativo (Andreazza et al, 2007a; Gergerlioglu et al, 2007; Machado-Vieira et al, 2007; Selek et al, 2007; Savas et al, 2006; Ozcan et al, 2004; Ranjekar et al, 2003; Kuloglu et al, 2002; Abdalla et al, 1986) quanto do dano ao DNA (Andreazza et al, 2007b; Benes et al, 2006; Butter et al, 2007; Simon et al, 2006) no TB.

Recentemente o nosso grupo realizou uma meta-análise para avaliar o poder dos resultados de estresse oxidativo no TB. Os dados confirmaram que o TB apresenta uma elevação nos índices de peroxidação lipídica (*random effect*; *pooled effect size*: 2.488; 95% CI = 1.41-3.56; p=0.001) e dos níveis de óxido nítrico (*random effect; pooled effect size*: 0.899; 95% CI = 0.14-1.66; p=0.02), porém os indícios de elevação na atividade da SOD (*random effect; pooled effect size*: -0.020; 95% CI = -1.33-1.29; p=0.976), de decaimento da catalase (*random effect; pooled effect size*: -1.246; 95% CI= -4.48-1.99; p=0.451) e da inaltereção da atividade da enzima GPx (*random effect; pooled effect size*: 0.134; 95% CI = -0.431-0.698; p=0.642) não foram confirmados (Andreazza et al., dados não publicados). Os artigos referentes ao dano ao DNA não foram avaliados nessa meta-análise, pois existem apenas 3 estudos, sendo que desses, 2 são estudos post-mortem e 1 em linfócitos de pacientes com TB (Tabela 2).

Tabela 1: Estudos sobre atividade de enzimas antioxidantes, peroxidação lipídica (TBARS) e níveis de óxido nitrico em pacinetes com trantosno bipolar.

Reference	Pacientes/Controles	Pacientes Bipolares			Fumo[2]	Amostra	Técnica	Resultados[4]
		Maniacos	Deprimidos	Eutimicos				
Superoxido Dismutase								
Andreazza et al, 2007a	85/32	32	21	32	0/85 (P) 0/32 (C)	Soro	Adrenochrome	aumentado em pacientes deprimidos e maniacos
Machado-Vieira et al,2007	30/30	30	0	0	NA	Soro	Adrenochrome	aumentado em pacientes livers de meidcação
Gergerlioglu et al, 2007	29/30	29	0	0	16/13 (P) 14/16 (C)	Soro	Nitroblue tetrazolium	diminuído
Selek et al, 2007	30/30	0	30	0	15/14 (P) 14/16 (C)	Soro	Nitroblue tetrazolium	diminuído
Savas et al, 2006	27/20	0	0	27	NA	Soro	Nitroblue tetrazolium	diminuído
Ozcan et al, 2004[1]	30/21	16	2	0	9/21 (P) 9/21 (C)	RBC[3]	Nitroblue tetrazolium	NS**
Ranjekar et al, 2003	10/31	NA*	NA	NA	NA	RBC	Nitroblue tetrazolium	NS
Kuloglu et al, 2002	23/20	NA	NA	NA	NA	RBC	Nitroblue tetrazolium	aumentado
Abdalla et al, 1986	20/58	NA	NA	NA	NA	RBC	Nitroblue tetrazolium	aumentado
Catalase								
Andreazza et al, 2007a	85/32	32	21	32	0/85 (P) 0/32 (C)	Soro	µmol of H_2O_2 consumed / min	diminuído em pacientes eutímicos e manícos
Machado-Vieira et al,2007	30/30	45	0	0	NA	Soro	µmol of H_2O_2 consumed / min	aumentado
Ozcan et al, 2004[1]	30/21	16	2	0	9/21 (P) 9/21 (C)	RBC[3]	µmol of H_2O_2 consumed / min	diminuido nos grupos pré e pós-tratamento
Ranjekar et al, 2003	10/31	NA	NA	NA	NA	RBC	µmol of H_2O_2 consumed / min	diminuido
Glutationa peroxidase								
Andreazza et al, 2007a	85/32	32	21	32	0/85 (P) 0/32 (C)	Soro	nmolNADPH oxidized / mim	aumentado em pacientes eutimicos
Ozcan et al, 2004[1]	30/21	16	2	0	9/21 (P) 9/21 (C)	RBC	nmolNADPH oxidized / mim	diminuido no grupo pré-tratamento
Ranjekar et al, 2003	10/31	NA	NA	NA	NA	RBC[3]	nmolNADPH oxidized / mim	NS
Kuloglu et al, 2002	23/20	NA	NA	NA	NA	RBC	nmolNADPH oxidized / mim	NS
Abdalla et al, 1986	20/58	NA	NA	NA	NA	RBC	nmolNADPH oxidized / mim	NS
TBARS								
Andreazza et al, 2007a	85/32	32	21	32	0/85 (P) 0/32 (C)	Soro	TBARS	aumetnado manicos, deprimidos e eutimicos
Machado-Vieira et al,2007	45/30	45	0	0	NA	Soro	TBARS	aumentados em pacientes livres de medicação
Savas et al, 2005	22/22	0	0	22	13/9 (P) 12/10 (C)	Soro	TBARS	aumentado
Ozcan et al, 2004[1]	30/21	16	2	0	9/21 (P) 9/21 (C)	Soro	TBARS	aumentado no pré e pós-tratamento
Ranjekar et al, 2003	10/31	NA	NA	NA	NA	Plasma	TBARS	NS
Kuloglu et al, 2002	23/20	NA	NA	NA	NA	Plasma	TBARS	aumentado
Oxido Nítrico								
Gergerlioglu et al, 2007	29/30	29	0	0	16/13 (P) 14/16 (C)	Soro	Greiss reaction	aumentado
Selek et al, 2007	30/30	0	30	0	15/14 (P) 14/16 (C)	Soro	Greiss reaction	aumentado
Savas et al, 2006	27/20	0	0	27	NA	Soro	Greiss reaction	aumentado
Savas et al, 2005	22/22	0	0	22	13/9 (P) 12/10 (C)	Soro	Greiss reaction	aumentado
Ozcan et al, 2004[1]	30/21	16	2	0	9/21 (P) 9/21 (C)	plasma	Greiss reaction	diminuído no grupo de pré-tratamento
Yanik et al, 2004	43/31	43	0	0	NA	plasma	Greiss reaction	aumentado
Savas et al, 2002	44/21	44	0	0	NA	plasma	Greiss reaction	aumentado
Dano ao DNA								
Andreazza et al, 2007b	32/32	0	0	32	0/32 (P) 0/32 (C)	linfocitos	Ensaio Cometa	aumentado
Benes et al, 2006	10/18	0	0	10	NA	CPF***	Teste de Klenow	NS
Butter et al, 2007	14/14	0	0	18	NA	NG[#]	Teste de Klenow	aumentado

