UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

NEUROTOXICIDADE DO METOTREXATO: UTILIZAÇÃO DA PROTEÍNA S100B COMO UM MARCADOR E ESTUDO DO ENVOLVIMENTO DO SISTEMA GLUTAMATÉRGICO.

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PORTO ALEGRE, 2005.

Siga confiante na direção dos seus sonhos. Viva a vida que imaginar.

Thoreau

Agradecimentos

Ao Prof Luis Valmor Cruz Portela por me orientar não somente neste trabalho, como também em etapas anteriores ao mestrado, por ajudar a construir minha carreira, pelos ensinamentos de vida, pela amizade e carinho.

Ao Prof Diogo O. Souza por me aceitar no seu laboratório, pelo incentivo e confiança e principalmente pela amizade.

À Profa Susana Wofchuk, pela ajuda, incentivo e pelo enorme carinho e amizade.

Aos professores e funcionários do Departamento de Bioquímica do ICBS, UFRGS, especialmente a Cléia e ao Valeri.

Ao Prof Sérgio Lamb, que não está mais convivendo conosco, por todo incentivo.

Ao Prof Francisco Lhullier, por acreditar em mim e me apresentar ao grupo do Diogo Souza.

Ao Diogo Lösch de Oliveira, André Prato Schmidt e Jean Pierre Oses pela intensa participação neste trabalho e amizade.

Às minhas amigas e colegas Ana Elisa Böhmer, Gabriele Ghisleni e Vanessa Kazlauckas Ghidini pelo incentivo, força e carinho.

Ao Thiago Ávila, Renan Sanna Jorge e à Alice Fischer pela grande ajuda.

A todos os meus colegas e amigos dos laboratórios 26, 28 e 35.

Aos meus pais e irmãs, pelo apoio nos momentos difíceis, por incentivarem meus estudos e pela confiança que depositam na minha escolha profissional. Obrigada por todos os ensinamentos e pelo amor.

A todos os meus amigos que sempre me apoiaram, especialmente à minha grande amiga Clarice Chemello, pelo carinho e cumplicidade.

Ao Marcos Rosemberg por estar sempre ao meu lado, pelo grande incentivo e amor.

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PRIMEIRA PARTE

Resumo

Neurotoxicidade do metotrexato: utilização da proteína S100B como um marcador e estudo do envolvimento do sistema glutamatérgico.

O metotrexato (MTX) é um antagonista do ácido fólico amplamente utilizado no tratamento de doenças neoplásicas e não neoplásicas. Entretanto, o uso terapêutico desse fármaco pode levar à neurotoxicidade que pode se manifestar na forma aguda, subaguda e crônica, abrangendo os seguintes sintomas: cefaléia, sonolência, confusão, edema cerebral, convulsões, encefalopatia, coma e prejuízo das funções cognitivas. Os achados neuropatológicos consistem de astrogliose reativa, desmielinização, dano axonal e necrose da substância branca. Em nível celular, o MTX parece afetar primeira e seletivamente os astrócitos, quando comparados com os neurônios.O exato mecanismo neurotóxico do MTX continua não esclarecido, e parece ser multifatorial. Visando ampliar os conhecimentos a respeito dos efeitos do MTX no SNC, foram desenvolvidos dois trabalhos com diferentes modelos em animais, nos quais foram estudados os possíveis mecanismos de ação tóxica desse fármaco, como também propomos a utilização do marcador bioquímico S100B na detecção de injúrias cerebrais associadas ao tratamento. A partir dos resultados obtidos nos experimentos de captação de glutamato *in vitro*, verificamos que o MTX interfere na remoção do glutamato da fenda sináptica, podendo levar à excitotoxicidade. Também, o aumento da proteína S100B auxilia no entendimento dos mecanismos de ação do MTX, pois sugere que os astrócitos estão respondendo a um insulto na tentativa de neuroproteção. Além disso, a S100B, aliada a outros marcadores neuroquímicos e técnicas de diagnóstico por imagem, seria muito importante no monitoramento terapêutico, pois poderia detectar alterações celulares sutis e ajudaria a prevenir a neurotoxicidade pelo MTX. Os resultados que obtivemos nos experimentos de convulsões induzidas pelo MTX demonstraram a participação do sistema glutamatérgico na neurotoxicidade desse fármaco. Especificamente, evidenciamos o envolvimento dos receptores inotrópicos glutamatérgicos na patogênese das convulsões. Porém, neste modelo experimental, a captação de glutamato possivelmente diminuiu em decorrência das manifestações das convulsões e não por uma ação direta, ou indireta, do MTX. O entendimento dos mecanismos de ação é muito importante para a clínica médica, pois permite que novas ferramentas sejam criadas no intuito de prevenir os danos tóxicos induzidos por fármacos. Assim, mais estudos devem ser realizados para tentar desvendar os mecanismos de neurotoxicidade do MTX, como também para estudar potenciais marcadores bioquímicos de injúria cerebral que auxiliem no monitoramento terapêutico.

Abstract

Methotrexate neurotoxicity: S100B measurement as a biochemical marker and study of glutamatergic system involvement.

Methotrexate (MTX), an acid folic antagonist, is widely used for the treatment of neoplasic and non-neoplasic diseases. However, neurotoxicity may occur and can be classified as acute, subacute and chronic and include the followed symptoms: headache, somnolence, confusion, cerebral edema, seizures, encephalopathy, coma and impairment of high cognitive functions. Central nervous system (CNS) morphological alterations as astrocytosis, demyelination, axonal damage and white matter necrosis have been related to MTX neurotoxicity. Moreover, at brain cellular level, it has been verified that astrocytes were primary and selectively affected by MTX rather than neurons. The mechanisms of MTX neurotoxicity are not totally understood, and appear to be multifactorial. Aiming to evaluate MTX effect against SNC, we developed two studies using animal models in which the involvement of glutamatergic system in MTX neurotoxicity were studied, as well as the measurement of S100B protein to detect brain injuries. The data from the *in vitro* experiments demonstrated that MTX, affect glutamate uptake and may lead to excitotixicity. Besides, we verified the increase of S100B CSF levels, which could be related to astrocyte response. Moreover, S100B can be better studied as a potential brain marker of MTX neurotoxicity. The measurement of this brain marker together with image diagnosis techniques could assist therapeutic drug monitoring and prevent MTX neurotoxicity. The data from glutamate uptake after MTX induced seizure demonstrated the involvement of glutamatergic system in MTX neurotoxicity. Moreover, we showed the involvement of ionotropic glutamatergic receptors in the pathogenesis of seizure. However, the decrease of glutamate uptake observed in this model possibly results from seizure manifestation rather than MTX direct action on glutamate transporters. Understand drugs mechanisms of action are very important to create new techniques of diagnosis and prevent drug toxicity. Therefore, more studies are necessary to understand the exact mechanism of MTX neurotoxicity as well as to suggest new potential brain markers of injury to assist therapeutic drug monitoring.

1. Introdução

1.1. O metotrexato

O metotrexato (4-NH_2-10-CH_3- ácido fólico; MTX) é um fármaco classificado como antagonista do ácido fólico (ou folato) pertencente à classe dos antimetabólicos (McGuire, 2003) (Fig.1). Na clínica médica, o MTX vem sendo utilizado desde a década de 50, principalmente no tratamento de diversos tipos de neoplasias que incluem as leucemias, linfomas não Hodking, coriocarcinomas, osteosarcoma, meningite neoplásica e tumores cerebrais e de pescoço (Mahoney et al, 1998; McGuire, 2003; Omuro et al, 2005). Além disso, o MTX tem sido largamente empregado como um agente antiinflamatório e imunossupressor na terapia de artrite reumatóide, psoríase e lupus eritematoso sistêmico (Smolenska et al, 1999; Ruiz-Irastorza et al, 2001; Chan e Cronstein, 2002).

1.2. Aspectos farmacocinéticos

O MTX pode ser administrado pelas vias oral, subcutânea, intramuscular, intravenosa e intratecal (Schröder e Stein, 2003). A absorção oral normalmente é rápida e parece estar relacionada com a dose administrada. Baixas doses são rapidamente absorvidas; entretanto, altas doses de MTX apresentam uma absorção ineficaz e por isso a biodisponibilidade pode variar de 12 a 77 % (Madden e Eaton, 1995; Calabresi e Chabner, 2001). A absorção pela via subcutânea é completa e atinge níveis séricos comparáveis com os da administração oral de baixas doses de MTX; já altas doses de MTX alcançam concentrações séricas semelhantes às da administração intravenosa. A via intramuscular apresenta uma boa absorção havendo, entretanto, uma variação na biodisponibilidade de 75

a 100 % (Madden e Eaton, 1995). As concentrações séricas máximas do MTX são alcançadas entre 0.5 a 2 horas após a administração intravenosa ou intramuscular, 1 a 4 horas após a administração oral e 2 horas após a administração intratecal (Madden e Eaton, 1995; Calabresi e Chabner, 2001).

No estado de equilíbrio, quando o fármaco encontra-se em concentração constante no sangue, o MTX é rapidamente distribuído para todo o organismo e pode se acumular em alguns tecidos, tais como o renal e o hepático (Schröder e Stein, 2003). Na corrente sangüínea o MTX encontra-se 50 % ligado às proteínas plasmáticas e apresenta um tempo de meia vida que varia de 3 a 5 horas (Calabresi e Chabner, 2001). Entretanto, quando altas doses são administradas, um segundo tempo de meia vida acontece entre 8 a 26 horas, que se caracteriza pela saída do MTX dos tecidos e cavidades nos quais se encontrava acumulado, para o sangue (Madden e Eaton, 1995; Calabresi e Chabner, 2001).

O MTX é metabolizado em 4-amino-4-desoxi-N^{10}-metilpteroico (DAMPA), 7-hidroximetotrexato (7-OH-MTX) e poliglutamatos de MTX (Madden e Eaton, 1995). O DAMPA é formado a partir da atividade de bactérias intestinais durante a circulação entero-hepática. O 7-OH-MTX, por sua vez, é proveniente da metabolização do MTX no fígado e apresenta solubilidade de até cinco vezes menor que o MTX (Madden e Eaton, 1995). Os poliglutamatos de MTX são considerados os principais metabólitos formados (Zeng et al, 2001). A enzima folilpoliglutamato sintetase adiciona moléculas de glutamato ao resíduo pré-existente do MTX intracelular, formando os poliglutamatos de MTX. Estes metabólitos são retidos dentro das células, podendo assim permanecer durante meses (Spinella et al, 1996; Zeng et al, 2001; Schroder e Stein, 2003).

A eliminação do MTX ocorre principalmente via renal, onde 24 horas após a administração intravenosa cerca de 90% do fármaco é excretado na sua forma inalterada na urina (Takeda et al, 2002 Schroder e Stein, 2003). Apenas uma pequena percentagem do MTX (10 %) é excretada pela secreção biliar e fezes (Madden e Eaton, 1995).

Estruturalmente o MTX apresenta alto peso molecular e baixa lipossolubilidade, e por isso necessita de mecanismos de transporte para alcançar o citosol das células (Spinella et al, 1996; Zeng et al, 2001). É interessante ressaltar que o MTX compartilha dos mesmos sistemas de transporte do folato. O principal sistema carreador é uma proteína integrante da família dos transportadores de glicoproteína P e glicose. Este carreador é responsável pela formação de gradiente transmembrana e apresenta grande afinidade pelo MTX e folatos reduzidos (ver adiante) (Zeng et al, 2001; McGuire, 2003). O segundo mecanismo de transporte é através de receptores de folato que se encontram ancorados na membrana e são internalizados por endocitose, mas este tipo de transporte apresenta uma baixa afinidade pelo MTX. Além disso, o MTX pode atravessar as membranas por difusão quando as concentrações extracelulares estão muito elevadas (Spinella et al, 1996; Zeng et al, 2001; Schroder e Stein, 2003).

1.3. Os folatos

Para a melhor compreensão dos mecanismos de ação do MTX é de fundamental importância entender como os folatos atuam no organismo humano. Para se tornarem biologicamente ativos, os folatos são reduzidos pela ação da enzima dihidrofolato redutase (DHFR) formando os intermediários dihidrofolato e tetrahidrofolato (Zeng et al, 2001; Schroder e Stein, 2003). Além disso, unidades de carbono que incluem grupamentos metil

(CH_3), metileno (CH_2), formil (-CHO-) ou formimino (-CHNH-), podem ser acopladas aos folatos para que dessa forma adquiram a função de coenzima em diversos sistemas enzimáticos, como carreadores de diferentes unidades de carbono (McGuire, 2003).