[1] Pacientes com transtornos afetivos: 16 maniacos-TB-1; 2 deprimios TB-1; 6 pacientes com depressao maior; 6 pacientes com esquizofrenia

[2] Fumantes/ Não-fumantes - Patientes (P) and Contorles ©

[3] Hemácias (RBC)

[4]Diferencas referentes ao grupo controle

* NA - não se aplica

** Não significante

***CPF= cortex pre-frontal

[#] NG= neurônios não-gabaérgicos

O estresse oxidativo ocorre em situações onde existe um desiquilíbrio entre a produção de radicais e a capacidade de removê-los pelos antioxidantes (enzimáticos ou não enzimáticos), causando, assim, um acúmulo de espécies reativas e, sonsequantemente, um aumento da chance de lesão oxidativa. A peroxidação lipídica ocorre pela reação dos radicias livres com as estruturas lipídicas (Halliwell e Gutteridge, 1999). Esse dano oxidativo aos lipídeos pode causar alteração da conformação de membranas lipídicas celulares, inciando um processo de perda de função celular (Poon et al., 2004), bem como a alteração da sinalização celular mediada por lipídeos (Bazan et al, 2005). Como mostrado pela meta-análise, anteriormente citada (Andreazza et al; dados não publicados), a peroxidação lipíca, avaliada através do ensaio TBARS, parece estar fortemente envolvida com o TB. Ainda, Andreazza et al (2007a) mostraram que o TBARS está aumetado tanto na fase de depressão como na fase de mania, o que sugere que a peroxidação lipídica pode ser um dano constatne no TB. Alguns autores comentam que o dano lipídico pode ser um marcador de progressão da doença (Dröge and Schipper, 2007; Berk et al, 2007), no entanto não temos na literatura estudos que tenham acompanahado a progressão da doença e verificado as alterações nos níveis de peroxidação lipídica por um período longo no TB para validar esse hipótese.

Os modelos animais são muito úteis para podermos avaliar o envolvimetno do estresse oxidativo e do dano ao DNA no SNC e, ainda, poder comparar esses danos com os resultados encontrados na circulação periférica. Além de nos auxiliarem a verificar a influência de diferentes medicações sobre rotas bioquímicas, o que é de fundamental imporatância uma vez que a maioria dos estudos são conduzidos com pacientes medicados (tabela 2). O nosso grupo tem investido no desenvolvimento de um modelo animal de mania, no qual fizemos a opção por usar a anfetamina (ANF) como indutora de hiperlocomoção (vide item 1.1.5 da introdução). Ainda nesse modelo, dividimos os animais em dois grupos: (1) Reversão: nesse grupo os ratos receberam anfetamiana por um período e nos seguintes 7

dias, lhes foram adiministrados os estabilizadores do humor, lítio (Li) ou valproato de sódio (VPT). (2) Prevenção: nesse modelo os ratos foram tratados nos primeiros 7 dias com Li ou VPT e nos seguintes 7 dias com ANF. Assim, esse modelo nos dá a oportunidade de olhar a ação da anfetamina no modelo, bem como o efeito do Li e VPT tanto na hiperlocomoção quanto nos parâmetros bioquímicos.

No primeiro estudo dessa tese, avaliamos a atividade das enzimas antioxidantes SOD e catalase, peroxidação lipídica e dano ao DNA recente e permanente no modelo animal. Os resultados mostraram que o Li e VPT foram capazes de reverter ou prevenir os danos induzidos pela anfetamina sobre as alterações das enzimas antioxidantes (SOD e catalase) e peroxidação lipídica. Quanto ao dano recente ao DNA, observamos que apenas o Li foi capaz de previnir ou reveter esse dano. Porém, nem Li e nem o VPT reverteram ou preveniram a formação de *micronucleus* (dano ao DNA permanente). Os resultados das enzimas antioxidantes e peroxidação estão de acordo com os publicados anteriormente por Frey et al (2006) usando o mesmo modelo. O interessante é que olhando juntamente os resultados de estresse oxidativo e o dano ao DNA, observamos que os níveis de TBARS correlacionam-se positivamente com os níveis de dano recente ao DNA (ensaio cometa) tanto no hipocampo quanto na periferia (soro e linfócitos).

Esses achados estão de acordo com a literatura que tem mostrado que o Li e VPT participam na regulação de diferentes mecanismos que estão envolvidos na resposta ao estresse oxidativo (Manji et al, 2000; Chuang et al, 2002). De Vasconcelos et al (2005) mostraram que o Li diminui a formação de radicais livres e aumenta a capacidade antioxidante total no hipocampo. Além disso, o Li e o VPT previnem o estresse oxidativo induzido pelo glutamato, a fragmentação do DNA (Shao et al, 2005) e aumentam a expressão da GST (Chuang et al, 2002) em culturas primárias de córtex cerebral de ratos. King e Jope (2005) mostaram que o Li atenua a indução das proteínas pró-apoptóticas Bax e caspase-3.

Ainda, Chen e Chuang (1999) mostram que o Li é capaz de aumentar a expressão da proteína anti-apoptótica Bcl-2 no córtex pré-frontal, hipocampo e estriado. A inibição da atividade da GSK-3b pela fosforilação da serina-9 também tem sido desmonstrada tanto pelo Li quanto pelo VPT (Rowe e Chuang, 2004). Beaulieu et al (2004) demonstraram que os efeitos do Li sobre o comportamento induzido pela ANF ocorrem em parte através da regulação da via de sinalização da Akt/GSK-3β, enquanto que Narita et al (2003) sugeriram que os efeitos do BDNF/trkB sobre o comportamento induzido pela ANF parecem envolver a ativação da PKC. Esses estudos sugerem que a regulação de cascatas de sinalização intracelular associadas a apoptose ou neuroplasticidade podem ser relevantes na terapêutica a longo prazo.

Os nossos resultados mostraram que o VPT não previne ou reverte o dano recente ao DNA. Tem sido demonstrado que o VPT possui atividade inbidora da histona desacetilase, o que promove a acetilação do DNA (Gottlicher et al, 2001; Phiel et al, 2001). Além disso, o VPT promove a indução da descondensação da cromatina o que aumenta a suceptibilidade do DNA à ação das nucleases e favorece a ligação de agentes intercalantes (Marchion et al, 2005). Um fato interessante é que o aumento da concentração de H_2O_2 induz a acetilação das histonas, bem como diminui a desacetilação (Marichon et al, 2005). Assim, pacientes bipolares que tenham elevada produção de H_2O_2 e façam uso de VPT podem estar aumentando as chances de dano ao DNA.

Seguindo os resultados deste estudo, observamos que a ANF induz a um aumento da peroxidação lipídica, da atividade das enzimas antioxidantes SOD e catalase, bem como aumenta a freqüência de dano recente e permanente ao DNA. A ANF atua principalmente facilitando a liberação de dopamina (DA) pelas vesículas pré-sinápticas, mas também age bloqueando a recaptação de DA pelos transportadores de DA pré-sinápticos (Sulzer et al. 1995). Dessa forma, a ANF aumenta significativamente o conteúdo de DA na fenda sináptica,

ativando a transmissão dopaminérgica cerebral. O envolvimento da via dopaminérgica no TB é apoiado por estudos farmacológicos, genéticos, pós-mortem e de neuroimagem como discutido na seção 1.1.2 da introdução. Além disso, a administração de substâncias dopaminérgicas, como ANF ou L-DOPA, induz sintomas maníacos em indivíduos com TB (Muphy et al 1971; Gerner et al, 1976). Assim, especulamos que a possível via de indução de estresse oxidativo no modelo animal de mania, usando anfetamina, ocorra pela via dopaminérgica. O aumento da concentração de DA na fenda sináptica ativa as rotas de oxidação da DA, as quais envolvem a formação de espécies reativas do oxigênio ou moléculas neurotóxicas como discutido abaixo (Ress et al, 2007; Chen et al, 2008). Além disso, Stumm et al (1999) mostraram que o tratamento de neurônios corticais de ratos com ANF induz a clivagem do DNA, bem como aumenta a expressão de proteínas pró-apoptóticas como a Bcl-x, o que está de acordo com os nossos resultados, onde encontramos um aumento da freqüência de micronúcleus demonstrando que o dano ao DNA não foi reparado eficientemente pelo sistema de reparo ao DNA, levando à divisão inadequada do material genético nuclear. É importante salientar que o dano permanente não foi prevenido ou revertido pelo Li ou VPA.

A dopamina sofre dois tipos de oxidação: enzimática, através das enzimas monoamino oxidase (MAO) e catecol-o-metil transferase (COMT), ou não-enzimática, também chamada de auto-oxidação (Obata 2002; Halliwell 2001; Asanuma et al, 2004). A auto-oxidação da dopamina ocorre em presença de íons Fe^{2+} livres e H_2O_2, promovendo a hidroxilação da dopamina na posição 6, formando a 6-hidroxidopamina que pode ser convertida, em torno de 20%, a o-quinona (Baez 1997). O sistema mais efetivo para detoxificação da o-quinona no cérebro é através da enzima glutationa S-transferase (GST), a qual utiliza glutationa (GSH) para formar um complexo estável com a o-quinona (Obata 2002; Baez 1997). A dopamina, quando oxidada pela enzima MAO, leva à formação do ácido

3,4 diidroxifenilacético e H_2O_2, capaz de gerar $OH^{\bullet}$ através da reação com íons de ferro (Asanuma et al 2004; Obata 2002). Essa via ocorre especialmente nos gânglios da base (putamen, núcleo caudal e substantia nigra) onde existem altas concentrações de dopamina, oxigênio e ferro (Qureshi et al 2004; Obata 2002) (figura 4).