Na circulação sanguínea a maior parte dos folatos se encontra na forma de metil - tetrahidrofolato com apenas um resíduo de glutamato (monoglutamatos). No entanto, no meio intracelular, as formas reduzidas de folatos são metabolizadas a poliglutamatos pela enzima folilpoliglutamato sintetase que insere até 12 resíduos de glutamato na molécula de folato (Spinella et al, 1996; Zeng et al, 2001; Schroder e Stein, 2003). Os folatos intracelulares se encontram predominantemente na forma de penta ou hexaglutamatos, e são retidos no interior das células para atuarem como coenzimas (Spinella et al, 1996; Zeng et al, 2001).

Os folatos são extremamente importantes para a síntese de timidina, purinas e aminoácidos que, conseqüentemente, são fundamentais para a síntese de RNA, DNA e proteinas (Spinella et al, 1996, McGuire, 2003; Cai e Horne, 2003).

1.4. Mecanismos de ação do MTX

Como descrito anteriormente, o MTX é um antagonista do acido fólico. Dessa maneira, este fármaco e seus metabólitos poliglutamatos inibem a DHFR através do bloqueio dos seus sítios catalíticos, acarretando na diminuição dos níveis intracelulares de tetrahidrofolatos e, conseqüentemente, na inibição das reações de transferência de unidades de carbono (Bruce-Gregorios et al, 1991a; Zeng et al, 2001; McGuire 2003). Dessa forma, a síntese de timidina, purinas e de certos aminoácidos são interrompidas, afetando a síntese

de DNA, RNA e divisão celular (Chan e Cronstein, 2002; Schroder e Stein, 2003; Vezmar et al, 2003) (Fig 2).

Sobre a síntese de purinas, o MTX atua inibindo glicinamida ribonucleotídeo (GAR) transformilase e a 5-aminoimidazol-4-carboxiamida ribonucleotideo (AICAR) transformilase, levando conseqüentemente ao acúmulo do substrato GAR e AICAR (Smolenska et al, 1999; Chan e Cronstein, 2002; Vezmar et al, 2003). O acúmulo do substrato AICAR, por sua vez, inibe as enzimas adenosina monofosfato deaminase e a adenosina deaminase, causando o aumento de adenosina intracelular (Van Ede et al, 2002; Chan e Cronstein, 2002; Smolenska et al, 1999).

A síntese de homocisteína também é diretamente afetada pelo MTX, pois uma vez estando baixas as concentrações intracelulares de metil-tetrahidrofolatos, existe a diminuição da formação de metionina e o acúmulo de homocisteína (Quinn et al, 1997; Kishi et al, 2003). Baixas concentrações de metionina também acarretam na inibição da síntese de S-adenosilmetionina, que é um importante doador de grupamento metil para o metabolismo celular (Drachtman et al, 2002; Vezmar et al, 2003). Além disso, o excesso de homocisteína pode ser direcionado para as reações de catabolização, formando o ácido cistéico e cisteína sulfídrica ácida, como também pode ser oxidada a ácido homocistéico e homocisteína sulfídrica ácida (Quinn et al, 1997; Vezmar et al, 2003). Estes compostos (ácido homocistéico, homocisteína sulfídrica ácida, ácido cistéico e cisteína sulfídrica ácida), conhecidos como aminoácidos excitatórios sulfurados, são estruturalmente semelhantes ao aspartato e glutamato e apresentam efeitos excitatórios (Quinn et al, 1997; Drachtman et al, 2002; Vezmar et al, 2003) (Fig. 2).

O MTX também interfere na síntese das biopterinas, pois impede a formação das tetrahidrobiopterinas a partir das dihidrobiopterinas. As tetrahidrobiopterinas são necessárias para a hidroxilação de tirosina, fenilalanina e triptofano, para posteriormente dar origem aos neurotransmissores dopamina e serotonina (Millot et al, 1995; Vezmar et al, 2003).

1.5. A neurotoxicidade do MTX

A neurotoxicidade induzida pelo MTX pode ocorrer em pacientes que fazem o uso terapêutico desse fármaco tanto para o tratamento de doenças neoplásicas como também inflamatórias, após a administração intratecal ou após baixas, intermediárias e altas doses sistêmicas de MTX (Mahoney et al, 1998; Drachtman et al, 2002; Renard et al, 2004). A neurotoxicidade pode se manifestar de forma aguda, subaguda e crônica e abrange os seguintes sintomas: cefaléia, sonolência, confusão, desordens afetivas, deficiências neurológicas focais, tremores, paraplegia, edema cerebral, convulsões, encefalopatia e coma (Quinn et al, 1997; Lo Nigro et al, 2000; Sánchez-Carpintero et al, 2001; Vezmar et al, 2003; Kuker et al, 2005).

Os achados neuropatológicos conseqüentes dos efeitos tóxicos do MTX consistem em lesões necrotizantes bilaterais, astrogliose reativa, desmielinização, necrose ou trombose dos vasos sangüíneos (Gregorios et al 1989; Kishi et al, 2003; Linnebank et al, 2005). Também já foi observada a degeneração das substâncias branca e cinzenta (Gregorios et al., 1989; Linnebank et al, 2005). Reduções no número de sinapses hipocampais pelo uso de MTX foram demonstradas em um modelo animal, que mimetizava as condições de neurodesenvolvimento de crianças (Igarashy et al, 1989). Além disso, já foi

demonstrado através de técnicas de diagnóstico por imagem que crianças com leucemia linfoblástica aguda, de idade inferior a cinco anos, apresentaram reduções no lobo posterior do cerebelo e do córtex pré-frontal devido ao tratamento intratecal com MTX (Lesnik et al, 1998).

No sistema nervoso central (SNC), a toxicidade do MTX parece afetar de modo diferenciado os neurônios e astrócitos. Gregorios et al, (1989) demonstram, através de um estudo morfológico de fatias de cérebros de ratos que receberam MTX via intraperitonial ou intratecal (10 e 100 mg/Kg), que os astrócitos são primeira e seletivamente afetados pelo MTX, quando comparados com os neurônios. Tanto *in vivo* como *in vitro* o MTX é capaz de induzir a uma resposta astrocitária, conhecida como astrogliose reativa, assim como a degeneração desse tipo celular (Gregorios et al, 1989; Bruce-Gregorios et al, 1991b). Essas alterações celulares aumentam o conteúdo citoplasmático da proteína ácida fibrilar glial (GFAP), a qual está diretamente relacionada com presença de astrogliose reativa (Bruce-Gregorios et al, 1991b). Especula-se que os astrócitos poderiam ser um sítio de captação e de metabolização de MTX, o que conseqüentemente elevaria os efeitos tóxicos a estas células (Gregorios et al, 1989; Zeng et al; 2001). De fato, hoje em dia sabe-se que os astrócitos fazem o transporte de formil-tetrahidrofolatos e que o MTX compartilha desses mesmos mecanismos (Cai e Horne, 2003).

O mecanismo exato pelo qual o MTX induz a neurotoxicidade não está totalmente esclarecido e parece ser multifatorial (Quinn et al, 1997; Lo Nigro et al, 2000; Vezmar et al, 2003). Um dos mecanismos propostos envolve o aumento das concentrações de homocisteína no SNC, conseqüente à inibição da formação de tetrahidrofolato (Quinn et al, 1997; Drachtman et al, 2002; Kishi et al, 2003). Uma vez que a homocisteína é tóxica para

o endotélio vascular, esta poderia ser a responsável pela microangiopatia mineralizante e isquemia da substância branca observadas em pacientes que manifestaram a neurotoxicidade pelo MTX (Drachtman et al, 2002; Kishi et al, 2003). Além disso, os aminoácidos excitatórios sulfurados, provenientes da homocisteína, são agonistas endógenos de receptores excitatórios e também promovem a liberação dos neurotransmissores glutamato e aspartato. Juntos, estes eventos poderiam justificar a patogênese da neurotoxicidade e, principalmente, das convulsões (Quinn et al, 1997; Drachtman et al 2002, Vezmar et al, 2003).

O MTX também atua diretamente sobre a síntese de aminas biogênicas, como já foi descrito. Um estudo demonstrou que pacientes tratados com MTX apresentavam diminuição dos níveis dos ácidos homovanílico e 5-hidroxindolacético, metabólitos da dopamina e serotonina, respectivamente (Millot et al, 1995). Além disso, em animais tratados com MTX foi detectada a diminuição significativa dos neurotransmissores dopamina, norepinefrina, serotonina e seu metabólito ácido 5-hidroxindolacético, em homogeneizados de hipocampo (Madhyastha et al, 2002).

Os efeitos tóxicos do MTX também estão relacionados com uma série de alterações cognitivas, como a diminuição do Quociente de Inteligência (QI) e prejuízos na memória e na atenção (Peckham et al 1988; Langer et al, 2002; Correa et al, 2004). Estas alterações cognitivas têm sido bastante observadas em crianças que fizeram o uso de MTX principalmente para tratar leucemia linfoblástica aguda e também meduloblastoma (Riva et al, 2002; Waber et al, 1995; Langer et al, 2002). Um estudo com crianças sobreviventes à leucemia linfoblástica aguda, que foram tratadas com terapia intratecal e radioterapia craniana, demonstrou que elas obtiveram escores de QI menores do que o esperado.

Também foi relatada dificuldade de concentração e alterações de memória e compreensão quando as crianças realizavam testes escolares (Peckham et al, 1988).

1.6. O sistema glutamatérgico

O glutamato é o principal neurotransmissor excitatório do SNC de mamíferos e está envolvido na maioria das funções cerebrais (Ozawa et al., 1998), tais como memória e aprendizado, (Izquierdo e Medina, 1997), desenvolvimento e envelhecimento (Segovia et al., 2001) e adaptação ao meio ambiente (Danbolt, 2001; Mattson et al., 2001). A sinalização glutamatérgica acontece pela ativação dos receptores de glutamato, sendo estes classificados em ionotrópicos e metabotrópicos. Os receptores ionotrópicos contêm canais iônicos e são subdivididos em AMPA (a-amino-3-hidroxi-5-metil-4-isoxazolepropriona to), cainato e NMDA (N-metil-D-aspartato). Os receptores metabotrópicos, por sua vez, são subdivididos em três grandes grupos (I, II e III) e modulam a produção intracelular de segundos mensageiros através da proteína G (Ozawa et al., 1998).

A captação de glutamato é o mecanismo responsável pela manutenção de baixos níveis desse neurotransmissor na fenda sináptica, uma vez que não existe um sistema enzimático extracelular que possa significativamente metabolizar o glutamato (Danbolt, 2001; Chen e Swanson, 2003; Maragakis e Rothstein, 2004). Até o presente momento, cinco diferentes transportadores de glutamato, de alta afinidade e dependentes de sódio, foram identificados em mamíferos. Os transportadores GLAST e GLT-1 são predominantemente astrocitários e os principais responsáveis pela captação de glutamato e manutenção de baixos níveis desse neurotransmissor na fenda sináptica (Danbolt, 2001, Chen e Swanson, 2003). O trasnportadores EAAC1, EAAT4 e EAAT5 são localizados nos

neurônios, porém pouco contribuem para a captação de glutamato (Maragakis e Rothstein, 2001-2004).

O glutamato pode agir como uma excitotoxina através da excessiva estimulação dos seus receptores, quando se encontra em altas concentrações na fenda sináptica (Chen e Swanson, 2003; Maragakis e Rothstein, 2001-2004). A excitotoxicidade do glutamato tem sido relacionada com a morte neuronal observada na isquemia, hipóxia, hipoglicemia e trauma, assim como em várias doenças crônicas do SNC, como a doença de Huntington e Alzheimer (Choi, 1988; Danbolt, 2001; Ingram et al., 2001; Maragakis e Rothstein, 2001; Segovia et al., 2001) Além disso, já se demonstrou que a excitotoxicidade do glutamato pode estar envolvida na patogênese do *Status epilepticus* (Meldrum, 1994-2000; Maragakis e Rothstein, 2001-2004).