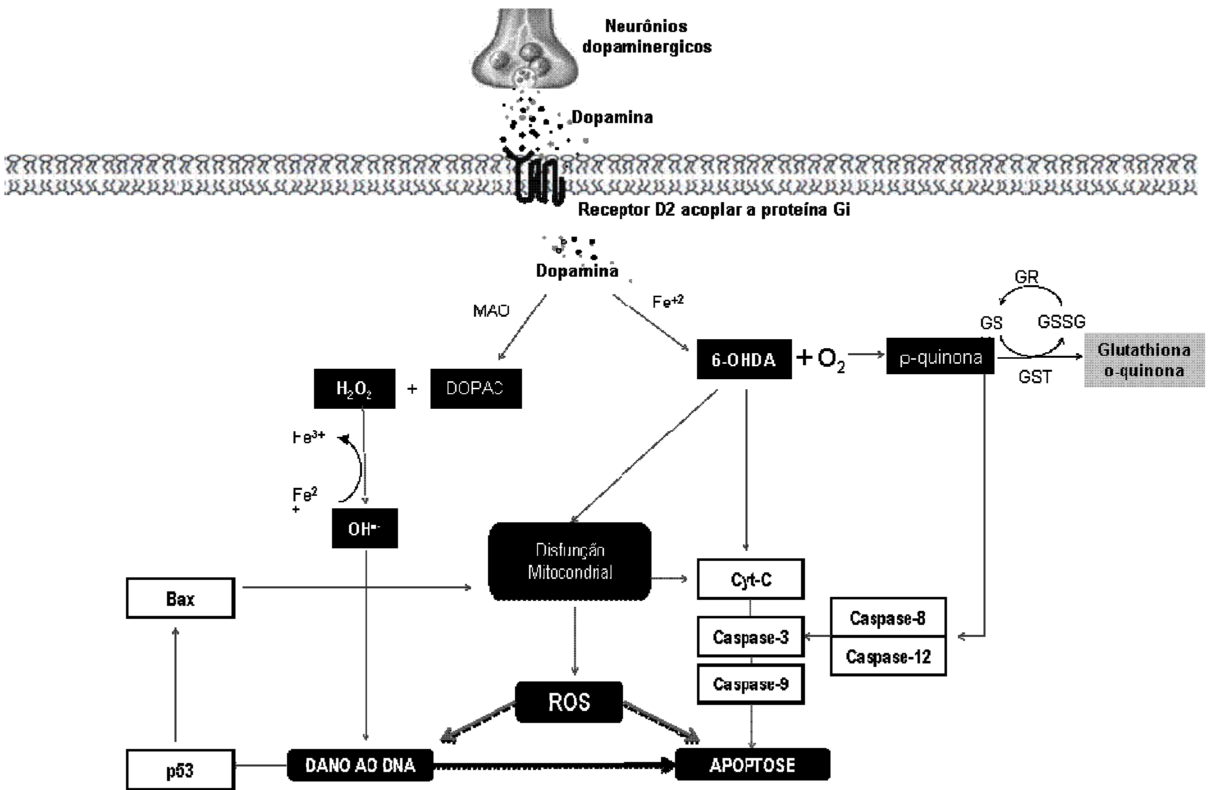

Figura 4: Rota de metabolização da dopamina via monoamino oxidase (MAO) e produção de radicais livres. A oxidação da dopamina pela enzima MAO leva a produção de peróxido de hidrogênio (H_2O_2) e ácido di-hidroxiacético (DOPAC). O H_2O_2 pode reagir com metais de transição, como o ferro (Fe^{+2}), levando a formação do radical hidroxila (OH^{-}) que pode oxidar e danificar diferentes moléculas como lipídios e o DNA. Outra via de oxidação da dopamina é a reação com Fe^{+2} formando a 6-hidroxidopamina que pode ser oxidada pelo oxigênio ou radical superóxido para 2-hydroxy-5-(2-aminoetil)-1,4-benzoquinona (p-quinona). A 6-hidroxidopamina pode causar a inibição do complexo 1 ou 4 da cadeia de elétrons mitocondrial levando ao aumentando da produção das espécies reativas do oxigênio (ERO). As ERO, em especial o radical OH^{-}, podem causar um dano oxidativo ao DNA que por sua vez, ativa o p53, induzindo a ativação de uma proteína pró apoptótica chamada “BAX”, com conseqüente liberação do Citocromo-c mitocondrial (Cyt-c) e de radicais livres. Inicia então a ativação da cascata das caspases, que pode induzir a célula a entrar em apoptose. A p-quinona por sua vez, pode induzir a ativação da caspase-8 ou caspase-12, que podem também induzir o processo apoptótico nas células.

Devido à hipótese de que o dano oxidativo causado pela anfetamina no modelo animal de mania ocorre, em parte, via oxidação da dopamina, um segundo objetivo da tese foi verifcar se o metilfenidato, também conhecido pelo seu papel sobre o sistema dopaminérgico, teria o mesmo efeito sobre dano ao DNA. O metilfenitado é o fármaco mais utilizado no tratamento de crianças e adolescentes que apresentam o TDAH. Este transtorno surge na infância, sendo diagnosticado por volta dos 7 anos de idade, apresentando como principais características os sintomas de desatenção, hiperatividade e impulsividade, em mais de um ambiente freqüentado pela criança/adolescente. O FDA (*Food and Drug Administration*) relatou em 2006 que 2,5 milhões de crianças, nos Estados Unidos, utilizam estimulantes (anfetamina e metilfenidato) para o tratamento do TDAH, sendo que destes, 10% são crianças com menos de 10 anos de idade. Ainda, o diagnóstico em adultos que apresentam o transtorno é relativamente um fenômeno recente e tem resultado em um crescimento na prescrição dessas medicações.

Em vista do crescimento do diagnóstico do TDAH no adulto, avaliamos o envolvimento do dano recente e permanente ao DNA induzidos pelo uso agudo e crônico de metilfenidato em ratos jovens (25 dias de idade) e adultos (60 dias de idade). Os resultados mostraram que o metilfenidato aumenta o dano recente ao DNA, tanto em ratos jovens como adultos, nos leucócitos, estriado e hipocampo. Porém, o metilfenidato não foi capaz de induzir à formação de um dano permanente ao DNA, verificado através da freqüência de micronúcleus. Isso sugere que no tratamento com metilfenidato o sistema de reparo pode estar reparando eficientemente o dano ao DNA, apresentando apenas efeitos genotóxicos transitórios. Os resultados estão de acordo com estudos prévios que mostraram que o metilfenidato não tem potencial mutagênico (Teo et al, 2003) ou clastogênico (Suter et al, 2006). A literatura, entretanto, não fornece estudos sobre o perfil do sistema de reparo ao DNA na presença de metilfenidato. Futuros estudos são necessários para verificar se o dano

ao DNA observado nesse modelo pode levar a mecanismos de indução à apoptose ou ainda outros tipos de mutações pontuais não verificadas pelo teste de micronucleus.

No terceiro artigo desta tese, avaliamos a freqüência de micronúcleus (marcador de dano permanete ao DNA), de pontes nucleoplasmáticas (NPB) e de apopotose em linfócitos de pacientes bipolares. Os resultados mostraram que a freqüência de micronucleus não está aumentada nos pacientes bipolares, porém a freqüência de células com presença de NPB e de apoptose está aumentada nos mesmos. A freqüência de apoptose foi verificada primeiramente na própria técnica para detecção de micronucleus como descrito por Fenech et al, 2003, onde as células apoptóticas são caracterizadas pela presença de fragmentação nuclear dentro de corpos apoptóticos, porém contém a membrana intacta. Posteriormente avaliamos a freqüência de apoptose pela marcação com anticorpo fluorescente conjugado a Annexina V. A annexina é um grupo de proteínas que se ligam às membranas fosfolipídicas na presença de cálcio. Durante o processo inicial da apoptose ocorre a perda da assimetria da membrana fosfolipídica, o que externa a fosfatidilserina, presente nessas membranas, passando da membrana interna para a externa, tornando-se passível de ser conjugada com a anexina, num processo mediado por cálcio. Com a intenção de confirmar a presença de apoptose nos linfócitos de pacientes com TB usamos a coloração com DAPI. As células em apoptose, detextadas pelo DAPI, apresentam um núcleo com condensação da cromatina e corpos apoptócos.

O genona humano é composto por três bilhões de pares de bases divididos em 23 cromossomos, onde temos de 30.000–50.000 genes (Ewing and Green, 2000; Venter et al., 2001). Ainda não conhecemos a função de cerca de 40% dos genes. Algumas partes do cromossoma são necessárias para manter a estrutura e regular a expressão gênica (Sadock e Sadock, 2000). As células estão continuamente expostas à ação de agentes exógenos e endógenos que podem lesar o DNA. (Barzilai e Yamamoto, 2004; Neeley e Essigmann,

2006). Dentre as reações endógenas que contribuem ao dano ao DNA estão depurinação, deaminação, metilação e oxidação (Wiseman e Halliwell, 1996; David et al, 2007). O dano ao DNA causado por reações de oxidação atrevés de ERO ou ERN parece ser o mais significante (Ames et al, 1993; Barzilai e Yamamoto 2004; David et al, 2007). Os diferentes tipos de dano ao DNA ativam diferentes mecanismos de resposta (Zhou e Elledge, 2000; Barzilai e Yamamoto, 2004). Essas respostas vão culminar com a ativação de *checkpoints* celulares ou, em determinados casos, podem induzir à apoptose nas células. (Iliakis et al, 2003).

O dano ao DNA no TB tem sido investigado nos últimos anos. Andreazza et al (2007b) mostraram que linfócitos de pacientes bipolares possuem um dano recente ao DNA. Butner et al (2007) mostraram que neurônios não-gabaérgicos provindos do córtex pré-frontal apresentam aumento da fragmentação do DNA. Além disso, Frey et al (2007), em um estudo com gêmeas com TB, mostraram que os marcadores de estresse oxidativo ocorrem em paralelo com o dano ao DNA. Ainda, Simon et al (2006) mostraram que pacientes com TB apresentam um aceleramento do encurtamento do telômero, o qual está muito correlacionado ao aumento do estresse oxidativo (Passos et al, 2007).

Os nossos resultados mostram que pacientes bipolares apresentam elevadas taxas de NPB, as quais ocorrem quando os centrômeros dos cromossomos dicêntricos são puxados para lados opostos durante a anáfase. A formação das NPB tem sido correlacionada positivamente ao aceleramento do encurtamento do telômero em modelos de roedores de câncer intestinal (Rudolph et al, 2001). Os nossos achados podem estar relacionados com os achados prévios de Simon et al (2006), que mostraram que o TB apresenta uma aceleração do encurtamento do telômero.

A apoptose é considerada a morte progamada das células e um processo altamente controlado que pode ser desencadeado por diversos estímulos (Rowe e Chuang, 2004).

Geralmente, a apoptose é regulada por dois mecanismos: (1) via extrínseca, onde ocorre a ativação de receptores de morte, como por exemplo, o Fas e o TNF, os quais têm domínios citosólicos (Lawen, 2003); (2) via intrínseca, também chamada via mitocondrial, que é ativada por estímulos pró-apoptóticos levando à liberação do citrocromo-c oxidase da mitoncôndria com respectiva geração de ERO e subseqüente ativação de caspases (figura 4) (Lawen, 2003). O balanço da expressão das proteínas pró e anti-apoptóticas da família Bcl-2 é um importante ponto de regulação da apoptose (Rowe e Chuang, 2004). A indução da Bax (proteína pró-apoptótica da família Bcl-2) é um dos principais responsáveis pela liberação do citocromo-c oxidase da mitocôndria (Rossé et al, 1998).

Interessantemente, a ativação da Bax pode ser induzida pelo fator p53 (Buckbinder et al., 1995; Miyashita e Reed, 1995), sendo esse ativado pelo estresse celular ou pelo dano ao DNA. Dessa maneira, podemos levantar a hipótese de que a indução da apoptose ocorrida no TB pode ser via p53, uma vez que resultados prévios mostram que o dano ao DNA está envolvido com o TB (Andreazza et al, 2007b; Buttner et al, 2007). Nossos resultados mostraram que não ocorre a formação de micronúcleus no TB, o que poderia ser justificado pelo fato de o dano ocorrido no DNA estar induzindo a célula a entrar em apoptose, assim impedindo uma lesão permanente ao DNA. Esses resultados ajudam a elucidar os mecanismos celulares envolvidos no TB, apontando para o envolvimento da apoptose, porém descartando o envolvimento da genotoxicidade celular.

Como último objetivo da tese, avaliamos o envolvimento do sistema antioxidante glutationa e da formação de danos protéicos em pacientes com 0-3 anos de doença e 10-20 anos de doença, sendo cada grupo pareado a um grupo controle. A escolha desses grupos deve-se a testarmos a hipótese se o oxidativo está envolvido desde o inicio da doença ou passa a ter inlfluência apenas mais tardiamente. Os nossos resultados mostraram que apenas os pacientes bipolares com 10-20 anos apresentam um aumento da atividade da enzima

glutationa redutase (GR) e da enzima glutationa-S-transferase (GST). A enzima glutationa peroxidase (GPx) não apresentou diferença entre os pacientes e o grupo controle. Quanto ao dano a proteínas não foram verificadas alterações na formação de grupos carbonil, porém a nitrotirosinação, avaliada pelo imunoconteúdo de 3-nitrotirosina, apresenta-se aumentada tanto nos pacientes com 0-3 anos de doença quanto nos pacientes com 10-20 anos de doença.