1.7. Proteína S100B- Marcador de neurotoxicidade

A neurotoxicidade do MTX, ocasionada pelo seu uso terapêutico, vem sendo relatada por diversos autores (Quinn et al, 1997, Drachtman et al, 2002; Vezmar et al, 2003; Correa et al, 2004). Porém, alterações no SNC decorrentes à ação tóxica deste fármaco normalmente são diagnosticadas quando existem danos cerebrais passíveis de serem detectados através de técnicas de diagnóstico por imagem, ou por manifestações neurológicas e comportamentais. As técnicas de neuroimagem rotineiramente utilizadas na clínica, como a tomografia computadorizada e a ressonância magnética nuclear, muitas vezes não são eficazes na detecção de alterações celulares sutis.

Visando a detecção de dano neurológico precoce, a utilização de marcadores bioquímicos de injúria ao SNC vem sendo proposta para auxiliar nas técnicas de

diagnóstico rotineiramente utilizadas na clínica. Neste sentido, os marcadores bioquímicos, como a proteína S100B, vem sendo estudados como possíveis ferramentas para avaliação de dano agudo ou crônico ao SNC (Lara et al. 2001; Portela et al. 2002; Klein et al, 2003).

A S100B é uma proteína ligante de cálcio de peso molecular de 21 kDa, expressa e secretada principalmente por astrócitos (Zimmer et al, 1995). No meio intracelular esta proteína está envolvida na modulação de proteínas do citoesqueleto, homeostase do cálcio e também atua sobre o ciclo celular (Donato, 2001). Já foi demonstrado que a S100B tem efeito modulatório sobre a morfologia dos astrócitos, uma vez que o bloqueio da sua atividade impede a formação de astrócitos estrelados. Dessa forma acredita-se que a S100B apresenta um papel regulador dos processos de polimerização e despolimerização de filamentos intermediáios do citoesqueleto, conseqüentemente modulando a plasticidade astrocitária (Ziegler et al, 1998; Frizzo et al, 2004 a-b). Extracelularmente, a S100B atua como um fator trófico sendo responsável pela regulação da proliferação e diferenciação das células do SNC (Zimmer et al, 1995; Donato, 2001).

Vários estudos têm demonstrado que o aumento das concentrações de S100B no plasma e no líquor se correlacionam com a intensidade e a extensão de injúrias ao SNC, tais como trauma, esquizofrenia e HTLV-I associado à mielopatia (Herrmann et al, 1999; Lara et al, 2001; Walz et al, 2000; Portela et al. 2002). Níveis aumentados da S100B podem refletir tanto um dano como uma resposta astrocitária um estímulo nocivo. A capacidade da S100B em ser benéfica ou deletéria para as funções cerebrais depende de como a glia responde ao estímulo nocivo e das concentrações extracelulares de S100B (Van Eldik e Wainwright, 2003).

2. Objetivos

2.1. Objetivo geral

- O objetivo deste trabalho foi estudar a toxicidade do metotrexato no SNC.

2.2. Objetivos específicos

- Estudar a neurotoxicidade do metotrexato através da utilização da proteína S100B.
- Estudar os mecanismos neurotóxicos do MTX sobre o sistema glutamatérgico, especificamente sobre a captação de glutamato.

SEGUNDA PARTE

CAPÍTULO I

Methotrexate increases rat CSF S100B levels and decreases glutamate uptake in brain slices

(Submetido à revista Brain Research Bulletin)

Methotrexate Increases Rat CSF S100B Levels and Decreases Glutamate Uptake in Brain Slices

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Abstract

Neurotoxicity induced by Methotrexate (MTX), a drug used for the treatment of malignant and inflammatory diseases, is an adverse effect frequently reported after systemic and intrathecal administration. It has been described that in central nervous system astrocytic cells are the primary target for MTX toxicity. In this work, it was evaluated MTX induced neurotoxicity in rats by measuring CSF S100B level and glutamate uptake, two well known astrocytic parameters. CSF S100B level increased significantly after *in vivo* treatment with 10.0 mg/kg of MTX ($P < 0.01$) and glutamate uptake decreased in cortical slices after *in vitro* exposure to 2.5 and 10.0 mg/mL of MTX ($P < 0.05$). Our results reinforce the postulation that astrocytes are affected by MTX and suggest a putative involvement of glutamate uptake in the neurotoxicity by MTX.

Key words: Methotrexate, Rat CSF, S100B, glutamate uptake.

1. Introduction

Methotrexate (MTX) is a folic acid antagonist widely used in the treatment of neoplasic and non-neoplasic disorders [8, 16]. Originally this drug was synthesized for the treatment of malignant diseases such as acute leukemia, lymphomas, breast and head cancer [16]. However, in the last decades, MTX has also been used as an anti-inflammatory and immunosuppressive agent for the treatment of rheumatoid arthritis, systemic lupus erithematosus and psoriasis [4, 16].

The therapeutic use of MTX can lead to adverse effects such as bone marrow suppression, nephrotoxicity and mucositis, common to other anti-neoplastic drugs [16]. Moreover, MTX neurotoxicity has been reported in patients treated for inflammatory and malignant diseases [8, 23, 28]. Motor and visual impairment, confusion, cerebral edema, encephalopathy, seizures and coma are neurological manifestations usually related to MTX induced neurotoxicity [13, 23, 28]. At brain cellular level, MTX was shown to affect primarily and selectively astrocytes rather than neurons [9]. Exposure of astrocytes cultures to MTX causes degenerative and reactive astroglial changes [3]. Concerning the neurotoxicity, folate depletion in brain tissue, high levels of homocysteine and excitatory amino acids [8, 23], and alterations in biopterine and adenosine metabolism [2, 17] are postulated as possible mechanisms underlying acute and chronic MTX neurotoxicity.

Dysfunction and damage of the human central nervous system (CNS) could be characterized with the help of biochemical markers [11]. S100B is a calcium binding protein mostly produced and released by astrocytes in the CNS [7]. Intracellularly, S100B modulates cytoskeletal proteins, calcium homeostasis and regulates the cell cycle. Extracellularly, S100B is involved in maintenance of CNS development [7]. Independent of

their putative functional role, S100B has been proposed as biochemical marker of brain injury, since increased serum and cerebrospinal fluid (CSF) levels are observed in some brain pathologies as traumatic brain injury, schizophrenia, HTLV-I associated myelopathy [10, 12, 21, 29]. The increase in S100B levels may reflect glial damage or astrocytic responses to neural injury, a process known as reactive astrogliosis [21].

Glutamate is the main excitatory neurotransmitter in the mammalian CNS, where it is involved in most brain functions [6, 19]. However, at high concentrations in the synaptic cleft this neurotransmitter acts as an excitotoxin through excessive stimulation of glutamate receptors [6, 15]. Glutamate toxicity has been related to neuronal death in acute and chronic neurodegenerative disorders of the CNS [6, 15]. Glutamate transporters perform regulation of glutamate levels in the synaptic clef and until now five high-affinity, sodium dependent glutamate transporters have been identified, distributed in both neurons and astroglia [5, 15]. Astroglial transporters are the predominant physiologic pathway responsible for the maintenance of low synaptic glutamate concentrations, preventing receptor desensitization and/or excitotoxicity [5, 6, 15].

In this study we evaluated MTX induced neurotoxicity by measuring the biochemical brain marker S100B, MTX levels and brain glutamate uptake.

2. Materials and methods

2.1 Chemicals

Reagents were purchased as follows: N-methyl-D-glucamine from Sigma (St. Louis, MO, USA); L-[^{3}H] glutamate (45 Ci/mmol) from Amersham Biosciences (Piscataway, NJ,

USA); MTX biometrox from Biosintética (São Paulo, SP, Brazil) and sodium thiopental from Cristalia (São Paulo, SP, Brazil). All other reagents were of analytical grade.

2.2 Animals

Female Wistar rats (60 days old; 200–250 g) were obtained from a local breeding colony. They were kept on a 12 h light/dark cycle (lights on at 07:00 a.m.) at constant temperature (22 ± 1°C), and housed in plastic cages (five animals per cage) with commercial food and water *ad libitum*. In all experiments, institutional protocols with animals were performed to minimize suffering and limit the number of animals sacrificed.

2.3 Drug treatment and sampling

Female rats were injected intraperitonially with 1.0, 3.0 or 10.0 mg/Kg of MTX or saline (control group) for three days. On the fourth day, the animals were anaesthetized with sodium thiopental (40 mg/Kg) for CSF sampling in the cisterna *magna* with an insulin syringe (27 gauge × 1/2 in. length). CSF samples were centrifuged at 5000 x g for 5 min, at 4°C to obtain cell-free supernatant and stored at -70ºC until S100B and MTX measurements.

After CSF sampling, rats were decapitated to obtain serum samples and brains. Cerebral cortices and hippocampus were immediately dissected and individually homogenized with 800 µL of salt solution using a motor-driven Teflonglass homogenizer, which was followed by the addition of 200 µL of Triton-X100 10 %. Afterwards tissue homogenates were centrifuged at 5000 x g for 5 min, at 4°C to obtain cell-free supernatant. Serum and tissue homogenates samples were stored at -70ºC until MTX measurements.

Approximately 60 rats were used in these experiments.

2.4 Measurement of MTX levels

MTX assay was performed in *TDxFLx*® immunoassay chemistry analyzer which uses a Fluorescence Polarization Immunoassay (FPIA) technology (Abbott Laboratories, IL, USA). FPIA is a type of homogeneous competitive fluorescence immunoassay that measures the intensity of the polarized, vertical light. The change in polarized light intensity, by molecules rotation, is proportional to the concentration of the MTX in the sample. The detection range is 0.02 - 1.0 μmol/L.

2.5 Measurement of S100B protein level

S100B assay was performed in a Lumat LB9507 luminometer (EG&G Berthold) using an immunoluminescent assay (LIA mat Sangtec 100, Dietzembach, Germany), which use an antibody labeled with isoluminol. The S100B standard curve was linear up to 20 μg/L and the coefficient of variation of the duplicates in all range levels of standards and samples were within 5 % [22].

2.6 Glutamate uptake

Glutamate uptake was performed as previously described by Oliveira et al, [18]. Untreated female Wistar rats (5 animals per group) were decapitated and the brains were immediately removed and humidified with Hank's balanced salt solution (HBSS), pH 7.2. Cortices were dissected and 400 μL coronal slices were obtained from the parietal area using a McIlwain tissue chopper. Cortical slices were separated and transferred to a 24

well-culture plates containing 500 μL of HBSS solution, immediately washed with 1.0 mL HBSS followed by the addiction of 500 μL HBSS per well and pre incubated for 30 minutes. Subsequently, the pre-incubating medium was changed by 160 μL of HBSS solution and the slices were incubated by 6 minutes in the absence (control) or presence of 0.1; 0.5; 2.5 and 10.0 mg/mL of MTX. After that, it was added 20 μL of 0.33 $Ci.mL^{-1}$ L-[^{3}H] glutamate with 100 μL unlabeled glutamate. Uptake was stopped after 7 minutes by two ice-cold washes with 1.0 mL HBSS followed by the addition of 300 μL of 0.5 N NaOH, which was kept overnight. Aliquots of lysates were taken for the determination of the intracellular content of L-[^{3}H] glutamate by scintillation counting. Sodium independent uptake was determined using N-methyl-D-glucamine instead of sodium chloride, which was subtracted from the total uptake to obtain the sodium-dependent uptake. Determination of protein was assessed using the method of Peterson [20]. The experiments were performed in triplicate.

2.7 Statistical analysis

S100B data are represented as absolute values (mean ± S.D) and glutamate uptake are represented as percentage of control (mean ± S.D). Statistical analysis was performed by ANOVA for multiple groups comparison, followed by the Tukey post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

Interestingly, some cytotoxic effects such as diarrhea, fur loss and decrease of locomotion activity were observed in rats that received 3.0 and 10.0 mg/Kg of MTX.

However, the 10 mg/kg group presented much worse symptoms than the 3 mg/Kg group and the highest dose have also caused death in around 40% of the animals, whereas no animal died in the 3 mg/Kg group. Furthermore, the 3 mg/Kg group presented less prominent symptoms (although they were qualitatively the same) and some of them presented no symptoms at all.