O sistema nervoso central é extremamente vulnerável ao dano oxidativo, em especial, por ser rico em substratos oxidáveis, como lipídeos, por possuir uma alta taxa de consumo de oxigênio e uma capacidade antioxidante relativamente baixa (Halliwell e Gutteridge, 1999; Takuma et al, 2004). A glutationa reduzida (GSH) possui um papel central na capacidade antioxidante cerebral (Dringen and Hirrlinger, 2003). A GSH é um tripeptídeo composto por glutamato, cisteína e glicina, a qual é usada como um doador de elétrons pela GPx, assim oxidando a glutationa (GSSG). Desse modo, a GR tem o papel de reduzir a GSSG para restabelecer o ciclo, como mostrado na figura 2. Além disso, a GSH também está envolvida na conjugação com moléculas estranhas ao organismo, reação essa catalisada pela enzima GST. Um importante papel da GST no cérebro é a detoxificação de o-quinonas formadas durante a oxidação da dopamina (Graham et al, 1978; Baez et al, 1997; Stokes et al, 1999; Obata et al, 2006).

O dano oxidativo a proteinas é um processo que merece atenção, uma vez que pode levar a alterações funcionais ou à inativação das mesmas. Quando as EROs causam oxidação dos resíduos de lisina, prolina, arginina e treonina, ocorre a formação de grupo carbonil nas proteínas (Berlett et al, 1998; Beal, 2002). Outro dano importante às proteínas é a nitração do resíduo tirosina, com conseqüente formação da 3-nitrotirosina, que ocorre via peroxinitrito (Beal, 2002). Ainda, a nitração de resíduos tirosina da enzima GST resulta no aumento de sua atividade (Ji e Bennet, 2003). Além disso, as modificações protéicas causadas por nitração

dos resíduos tirosina têm sido associadas aos eventos precoces do dano neuronal induzido pela dopamina (Ara et al, 1998).

Como já foi discutido previamente, estudos de neuroimagem sugerem o envolvimento do sistema dopaminérgico na fisiopatologia do TB (Yatham et al, 2005; Berk et al, 2007). A dopamina, na presença de Fe^{+2} e H_2O_2, pode sofrer hidroxilação na posição 6, levando à formação da 6-hidroxidopamina, a qual é altamente tóxica ao SNC. Os mecanismos de sua toxicidade incluem: indução de estresse no retículo endoplasmático, ativação da GSK-3b pela fosforilação da tirosina 216 e inibição da proteína AKT pela fosforilação da Ser437 (Chen et al, 2004). Além disso, Ferger et al (2001) sugerem que a 6-hidroxidopamina pode aumentar a formação da 3-nitrotirosina. Interessantemente, tanto a 3-nitrotirosina quanto a 6-hidroxidopamina podem inibir a atividade do complexo 1 da cadeia de transporte de elétrons, sugerindo, mais uma vez, o envolvimento da disfunção mitocondrial no TB.

Alterações do sistema glutamatérgico também têm sido associadas à fisiopatologia do TB (Yildiz-yesiologlu e Ankerst, 2006). Um dos mecanismos de neurotoxicidade do glutamato é o excesso de influxo de Ca^{+2} pela hiperestimulação dos receptores iônicos. O cálcio irá ativar a calmudolina cinase IV, que ativa a óxido nítrico sintase aumentando a produção de NO pela conversão de L-arginina em L-citrulina (Duchen et al, 2000). Dando suporte a essas evidências, os níveis intracelulares de cálcio mostraram-se aumentados no TB (Kato, 2008). Ainda, o cálcio necessita ser tamponado pela mitocôndria e, quando em excesso, pode induzir à disfunção mitocondrial, diminuindo assim seu tamponamento e aumentando a produção de ERO (Kato, 2008). Diversos estudos conduzidos em tecido cerebral post-mortem estão de acordo com os estudos de neuroimagem abordados na seção anterior, corroborando com a hipótese de disfunção mitocondrial no TB. Por exemplo, Konradi et al (2004) demonstraram que a expressão de genes mitocondriais está diminuída no hipocampo de pacientes bipolares em relação a voluntários normais, enquanto outros dois

estudos encontraram o mesmo resultado na região do córtex pré-frontal dorsolateral (Iwamoto et al, 2005; Sun et al, 2006). Um recente estudo do nosso grupo, ainda não publicado, mostrou que o córtex pré-frontal de pacientes bipolares apresenta uma diminuição de duas vezes na atividade do complexo I da cadeia de transporte de elétrons da mitocôndria (Andreazza et al dados não publicados). Essa inbição do complexo I induz a mitocôndria a liberar mais radical superóxido, o qual pode reagir com o NO formando o $ONOO^-$ aumentando assim as chances de nitração das proteínas. Na figura 5 mostramos um esquema que resume os achados e hipóteses desse artigo.

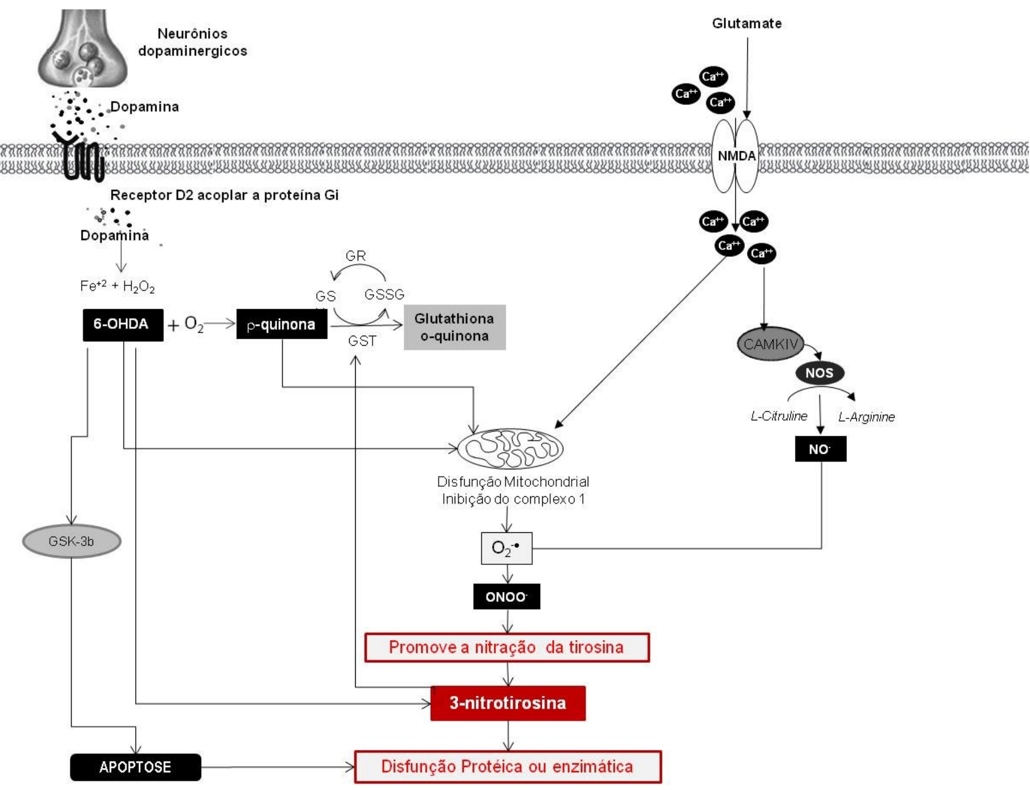

Figura 5: Possíveis vias que induzem a nitração das proteínas e formação da 3-nitrotirosina.

Os resultados também mostraram que somente os pacientes com 10-20 anos de doença possuem um aumento da atividade das enzimas GR e GST, porém o aumento da 3-nitrotirosina foi presente tanto nos pacientes com 0-3 anos de doença como 10-20 anos, sugerindo a presença de um mecanismo compensatório no TB. Na mesma linha, Tirmenstein

et al (2005), usando células de neuroblastoma, mostraram que após 4h de tratamento com a 6-hidroxidopamina, essa é capaz de depletar as concentrações celulares de GSH e ATP, enquanto que após 24h essa induz a um aumento na concentração de GSH, o que pode sugerir um processo adaptativo.

5. CONSIDERAÇÕES FINAIS

Avaliando os resultados conjuntamente, podemos dizer que o dano recente ao DNA está associado ao mecanismo de ação tanto da anfetamina como do metilfenidato. Porém, a formação de micronucleus, que indica um potencial genotóxico, está associado apenas à administração de anfetamina. Ainda no modelo animal, verificamos que o lítio fornece proteção ao dano recente ao DNA, induzido pela anfetamina, e que o valproato apenas foi capaz de modular a atividade das enzimas antioxidantes SOD e catalase. No tratamento com metilfenidato, para nossa surpresa, podemos constatar que os ratos adultos também apresentaram dano recente ao DNA. Nos resultados em pacientes com TB podemos verificar que o dano permanente ao DNA não está aumentado, o que contradiz o resultado encontrado no modelo animal. Isso mostra que uma das limitações do modelo animal é o fato de que a hiperatividade está sendo induzida por uma droga e sempre temos que levar em consideração a influência de sua ação nos resultados. Nos pacientes com TB verificamos um aumento da frequência de apotose que pode ser explicada, pelo menos em parte, pelo dano ao DNA o qual ativa rotas de sinalização, como a via do p53, para a célula entrar em processo apoptótico. Esses resultados estão de acordo com os estudos prévios do nosso grupo que mostraram que pacientes bipolares possuem 10x aumentado o dano recente ao DNA (Andreazza et al, 2007b), além de estarem de acordo com os resultados encontrados no modelo animal de mania. Ainda, verificamos que o dano oxidativo a proteínas ocorre por nitração da tirosina, e que este pode ativar a enzima GST, aumentada nos pacientes bipolares

com maior tempo de doença, indicando o envolvimento de um mecanismo compensatório. É importante considerar que os pacientes desses estudos estavam fazendo uso de medicações psiquiátricas, e como visto pelos resultados dessa tese no estudo no modelo animal de mania, bem como em cultura celular (Shao et al, 2005; Brunello, 2004; Chuang et al, 2002) os estabilizadores do humor possuem ação sobre diferentes mecanismos neuroprotetores.

Recentemente o nosso grupo tem sugerido o envolvimento da carga alostática no TB (Kapczinski et al, 2008). O termo alostase se refere ao esforço extra que o organismo emprega na adaptação dos sistemas fisiológicos as diversas situações de estresse (agudos e crônicos). Alostase literalmente quer dizer “estabilidade através da mudança”. A constante ativação deste mecanismo de resgate gera um desgaste cumulativo. Este desgaste é chamado de carga alostática. Este modelo tem sido associado a processos patológicos envolvendo o sistema cardiológico, endocrinológico, imunológico e cerebral. O modelo proposto por Kapczinski baseia-se na complexa interação entre as alterações genéticas e ambientais que induzem mudanças já documentadas no eixo hipotálamo-hipofise-adrenal, sistema imunológico, aumento do estresse oxidativo e alterações em neurotrofinas. Tais alterações culminariam em alterações na transmissão entre a conexão CPF-sistema límbico cerebral, e conseqüente aparecimento ou piora dos sintomas cognitivos e de humor (Figura 6).