Rat CSF S100B levels increased significantly in 10.0 mg/Kg MTX group (9.23 ± 5.54 μg/L, n=9) when compared to 1.0 mg/kg (1.47 ± 0.75 μg/L, n=5) and 3.0 mg/Kg (2.80 ±1.54 μg/L, n=6) groups, as well as to control group (3.38 ± 1.71 μg/L, n=9) ($P < 0.01$) (Figure 1). Initially, the number of animals for each group was equal; however, as CSF samples containing blood cells were rejected for S100B analysis, and as some animals treated with the highest dose did not survive, we needed to treat more animals, accounting for the unequal sample sizes.

MTX was not detected in serum, CSF, cortical and hippocampal samples when sampling was performed in the fourth day. However, 1.0 μmol/L of MTX in cortical homogenates and 0.06-0.09 μmol/L in serum samples were detected in the treatment with 3.0 and 10.0 mg/Kg when sampling occurred 3 hours after the last dose. MTX was not detected in CSF in any protocol used.

In vitro, 2.5 and 10.0 mg/mL of MTX decreased glutamate uptake in cortical slices in 23.67 ± 7.78 % (n=5) and 48.45 ± 13.83 % (n=5) respectively, when compared to control group (100 %, n=5) ($P < 0.05$). Moreover, glutamate uptake values in 10.0 mg/mL MTX treated slices were significantly different from 0.1 mg/mL (17.58 ± 16.84 %, n=5) and 0.5 mg/mL (21.45 ± 13.23 %, n=5) groups ($P < 0.05$) (Figure 2). We also measured glutamate uptake, accordingly Oliveira et al. [18], using ex vivo preparations from the 10 mg/kg of

MTX and control groups after 24 hours of the last dose, but no differences were found between these groups (data not shown).

4. Discussion

MTX neurotoxicity has been widely described in the last years. Patients treated for both malignant and inflammatory diseases could manifest neurological disorders after administration of low, intermediate and high doses [8, 23]. The neurotoxic manifestations can be classified as acute, subacute and late forms. Acute toxicity happens hours after MTX treatment, with symptoms as nausea, headaches, somnolence, confusion and seizures. Subacute neurotoxicity occurs days or weeks after MTX exposure and could be manifested as seizures, affective and focal neurologic disturbances. Late neurotoxicity occurs months after MTX treatment and is associated to leucoencephalopathy and impairment of higher cognitive functions [23, 28].

The neurological symptoms are related to the presence of CNS morphological alterations as astrocytosis, demyelination, axonal swelling and gray and white matter necrosis [9, 28]. MTX induce degenerative and reactive changes in astrocytes *in vivo* and *in vitro*, and these neuropathological findings were similar to those observed in human brain studies [3, 9]. Actually, astrocytes were shown to be primarily and selectively affected by MTX rather than neurons [9]. Nevertheless, taking into account the closer structural and functional relationship between astrocytes and neurons is possible to suggest that depending on the time course of treatment both cells can be affected.

Folic acid is a crucial molecule for the synthesis of purines and pyrimidines bases and consequently for the DNA and RNA synthesis [25]. Within the cell, folates are

metabolized to polyglutamates, which are essential for folate retention. Intracellularly, MTX acts competitively with folates and it is also metabolized to form MTX polyglutamates. Polyglutamylation has been shown to play a key role in the citotoxic and selective action of MTX [25]. It is believed that astrocytes are a site for uptake and polyglutamylation of MTX and consequent drug accumulation inside the cells would increase the toxicity [9].

S100B protein is considered a sensitive biochemical marker of astrocyte injury or reactive gliosis, and increase on its levels has been demonstrated in several acute and chronic CNS diseases [26]. However, to our knowledge, the measurement of S100B to investigate neurotoxicity induced by a pharmacological agent has not been performed up to the moment. Our results showed that 10.0 mg/Kg of MTX administered intraperitonially increased significantly rat CSF S100B levels 24 hours after exposure. Furthermore, MTX brain levels were only detected 3 hours after administration, indicating a rapid drug metabolism. In this study we cannot discard the possibility of central effects for all doses. Our experimental model was adapted from Gregorios et al. [9], which well characterized, through light and electron microscopy, clear morphological alterations in astrocytes after intraperitoneal and intraventricular MTX treatment with 10 and 100 mg/kg doses.

Whether the increase in S100B levels is due to astrocytic death or may indicate astrocytic responses is unclear. The capacity of S100B to be beneficial or detrimental to brain functions depends on how glia respond to injurious stimuli, the concentration of S100B reached and repertoire of other glial derived molecules present [26].

Independently of its functional roles, the increase in extracellular S100B levels can be pointed as a potential brain marker of MTX neurotoxicity. Regarding to neuronal

damage after MTX treatment, Van Gool et al. [27] demonstrated that the neuronal markers Tau, Phospho-Tau and Neuromodulin are increased in CSF of children with non-B-cell acute lymphoblastic leukemia. This increase was related to pathophysiological mechanisms of disease-induced brain damage and chemotherapy-induced neurotoxicity. The measurement of neuronal and glial brain markers could assist therapeutic drug monitoring especially considering that MTX neurotoxicity persists from days to months despite of its depuration from CNS.

Glutamate uptake is a fundamental physiological process exerted by astrocytes to avoid toxic glutamate concentrations in synaptic cleft. Therefore, an impaired function of astrocytes could lead to a disturbance of neuronal activity or even neuronal damage [5]. As MTX *in vivo* increased CSF S100B levels and the integrity of astrocytes are extremely important for glutamate uptake, we additionally investigate the *in vitro* effect of MTX on glutamate uptake. This work demonstrated a decreased function of glutamate uptake in cortical slices, suggesting an involvement of this neurotransmitter in MTX neurotoxicity.

Interestingly, S100B protect cultured neurons against glutamate excitotoxicity [1, 11]. It is possible that our data reflect astrocytic reaction to a deleterious environment where the increased levels of glutamate are contributing for brain toxicity. Unfortunately the exact mechanism of MTX neurotoxicity is not totally understood and is likely to be multifactorial.

In summary, our results corroborate with the postulation pointing to astrocytes as a target of MTX toxicity. In addition, we speculate that the impaired glutamate uptake, which raises the glutamate levels in the synaptic cleft, could contribute to the mechanisms of MTX neurotoxicity.

References

1. B. Ahlemeyer, H. Beier, I. Semkova, C. Schaper, J. Krieglstein, S-100beta protects cultured neurons against glutamate- and staurosporine-induced damage and is involved in the antiapoptotic action of the 5 HT(1A)-receptor agonist, Bay x 3702, Brain Res. 858 (2000) 121–128.
2. J.H. Bruce-Gregorios, D.M. Soucy, M.G. Chen, M.D. Norenberg, Effect of methotrexate on glial fibrillary acid protein content of astrocytes in primary culture, J Neuropathol Exp Neurol. 50 (1991) 118-125.
3. J.C. Bernini, D.W. Fort, J.C. Griener, B.J. Kane, W.B. Chappell, B.A. Kamen, Aminophylline for methotrexate-induced neurotoxicity. Lancet. 345 (1995) 544-547.
4. E.S.L. Chan, B.N. Cronstein, Molecular action of methotrexate in inflammatory diseases, Arthritis Res. 4 (2002) 266-273.
5. Y. Chen, R.A. Swanson, Astrocytes and brain injury, J Cereb Blood Flow Metab. 23 (2003) 137-49.
6. N.C. Danbolt, Glutamate uptake, Prog Neurobiol 65 (2001) 1-105.
7. R. Donato, S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles, Int J Biochem Cell Biol. 33 (2001) 637-668.
8. R.A. Drachtman, P.D. Cole, C.B. Golden, S.J. James, S. Melnyk, J. Aisner, B.A. Kamen, Dextromethorphan is effective in treatment of subacute methotrexate neurotoxicity, Pediatr Hematol Oncol. 19 (2002) 319-327.
9. J.B. Gregorios, A.B. Gregorios, J. Mora, A. Marcillo, R.M. Fojaco, B. Gren, Morfologic alterations in rat brain following systemic & intraventricular methotrexate

injection: light and electron microscopic studies, J Neuropathol Exp Neurol. 48 (1989) 33- 47.

10. M. Herrmann, N. Curio, S. Jost, M.T. Wunderlich, H. Synowitz, C.W. Wallesch, Protein S-100B and neuron specific enolase as early neurobiochemical markers of the severity of traumatic brain injury, Restor Neurol Neurosci. 14 (1999) 109–114.
11. T.O. Kleine, L. Benes, P. Zöfel, Studies of the brain specificity of S100B and neuron-specific enolase (NSE) in blood serum of acute care patients, Brain Res Bull 61 (2003) 265–279.
12. D. Kogel, M. Peters, H.G. König, S.M. Hashemi, N.T. Bui, V. Arolt, M. Rothermund, J.H. Prehn, S100B potently activates p65/c-Rel transcriptional complexes in hippocampal neurons: clinical implications for the role of s100b in excitotoxic brain injury, Neuroscience. 127 (2004) 913–920.
13. W. Kuker, P. Bader, U. Herrlinger, S. Heckl, T. Nagele, Transient encephalopathy after intrathekal methotrexate chemotherapy: diffusion-weighted MRI, J Neurooncol. 73 (2005) 47-9.
14. D.R. Lara, C.S. Gama, P. Belmonte-de-Abreu, L.V. Portela, C.A. Goncalves, M. Fonseca, S. Hauck, D.O. Souza, Increased serum S100B protein in schizophrenia: a study in medication-free patients, J Psychiatr Res. 35 (2001) 11-14.
15. N.J. Maragakis, J.D. Rothstein, Glutamate transporters: animal models to neurologic disease, Neurobiol Dis. 15 (2004) 461- 473.
16. J.J. McGuire, Anticancer antifolates: current status and future directions, Curr Pharm Des. 9 (2003) 2593- 2613.

17. Millot F., Dhondt J.L., Mazingue F., Mechinaud F., Ingrand P., Guilhot F., 1995. Changes of cerebral biopterin and biogenic amine metabolism in leukemic children receiving 5g/m2 intravenous methotrexate. Pediatr. Res. 37, 151-154.

18. D.L. Oliveira, J.F. Horn, J.M. Rodrigues, M.E. Frizzo, E. Moriguchi, D.O. Souza, Wofchuk S, Quinolinic acid promotes seizures and decreases glutamate uptake in young rats: reversal by orally administered guanosine, Brain Res. 1018 (2004) 48-54.

19. S. Ozawa, H. Kamiya, K. Tsuzuki, Glutamate receptors in the mammalian central nervous system, Prog. Neurobiol. 54 (1998) 581–618.

20. G.L. Peterson Jr., A simplification of the protein assay method of Lowry, which is more generally applicable, Anal Biochem. 83 (1977) 346- 356.

21. A. Petzold, G. Keira, D. Limb, M. Smith, E.J. Thompson, Cerebrospinal fluid (CSF) and serum S100B: release and wash-out pattern, Brain Research Bulletin 61 (2003) 281–285.

22. L.V. Portela, A.B. Tort, D.V. Schaf, L. Ribeiro, D.B. Nora, R. Walz, L.N. Rotta, C.T. Silva, J.V. Busnello, F. Kapczinski, C.A. Goncalves, D.O. Souza, The serum S100B concentration is age dependent, Clin Chem. 48 (2002) 950-952.

23. C.T Quinn, J.C. Griener, T. Bottiglieri, K. Hyland, A. Farrow, B.A. Karmen, Elevation of homocysteine and excitatory amino acid neurotransmitters in CSF of children who receive methotrexate for the treatment of cancer, J Clin Oncol. 15 (1997) 2800- 2806.

24. R. Sánchez-Carpintero, J. Narbona, R. López de Mesa, J. Arbizu, L. Sierrasesúmaga, Transient posterior encephalopathy induced by chemotherapy in children, Pediatr Neurol. 24 (2001) 145-148.

25. M.J. Spinella, K.E. Brigle, S.J. Freemantle, E.E. Sierra, I.D. Goldman, Comparison of methotrexate poliglutamylation in L1210 leukemia cells when influx is mediated by the reduced folate carrier or the folate receptor, Biochem Pharmacol. 52 (1996) 703- 712.
26. L.J. Van Eldik, M.S. Wainwright, The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain, Restor Neurol Neurosci. 21 (2003) 97-108.
27. S.W. Van Gool, G. De Meyer, A. Van de Voorde, E. Vanmechelen, H. Vanderstichele, Neurotoxicity marker profiles in the CSF are not age-dependent but show variation in children treated for acute lymphoblastic leukemia, Neurotoxicology. 25 (2004) 471-480.
28. S. Vezmar, A. Becker, U. Bode, U. Jaehde, Biochemical and clinical aspects of methotrexate neurotoxicity, Chemotherapy. 49 (2003) 92-104.
29. R. Walz, L.V. Portela, A.B. Tort, E.C. Neto, L.N. Fernandes, C.A. Goncalves, D.O. Souza, Serum S100B levels in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis, Neurology. 54 (2000) 2021-2022.