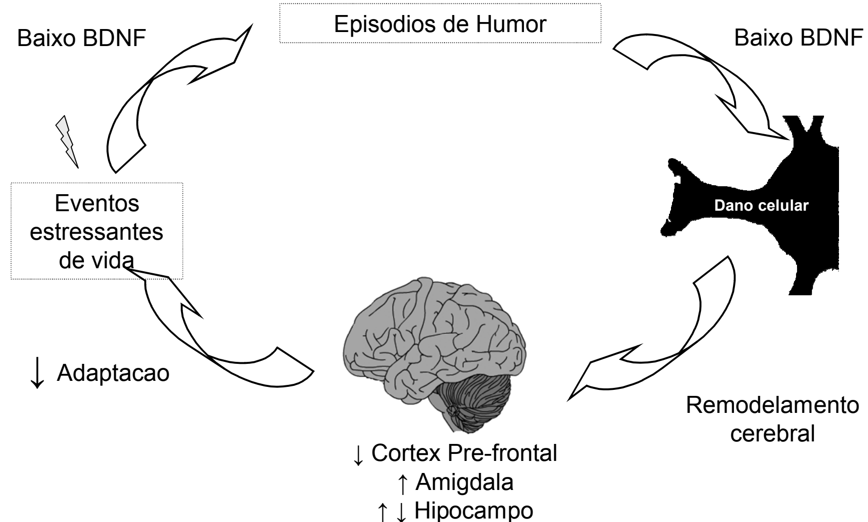

Figura 6. Ciclo proposto para o dano pelos múltiplos episódios. O estresse ambiental e os episódios de humor recorrentes estariam associados a diminuição dos níveis de BDNF. Esta alteração estaria associada a uma maior propensão ao dano celular o que, por sua vez, levaria a um remodelamento cerebral e conseqüente alteração nas regiões criticamente envolvidas na regulação do humor. Tais alterações diminuem a capacidade de adaptação ao estresse e o ciclo se instala (adaptada de Kapczinski et al, 2008).

Talvez mais importante seja o fato de que este modelo oferece uma perspectiva de que a detecção e tratamento precoce teriam potencialmente um efeito benéfico de diminuir a carga alostática, o que reduziria os efeitos deletérios observados a curto e, principalmente, a longo prazo. Neste sentido, o conceito de carga alostática permite um elo entre as alterações moleculares associadas a eventos estressores biológicos e ambientais (vide interação gene X ambiente), que ocorrem a nível celular (neuronal) e que podem ocasionar um rompimento em circuitos intimamente associados à regulação do humor e subseqüente exacerbação dos sintomas de humor. Além disso, esse modelo sugere que a somação de eventos que perturbem a homeostase, como trauma e uso de substâncias ilícitas, associados a uma maior vulnerabilidade genética, podem estar associados a quadros mais graves, maior refratariedade a intervenções terapêuticas, tendência a um curso mais crônico, deterioro cognitivo e pior

funcionamento sócio-ocupacional. Deste modo, fica claro o entendimento de que estudos futuros devem idealmente combinar a avaliação clinico-farmacológica com marcadores biológicos laboratoriais e/ou de neuroimagem de maneira prospectiva. Ainda, a busca por modelos animais mais refinados é fundamental para a geração de hipóteses e para o desenvolvimento de tratamentos mais eficazes.

6. CONCLUSÕES

- Os objetivos dessa tese foram comtemplados e podemos confirmar que: tanto o modelo animal de mania como os pacientes bipolares apresentam envolvimento do estresse oxidativo e do dano ao DNA.
- No primeiro artigo dessa tese, podemos concluir que os animais tratados com anfetamina possuem um aumento da hiperlocomoção, a qual foi revertida pela administração de lítio ou valproato. Observamos também, que a anfetamina induz um aumento tanto dos marcadores de estresse oxidativo como do dano recente e permanete ao DNA sendo que o lítio e valproato foram capazes de pervenir ou reverter o estresse oxidativo, porém apenas o lítio conseguiu prevenir o dano recente ao DNA. O nos leva a concluir que o mecanismo envolvido no dano recente ao DNA pode ser inibido pelo mecanismo de ação do lítio, apontando para diferentes mecanismos de ação entre lítio e valproato.
- No segundo artigo dessa tese, observamos que administração de metilfenidato a ratos jovens e adultos, tanto de forma crônica ou aguda induzem ao dano recente ao DNA e não permanete. O que sugere que o metilfenidato apresenta um papel genotóxico transitório, porém não mutagênico.
- No terceiro artigo dessa tese, obesrvamos que os linfócitos de pacientes bipolares apresentam um aumento da freqüência de apoptose e não de apresetam um aumento da freqüência de micronucles. Apontado que o dano ao DNA presente no trantorno bipolar,

como verficado por estudos anteriores, induz a ativação de rotas que induzem a apoptese, provelvelmente por ativação da proteína p53, não permitando um dano permaneto ao DNA nos pacientes com transtono bipolar.

- No ultimo artigo dessa tese, verificamos que os tanto pacientes bipolares no início da doença como pacientes com mais de 10 anos de doença apresentam aumento do conteúdo de 3-nitrotirosina o que indica um envolvimento de lesão protéica via espécies reativas do nitrogênio. Apenas nos pacientes com mais de 10 anos de doença observamos um aumento da atividade da GST e da GR o que aponta para o envolvimento de um mecanismo compensatório no TB.

7. PERSPECTIVAS

1. Avaliar o envolvimento do dano oxidativo ao DNA pelo radical hidroxila através da quantificação dos níveis de 8-hidroxi-desoxiguanosina em pacientes bipolares.
2. Complementar os estudos de apoptose pela quantificação dos níveis de citocromo-c oxidase, caspase-3, Bcl-2, bax, bad e Bin, a fim de nos certificarmos do aumento da freqüência de apoptose e qual via está associada ao TB.
3. Avaliar a funcionalidade, em pacientes com transtorno bipolar, das principais enzimas de reconhecimento e incisão de danos, envolvidas nas vias de reparação de excisão de DNA.
4. Avaliar a freqüência do polimorfismo, expressão gênica e atividade das enzimas antioxidantes (SOD-1, SOD-3, catalase, GPx-1, GPx-3, GST-A1, GST-P4) em pacientes bipolares responsivos ou não ao tratamento com N-acetilcisteína.

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