Acknowledgements

This work was supported by grants from CNPq-Brasil and CAPES. We are grateful to Weinmann Laboratory for the scientific and technical support.

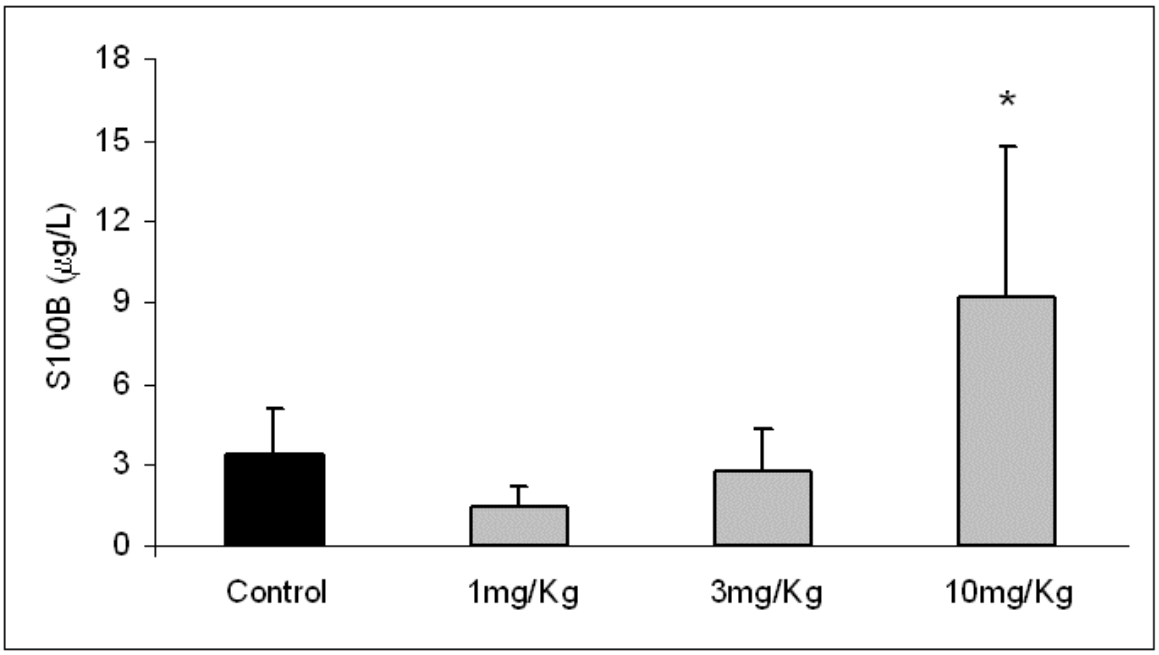

Fig. 1: S100B protein levels in rat CSF after MTX treatment (1.0, 3.0 and 10.0 mg/Kg). The data represent mean ± S.D. (n>5). *$P < 0.01$, when compared to all groups using ANOVA one-way test t followed by Tukey as *post hoc* test.

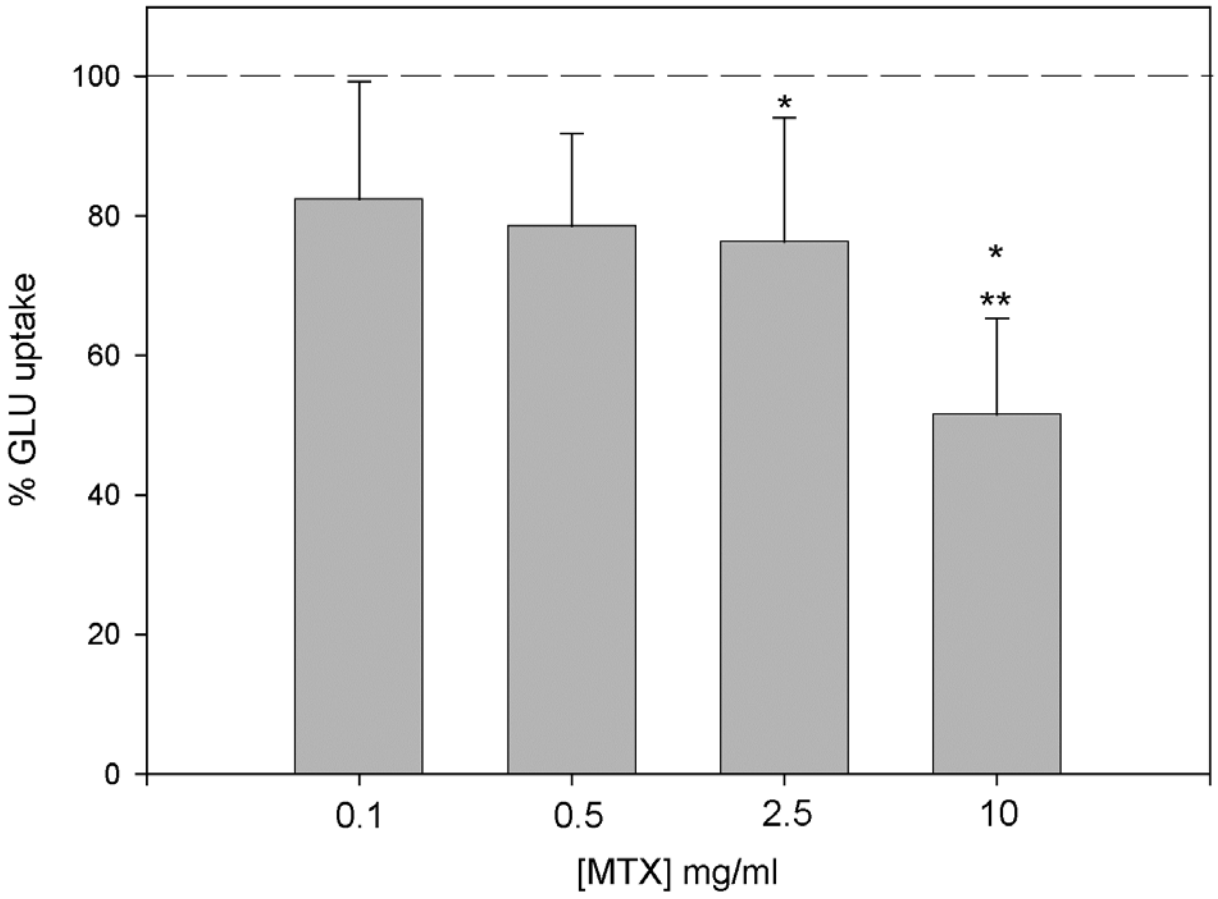

Fig. 2: Glutamate uptake in cortical slices after *in vitro* treatment (0.1, 0.5, 2.5 and 10.0 mg/mL of MTX). The data represent mean ± S.D. (n=5). *$P < 0.05$, when compared to control (100% - dot line); **$P < 0.05$, when compared to 0.1 and 0.5 mg/mL of MTX, using ANOVA one-way test t followed by Tukey as *post hoc* test.

CAPÍTULO II

Methotrexate induces seizure and decreases glutamate uptake in brain slices: Prevention by ionotropic glutamate receptors antagonists and adenosine.

(Submetido à revista Experimental Neurology)

Methotrexate induces seizure and decreases glutamate uptake in brain slices: Prevention by ionotropic glutamate receptors antagonists and adenosine.

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Abstract

Methotrexate (MTX) induced neurotoxicity may occur after intrathecal or systemic administration at low, intermediate and high doses for the treatment of malignant or inflammatory diseases. The mechanisms of MTX neurotoxicity are not totally understood, and appear to be multifactorial. In this study MTX induced tonic-clonic seizures in approximately 95.00 % of animals and pre-treatment with MK-801, DNQX and adenosine prevented seizure in 80.00 %, 62.50 % and 50.00 % of animals, respectively. Moreover, MTX lead 58.82 % of mice to death, which was prevented in 100.00 % and 93.75 % when animals received MK-801 and DNQX, respectively. Glutamate uptake decreased by 20% to 30 % in cortical slices after MTX-induced seizure. Interestingly, when seizures were prevented by MK-801, DNQX and adenosine, glutamate uptake activity remained at the same level as the control group. Thus, our results demonstrate the involvement of the glutamatergic system in MTX-induced seizures.

Key words: Methotrexate, glutamate uptake, seizure, MK-801, DNQX, adenosine.

Introduction

Methotrexate (MTX), a folic acid antagonist, is widely used for the treatment of malignant and inflammatory diseases (Chan and Cronstein, 2002; McGuire, 2003). However, MTX therapeutic use has been associated to neurotoxic effects after intrathecal or systemic administration at low, intermediate and high doses (Drachtman et al., 2002). The neurotoxic effects can be classified as acute, subacute and chronic, and the symptoms include nausea, headaches, somnolence, confusion, cerebral edema, impairment of higher cognitive functions and seizures (Quinn et al., 1997; Sánchez-Carpintero et al., 2001; Vezmar et al., 2003; Kuker et al., 2005).

Regarding seizures, this neurotoxic effect is more frequently observed in patients who received high intravenous doses and intrathecal therapy of MTX for the treatment of malignant diseases (Millot et al., 1995; Lo Nigro et al., 2000; Vezmar et al., 2003; Atra et al., 2004). The incidence of seizure or other acute neurotoxicity during the treatment of acute lymphoblastic leukemia has been reported at 3% to 13%. This variability is a consequence of the failure in recognizes uncomplicated seizures related to MTX treatment (Mahoney et al., 1998). However, the exact mechanism of MTX-induced neurotoxicity is still unclear. Given this, some studies have suggested that higher levels of homocysteine and its metabolites in cerebrospinal fluid (CSF) are related to the development of seizures (Quinn et al. 1997; Drachtman et al., 2002). Moreover, is believed that MTX decreases the bioamine synthesis, so leading to an imbalance in some neurotransmitter systems (Millot et al., 1995; Madhyastha et al., 2002).

Glutamate is the main excitatory neurotransmitter in the mammalian CNS, where it is involved in most brain functions (Ozawa et al., 1998), including memory and learning

(Izquierdo and Medina, 1997), development and aging (Segovia et al., 2001), and environmental adaptation (Danbolt, 2001; Mattson et al., 2001). Glutamate signalling occurs by the activation of ionotropic and metabotropic glutamate receptors. Ionotropic receptors contain cation ion channels and are subdivided into AMPA (a-amino-3-hydroxi-5-metyl-4-isoxazolepropionate), kainate and NMDA (N-metyl-D-aspartate) receptors. Metabotropic receptors (mGluRs1-8) are subdivided into 3 groups (I, II and III) modulating the production of intracellular messengers through G proteins (Ozawa et al., 1998). In contrast with its physiological roles, when present at high concentrations in the synaptic cleft glutamate acts as an excitotoxin through excessive stimulation of glutamate receptors (Chen and Swanson, 2003; Maragakis and Rothstein, 2001-2004). Glutamate excitotoxicity has been linked to various acute and chronic CNS disorders, including status epileptics (Meldrum, 2000; Maragakis and Rothstein, 2001-2004).

Astrocytic glutamate uptake is the predominant pathway for the maintenance of synaptic glutamate concentrations below excitotoxic levels (Danbolt, 2001; Chen and Swanson, 2003; Maragakis and Rothstein, 2004). It has been suggested that impaired astrocytic glutamate uptake may be involved in epileptogenesis. Studies with epileptic mice and rats presenting chronic seizures induced by kainic acid showed a decrease in astrocytic glutamate transporters GLT-1 and GLAST (Ingram et al., 2001; Ueda et al., 2001). Moreover, quinolinic acid-induced seizures decrease brain glutamate uptake in young and adult rats (Oliveira et al., 2004; Vinade et al., 2005).

In this study we characterised a model of MTX-induced seizures in mice to evaluate the convulsive and toxic MTX properties. Additionally, we investigated the effect of MTX-

induced seizures on the activity of glutamate transporters, as well as the anticonvulsant role of MK-801, DNQX and adenosine on glutamate uptake in brain slices.

Materials and methods

Chemicals

Methotrexate (MTX) was obtained from Biossintética (São Paulo, SP, Brazil); Phenobarbital, adenosine (ADO) and 6,7-dinitroquinnoxaline-2,3-dione (DNQX), N-methyl-D-glucamine were obtained from Sigma Chemicals (St Louis, MO, USA). 5-methyl-10-11-dihydro-5H-dibenzo[a,b]cyclohepta-5-10-imine maleate (MK-801) was obtained from RBI-Research Biochemicals International (Natick, MA, USA); L-[^{3}H] glutamate (45 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ, USA). The anaesthetic sodium thiopental was obtained from Cristália (SP, Brazil). All solutions were dissolved in saline 0.9% and buffered with 0.1 N NaOH or 0.1 N HCl to achieve pH 7.4, when necessary. All other reagents were of analytical grade.

Animals

Male adult Swiss albino mice (20-30 g) were kept on a 12 hour light/dark cycle (light on at 7:00 am) at a temperature of 22 ± 1ºC. They were housed in plastic cages (five per cage) with commercial food and tap water ad libitum. Our institutional protocols for experiments with animals, designed to minimise suffering and limit the number of animals sacrificed, were followed throughout. All behavioural procedures were conducted between 1:00 and 5:00 p.m.

Surgical procedure and drugs administration

Surgery and i.c.v. infusion techniques were adapted from Haley and McCormick (Haley and McCormick, 1957). Animals were anaesthetised with sodium thiopental (60 mg/Kg, 10 mL/Kg, i.p.). In a stereotaxic apparatus, the skin of the skull was removed and a 27 gauge 7 mm guide cannula was placed at 1 mm posterior to bregma, 1 mm right from of midline and 1 mm above the lateral brain ventricle. Through a 2 mm hole made at the cranial bone, the cannula was implanted 1.5 mm ventral to the superior surface of the skull, and fixed with jeweller's acrylic cement. Experiments were performed 48 hours after surgery.

A 30-gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a microsyringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula targeting the lateral right brain ventricle. Then, animals were pre-treated with an i.c.v. infusion of vehicle (saline 0.9 %), phenobarbital (1.16 μg), MK-801 (5 μg), DNQX (2.5 μg) or adenosine (10 μg). After 5 minutes, an i.c.v. infusion of either 4 μL of vehicle or MTX (90 μg) was performed and the behaviour observed for 30 minutes. For the glutamate uptake experiments, mice were observed for 10 minutes after MTX administration and immediately decapitated for brain dissection. The MTX dose used was adapted from previous studies with rats (Mullenix et al., 1994; Madhyastha et al., 2002).

Evaluation of seizures and toxicity

Mice were observed for 30 minutes in Plexiglas chambers for the occurrence of tonic-clonic seizures. Latency to start seizures, time of seizures and death were also evaluated. Animals not displaying seizures during these 30 minutes were considered

protected. Methylene blue (4 μL) was injected through the cannula and animals without dye in the lateral brain ventricle were discarded.

Glutamate uptake

Mice were decapitated 10 minutes after vehicle or MTX administration, with or with out the pre-treatment, and brains were immediately removed and humidified with Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, adjusted to pH 7.2. Cortices and hippocampi from the right hemisphere were dissected and slices (400 μm) were obtained using a McIlwain tissue chopper (coronal slices from the cortex parietal area were used). Glutamate uptake was performed at 35ºC as in previous reports (Frizzo et al., 2002; Oliveira et al., 2004; Thomazi et al., 2004). Cortical and hippocampal slices were separated and transferred to 24 well-culture plates containing 500 μL of HBSS solution and pre incubated for 15 minutes. Subsequently, the pre-incubating medium was changed for 280 μL of HBSS solution followed by the addition of 20 μL of 0.66 and 0.33 Ci.mL-1 L-[^{3}H] glutamate for hippocampal and cortical slices, respectively, containing 100 uM (final concentration) of unlabeled glutamate. Uptake was stopped after 5 and 7 minutes for hippocampal and cortical slices, respectively, by two ice-cold washes with 1.0 mL HBSS. NaOH (0.5 N, 200 μL for hippocampal and 300 μL for cortical slices) was used to lyse the tissues. Aliquots were taken for the determination of the intracellular content of L-[^{3}H] glutamate by scintillation counting. Sodium independent uptake was determined using N-methyl-D-glucamine instead of sodium chloride, which was subtracted from the total uptake to obtain the sodium-dependent uptake. Determination of protein was

assessed using the method of Peterson (Peterson, 1977). The experiments were performed in triplicate.

Statistical analysis

Toxicity data are expressed as a percentage of animals presenting seizures and death. Glutamate uptake data is expressed as mean ± S.D in percentage from the vehicle group. Statistical analysis was performed by the Fisher exact test for the occurrence of seizures and death and by ANOVA plus Tukey's post hoc test for latency to start first seizure, time and number of seizures and glutamate uptake. All results with $P < 0.05$ were considered statistically significant.

Results

Seizures and death

Administration of 90 μg i.c.v of MTX induced tonic-clonic seizures in 94.12 % (n=17) of animals. Pre-treatment with MK-801, DNQX and adenosine significantly prevented the manifestation of seizure in 80.00 % (n=5), 62.50 % (n=16) and 50.00 % (n=10) of animals, respectively ($P < 0.05$), while pre-treatment with phenobarbital had no effect (Fig 1A). There was no difference in the latency to start seizure and the duration of seizure between all groups.

MTX triggered death in 58.82 % (n=17), and pre-treatment with MK-801 and DNQX significantly prevented death in 100.00 % (n=5) and 93.75 % (n=16), respectively ($P < 0.05$) (Fig 1B). Adenosine and phenobarbital did not demonstrate any protective effect

against MTX-induced death (Fig 1B). There was no difference in the time to death between all groups.

Effect of MTX-induced seizures on glutamate uptake in brain slices

We evaluated the effect of MTX-induced seizures on glutamate uptake in hippocampal and cortical slices. As control groups we used the vehicle group (NaCl 0.9 g %), and the naive group, whose animals were not submitted to surgical procedure or to drugs administration. At this time, MTX or vehicle were administered without the pre-treatment and 10 minutes later mice were decapitated for brain dissection. MTX administration and seizure induction did not affect glutamate uptake in hippocampal slices (90.89 ± 33.99 %, n=8) when compared to vehicle (100.00 ± 28.25 %, n=7) and naive (108.37 ± 22.68 %, n=7) control groups (Fig 2A). However, in cortical slices, MTX-induced seizures significantly decreased glutamate uptake (30.11 ± 15.72 %, n=8, $P < 0.05$) when compared to vehicle (100.00 ± 22.45 %, n=7) and naive groups (110.68 ± 21.90 %, n=7) (Fig 2B).

MK-801, DNQX and adenosine prevented MTX-induced decrease in glutamate uptake

Since MK-801, DNQX and adenosine prevented MTX-induced seizures, we decided to evaluate their effects on glutamate uptake. Firstly, we compared the glutamate uptake in animals treated with a single injection of MTX with animals that received vehicle followed by MTX. No statistical difference in glutamate uptake was found between these groups (data not shown). As hippocampal slices did not demonstrate any difference in the glutamate uptake, only cortical slices were used.

Glutamate uptake in cortical slices decreased 21.83 ± 19.44 % (n=14) after MTX-induced seizures when compared to vehicle group (100.00 ± 9.18 %, n=20, $P < 0.05$). Pre-treatment with MK-801 significantly prevented the decrease in glutamate uptake (102.68 ± 10.90 %, n=7, $P < 0.05$) when compared to MTX group (78.17 ± 19.44 %) (Fig. 3A). DNQX seemed to prevent the decrease in glutamate uptake (93.36 ± 12.45 %, n=7), however, no statistical significance was found when compared to MTX group (78.17 ± 19.44 %) (Fig. 3B). Adenosine has a dual response; as it has an anticonvulsant effect, the decrease of glutamate uptake was not observed (98.48 ± 19.02 %, n=8, $P < 0.05$), when compared to MTX group (78.17 ± 19.44 %). However, when seizures were not prevented by adenosine, there was a slight but not significant decrease in glutamate uptake (85.92 ± 15.74 %, n=11) (Fig. 3C). No effect on glutamate uptake was observed when mice received MK-801 (96.37 ± 13.08 %, n=6), DNQX (94.85 ± 4.38 %, n=3) and adenosine (94.50 ± 20.18 %, n=8) followed by vehicle, when compared to vehicle group (100.00 ± 9.18 %,) (Fig 3D).

Discussion

Methotrexate (MTX) has been widely used for the treatment of neoplasic diseases such as acute leukaemia, lymphomas, osteosarcoma, breast and head cancer (Quinn et al., 1997; Drachtman et al., 2002; McGuire, 2003) Moreover, MTX has also been used as an anti-inflammatory agent for the treatment of rheumatoid arthritis, systemic lupus erythematosus and psoriasis (Chan and Cronstein, 2002; Kinder et al., 2005). However, neurotoxicity is one of the most important adverse effects of MTX and has been extensively described in recent years (Quinn et al., 1997; Vezmar et al., 2003). Moreover, CNS

morphological alterations such as astrocytosis, demyelination, axonal damage and white matter necrosis have been associated with MTX neurotoxicity (Gregorios et al., 1989; Bruce-Gregorios et al., 1991, Linnebanck et al., 2005).

Seizure is an adverse effect that may be manifested in acute, subacute and late forms of MTX neurotoxicity, in all ages. The mechanism of MTX-induced neurotoxicity is not totally understood, and seems to be multifactorial (Quinn et al., 1997). Increased homocysteine levels in serum and CSF have been described in patients who developed MTX-induced neurotoxicity (Quinn et al., 1997; Drachtman et al., 2002; Kishi et al., 2003). Homocysteine is directly toxic to vascular endothelium and has the potential to cause ischemic microvascular disease, being related to the pathogenesis of MTX-induced neurotoxicity (Quinn et al., 1997; Kishi et al., 2003). Besides, studies demonstrate that sulphur-containing excitatory amino acids, derived from homocysteine, act directly on excitatory receptors and promote glutamate and aspartate release. Moreover, inhibition of the reuptake of these neurotransmitters in neuronal and glial plasma membranes was observed. These events could be involved in the pathogenesis of seizure (Vezmar et al., 2003).

At brain cellular level, it has been verified that astrocytes rather than neurons were primarily affected by MTX (Gregorios et al., 1989). Therefore, impaired astrocytic function might disturb glutamatergic synapses and damage neurones (Chen and Swanson, 2003). Since there is evidence to show that MTX impairs astrocytes and affects the glutamatergic system through sulphur-containing amino acids, we used an animal model with the aim of investigating putative mechanisms of MTX-induced neurotoxicity. In our study, MTX induced tonic-clonic seizure in almost all animals, and approximately 59 % of them died

within 30 minutes. MK-801 and DNQX prevented the seizures, demonstrating the involvement of ionotropic glutamatergic receptors (NMDA, AMPA and kainate) in the pathogenesis of seizures. Moreover, death was avoided in 100 and 93.75 % of animals by MK-801 and DNQX, respectively.

MK-801 is a non-competitive NMDA antagonist, which inhibits glutamatergic signalling and also promotes glutamate release from the pre-synaptic vesicles (Moghaddam et al., 1998). MK-801 had a protective effect against MTX-induced seizures by reducing the excitatory neurotransmission. Our results are in accordance with previous studies that propose NMDA receptor activation is implicated with seizure development (Vezmar et al., 2003). Furthermore, the NMDA antagonist dextromethorphan has been successfully used for the treatment of MTX neurotoxic effects in patients with malignant or inflammatory diseases (Drachtman et al., 2002). Dextromethorphan is a non-opioid antitussive that has been shown to have both anticonvulsant and neuroprotective effects (Chou et al., 1999).

Concerning DNQX, it is a competitive antagonist of the non-NMDA receptors, AMPA and kainate. DNQX has demonstrated to be effective against seizures induced by electroshock or pentylenetetrazol, and also by picrotoxin (Sierra-Paredes et al., 2000). As observed with MK-801, the decrease in glutamatergic receptors stimuli could be implicated in the DNQX neuroprotective effect. Additionally, it is possible that the blockade of AMPA receptors could also decrease NMDA receptor activation (Ozawa, 1998). Although our study points to the involvement of the glutamatergic system in the MTX-induced seizures, it is still not known whether this drug acts directly on glutamate receptors.

Adenosine is a purine nucleoside, essential for every living cell, which acts by activating four different types of adenosine receptors, named: A_1, A_2A, A_2B and A_3, which

regulate many physiological processes. Through A_1 and A_3 receptors adenosine mainly mediates the inhibitory functions; in contrast, A_2A and A_2B receptors mediate the stimulatory effects of adenosine (Cunha, 2001-2005; Boison, 2005). In fact, adenosine is known as a neuromodulator of the glutamatergic system through a presynaptic inhibition of glutamate release by A_1 receptor activation (Cunha, 2001-2005). Adenosine receptors agonists, especially A_1 types, display anticonvulsive effects in electrical and chemical seizure models (Zuchora et al., 2005, Vianna et al., 2005). Here, adenosine prevented 50 % of MTX-induced seizures, however death was not avoided.

Phenobarbital is a specific $GABA_A$ receptor agonist and is used as an anticonvulsant in some gabaergic-induced animal models of seizure (Lapin et al., 1986). In this study, the lack of anticonvulsant effect of $GABA_A$ points to excitatory ionotropic receptors as potential target of MTX induced seizures and toxicity.

Glutamate uptake is a fundamental physiological process exerted mainly by astrocytes responsible for maintaining the glutamate concentrations in the synaptic cleft below the toxic levels. As studies have shown that astrocytes are damaged by MTX exposure (Gregorios et al., 1989, Bruce-Gregorios et al., 1991) and we demonstrated that MTX-induced seizures affected the glutamate system, we further investigated the effect of MTX- induced seizures on glutamate uptake. We verified that MTX decreased glutamate uptake by 20%-30 % in cortical slices and did not affect glutamate uptake in hippocampal slices.

Some studies have shown that decreased glutamate transporter activity is linked to seizures (Meldrum et al., 1994; Ingram et al., 2001; Maragakis and Rothstein, 2001). In our study, pre-treatment with MK-801, DNQX and adenosine significantly prevented the

decrease of glutamate uptake when seizures were avoided. Moreover, when seizures were not prevented by adenosine, there was a slight but not significant decrease in glutamate uptake. These data suggest that the decrease in glutamate uptake probably results from seizure manifestation rather than the direct action of MTX on glutamate transporters.

In summary, it was demonstrated that glutamatergic ionotropic antagonists and the neuromodulator adenosine prevented MTX-induced seizures. Furthermore, MTX-induced seizures slightly decreased glutamate uptake in cortical, but not hippocampal, slices, and pre-treatment with MK-801, DNQX and adenosine prevented these decreases. Therefore, our results demonstrate the involvement of the glutamatergic system in MTX-induced seizures.

Acknowledgements

This work was supported by grants from CNPq-Brazil and CAPES.

References

Atra, A., Pinkerton, C.R., Bouffet, E., Norton, A., Hobson, R., Imeson, J.D., Gerrard, M., 2004. Acute neurotoxicity in children with advanced stage B-non-Hodgkin's lymphoma and B-acute lymphoblastic leukaemia treated with the United Kingdom children cancer study group 9002/9003 protocols. Eur J Cancer. 40, 1346-13501.

Boison, D., 2005. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. Neuroscientist.11, 25-36.

Bruce-Gregorios, J.H., Soucy, D.M., Chen, M.G, Norenberg, M.D., 1991. Effect of methotrexate on glial fibrillary acid protein content of astrocytes in primary culture. J Neuropathol Exp Neurol. 50, 118-125.

Chan, E.S.L., Cronstein, B.N., 2002. Molecular action of methotrexate in inflammatory diseases. Arthritis Res. 4, 266-273.

Chen, Y., Swanson, R.A., 2003. Astrocytes and brain injury. J Cereb Blood Flow Metab. 23, 137-149.

Chou, Y.C., Liao, J.F., Chang, W.Y., Lin, M.F., Chen, C.F., 1999. Binding of dimemorfan to sigma-1 receptor and its anticonvulsant and locomotor effects in mice, compared with dextromethorphan and dextrorphan. Brain Res. 821, 516–519.

Cunha, RA., 2001. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem Int. 38, 107-125.

Cunha, RA., 2005. Neuroprotection by adenosine in the brain: From A1 receptor activation to A2A receptor blockade. Purinergic Signal. 1, 111-134.

Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65, 1 –105.

Drachtman, R.A., Cole, P.D., Golden, C.B., James, S.J., Melnyk, S., Aisner, J., Kamen, B.A., 2002. Dextromethorphan is effective in treatment of subacute methotrexate neurotoxicity. Pediatr Hematol Oncol. 19, 319-327.

Frizzo, M.E.S., Lara, D.R., Prokopiuk, A.S., Vargas, C.R., Salbego, C.G.,Wajner, M., Souza, D.O., 2002. Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. Cell. Mol. Neurobiol. 22, 353– 363.

Gregorios, J.B., Gregorios, A.B., Mora, J., Marcillo, A., Fojaco, R.M., Gren, B., 1989. Morphologic alterations in rat brain following systemic & intraventricular methotrexate injection: light and electron microscopic studies. J Neuropathol Exp Neurol. 48, 33- 47.

Haley, T.J., and McCormick, W.G. 1957. Pharmacologic effects of intracerebral injection of drugs in conscious mouse. Br. J. Pharmacol. 12, 12-15.

Ingram, E.M., Wiseman, J.W., Shoshi, T., Emson, P.C., 2001. Reduction of glial glutamate transporters in the parietal cortex and hippocampus of EL mouse. J. Neurochem. 79, 564–575.

Izquierdo, I., Medina, J.H., 1997. Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiol. Learn. Mem. 68, 285– 316.

Kinder, A.J., Hassell, A.B., Brand, J., Brownfield, A., Grove, M., Shadforth, M.F., 2005. The treatment of inflammatory arthritis with methotrexate in clinical practice: treatment duration and incidence of adverse drug reactions. Rheumatology 44, 61-66.

Kishi, S., Griener, J., Cheng, C., Das, S., Cook, E.H., Pei, D., Hudson, M., Rubnitz, J., Sandlund, J.T., Pui, C.H., Relling, M.V., 2003. Homocysteine, pharmacogenetics, and neurotoxicity in children with leukemia. J Clin Oncol. 21, 3084-3091.

Kuker, W., Bader, P., Herrlinger, U., Heckl, S., Nagele, T., 2005. Transient encephalopathy after intrathecal methotrexate chemotherapy: diffusion-weighted MRI. J Neurooncol. 73, 47-49.

Lapin, I.P., Prakhie, I.B., Kiseleva, I.P., 1986. Antagonism of seizures induced by the administration of the endogenous convulsant quinolinic acid into rat brain ventricles, J. Neural Transm. 65, 177- 185.

Linnebank, M., Pels, H., Kleczar, N., Farmand, S., Fliessbach, K., Urbach, H., Orlopp, K., Klockgether, T., Schmidt-Wolf, I.G., Schlegel, U., 2005. MTX-induced white matter changes are associated with polymorphisms of methionine metabolism. Neurology 64, 912-913.

Lo Nigro, L., Di Cataldo, A., Schiliro, G., 2000. Acute neurotoxicity in Children with b-lineage acute lymphoblastic leukemia (B-ALL) treated with intermediate risk protocols. Med. Pediatr. Oncol. 35, 449-455.

Madhyastha, S., Somayaji, S.N., Rao, M.S., Nalini, K., Bairy, K.L., 2002. Hippocampal brain amines in methotrexate-induced learning and memory deficit. Can. J. Physiol. Pharmacol. 80, 1076-1084.

Mahoney, D.H. Jr, Shuster, J.J., Nitschke, R., Lauer, S.J., Steuber, C.P., Winick, N., Camitta, B., 1998. Acute neurotoxicity in children with B-precursor acute lymphoid leukemia: an association with intermediate-dose intravenous methotrexate and

intrathecal triple therapy- a Pediatric Oncology Group study. J Clin Oncol. 16, 1712-22.

Maragakis, N.J., Rothstein, J.D., 2001. Glutamate transporters in neurologic disease. Arch. Neurol. 58, 365–370.

Maragakis, N.J., Rothstein, J.D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiol. Dis. 15, 461–473.

Mattson, M.P., Chan, S.L., Duan W., 2002. Modification of brain aging and neurodegenerative disorders by genes, diet, and behavior. Phys. Rev. 82, 637–672.

McGuire, J.J., 2003. Anticancer antifolates: current status and future directions. Curr Pharm Des. 9, 2593- 2613.

Meldrum, B.S., 1994. The role of glutamate in epilepsy and other CNS disorders. Neurology 44, S14– S23.

Meldrum, B.S., 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J. Nutr. 130, 1007S– 1015S.

Millot F., Dhondt J.L., Mazingue F., Mechinaud F., Ingrand P., Guilhot F., 1995. Changes of cerebral biopterin and biogenic amine metabolism in leukemic children receiving 5g/m2 intravenous methotrexate. Pediatr. Res. 37, 151-154.

Moghaddam, B., Adams, B.W, 1998. Reversal of phencyclidine effects by a group II metabotropic glutamate receptor agonist in rats. Science 281, 1349–1352.

Mullenix, P.J., Kernan, W.J., Schunior, A., Howes, A., Waber, D.P., Sallan, S.E., Tarbell, N.J., 1994. Interactions of steroid, methotrexate, and radiation determine neurotoxicity in an animal model to study therapy for childhood leukemia. Pediatr. Res. 35, 171-178.

Oliveira, D.L., Horn, J.F., Rodrigues, J.M., Frizzo, M.E.S., Moriguchi, E., Souza, D.O., Wofchuk, S., 2004. Quinolic acid promotes seizures and decreases glutamate uptake in young rats: Reversal by orally administered guanosine. Brain Res. 1018, 48– 54.

Ozawa, S., Kamiya, H., Tsuzuki, K., 1998. Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol. 54, 581– 618.

Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83, 346– 356.

Quinn, C.T., Griener, J.C., Bottiglieri, T., Hyland, K., Farrow, A., Karmen, B.A., 1997. Elevation of homocysteine and excitatory amino acid neurotransmitters in CSF of children who receive methotrexate for the treatment of cancer. J Clin Oncol. 15, 2800- 2806.

Sánchez-Carpintero, R., Narbona, J., López de Mesa, R., Arbizu, J., Sierrasesúmaga, L., 2001. Transient posterior encephalopathy induced by chemotherapy in children. Pediatr Neurol. 24, 145-148.

Segovia, G., Porras, A., Del Arco, A., Mora, F., 2001. Glutamatergic neurotransmission in aging: a critical perspective. Mec. Ageing Dev.122, 1 – 29.

Sierra-Paredes, G., Galan-Valiente, J., Vazquez-Illanes, M.D., Aguilar-Veiga, E., Sierra-Marcuno, G., 2000. Effect of ionotropic glutamate receptors antagonists on the modifications in extracellular glutamate and aspartate levels during picrotoxin seizures: a microdialysis study in freely moving rats. Neurochem Int. 37, 377-386.

Thomazi, A.P., Godinho, G.F.R.S., Rodrigues, J.M., Schwalm, F.D., Frizzo, M.E., Moriguchi, E., Souza, D.O., Wofchuk, S.T., 2004. Ontogenetic profile of glutamate

uptake in brain structure slices from rats: sensitivity to guanosine. Mech. Ageing Dev. 125, 475– 481.

Ueda, Y., Doi, T., Tokumaru, J., Yokoyama, H., Nakajima, A., Mitsuyama, Y., Ohya-Nishiguchi, H., Kamada, H., Willmore, L.J., 2001. Collapse of extracellular glutamate regulation during epileptogenesis: down-regulation and functional failure of glutamate transporter function in rats with chronic seizures induced by kainic acid. J. Neurochem. 76, 892–900.

Vezmar, S., Becker, A., Bode, U., Jaehde, U., 2003. Biochemical and clinical aspects of methotrexate neurotoxicity. Chemotherapy 49, 92-104.

Vianna, E.P., Ferreira, A.T., Dona, F., Cavalheiro, E.A., da Silva Fernandes, M.J., 2005. Modulation of seizures and synaptic plasticity by adenosinergic receptors in an experimental model of temporal lobe epilepsy induced by pilocarpine in rats. Epilepsia 46, 166S-173S.

Vinade, E.R., Schmidt, A.P., Frizzo, M.E., Portela, L.V., Soares, F.A., Schwalm, F.D., Elisabetsky, E., Izquierdo, I., Souza, D.O., 2005. Effects of chronic administered guanosine on behavioral parameters and brain glutamate uptake in rats. J Neurosci Res. 79, 248-253.

Zuchora, B., Wielosz, M., Urbanska, E.M., 2005. Adenosine A1 receptors and the anticonvulsant potential of drugs effective in the model of 3-nitropropionic acid-induced seizures in mice. Eur Neuropsychopharmacol. 15, 85-93.

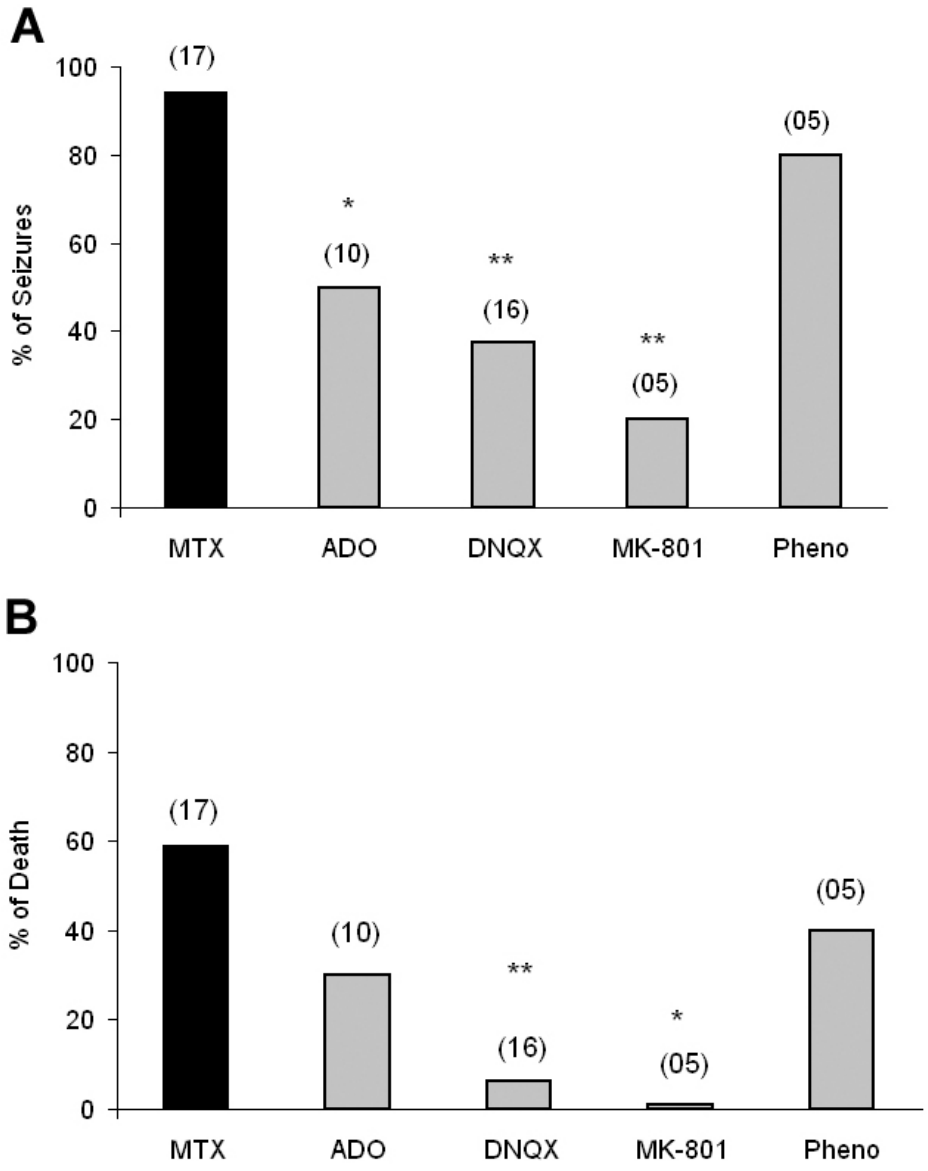

Fig. 1: Evaluation of MTX- induced seizures (A) and death (B), and the effect of adenosine (ADO) MK-801, DNQX and phenobarbital (pheno) on these parameters. ** $P < 0.01$ and * $P < 0.05$ by Fisher exact test when compared to MTX group.

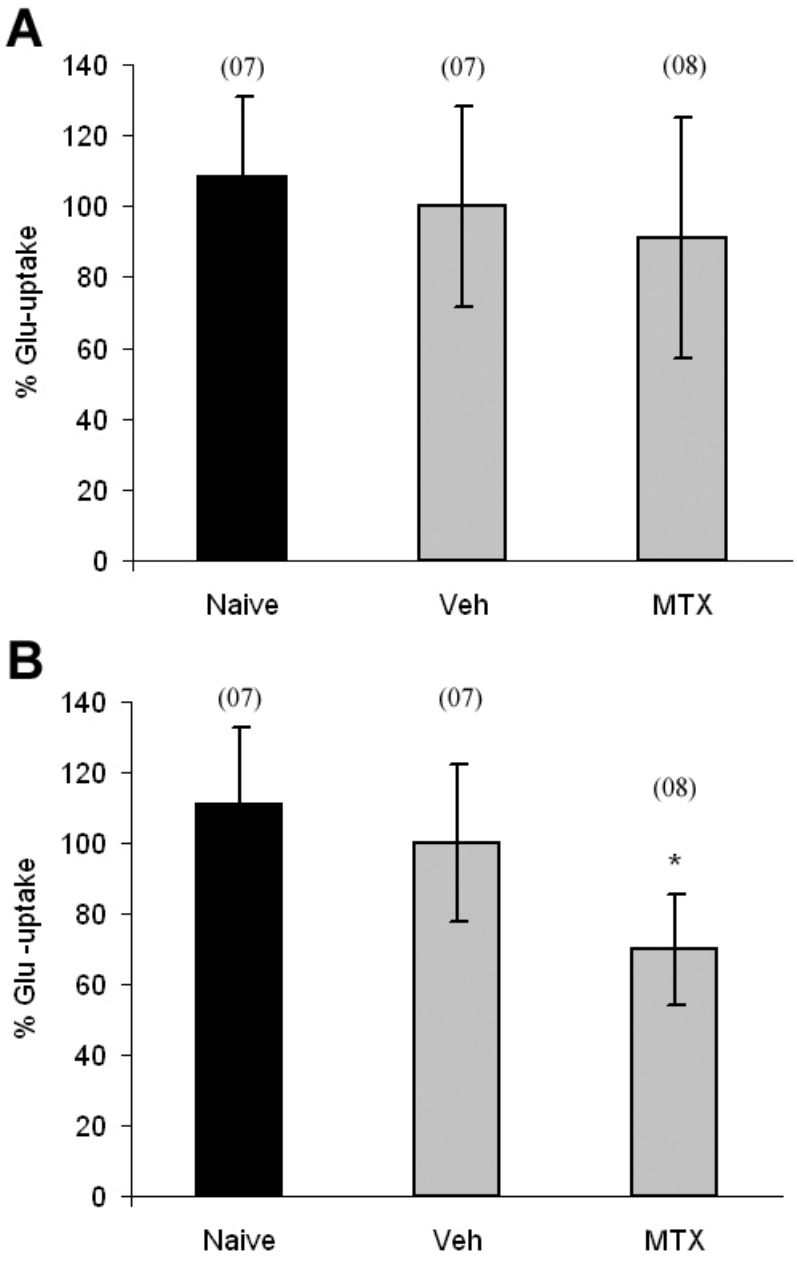

Fig. 2: Glutamate uptake in hippocampal (A) and cortical (B) slices after MTX-induced seizure. The data are presented as mean + S.D. in percentage of vehicle group (Veh). * $P <$ 0.05 when compared to all groups using ANOVA one-way followed by post hoc Tukey test.

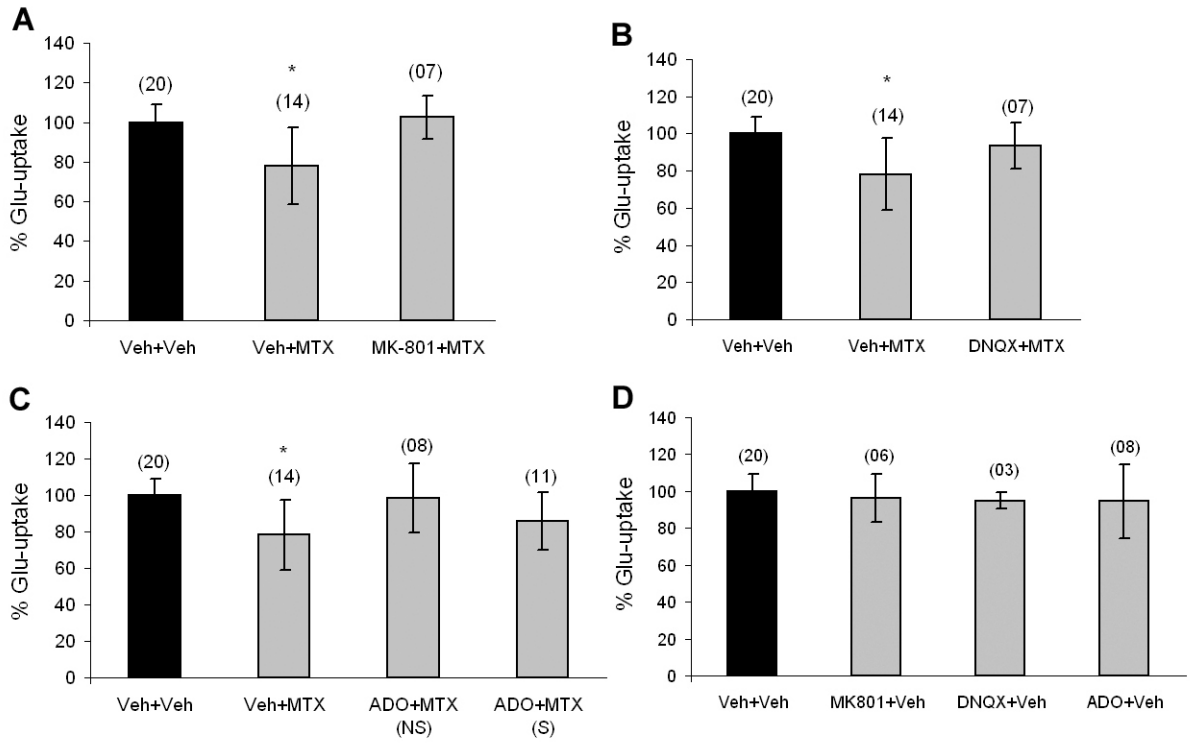

Fig. 3: Glutamate uptake in cortical slices after pre-treatment with vehicle or MK-801, DNQX and adenosine and subsequent i.c.v. administration of vehicle or MTX. The data are presented as mean + S.D. in percentage of vehicle group (Veh+Veh). Statistical significance (* $P < 0.05$) was determined using one-way ANOVA followed by post hoc Tukey test. (A) MK-801 (MK801+MTX) prevents the decrease of glutamate uptake, * $P < 0.05$ when compared to the vehicle and MK801+MTX groups. (B) DNQX (DNQX+MTX) prevents the decrease of glutamate uptake, * $P < 0.05$ when compared to the vehicle group. (C) Adenosine prevents the decrease of glutamate uptake when seizure was avoided (ADO +MTX (NS)) and there was a slightly decrease when seizure were not avoided (ADO+MTX (S)), * $P < 0.05$ when compared to the vehicle and to ADO +MTX (NS) groups. (D) MK-801, DNQX and ADO do not affect glutamate uptake.

TERCEIRA PARTE

Discussão

O Metotrexato (MTX) é um fármaco amplamente utilizado na clínica médica para o tratamento de diversas doenças neoplásicas e inflamatórias (Chan e Croinstein, 2002; McGuire, 2003). Entretanto, apesar de sua comprovada eficácia clínica, o uso terapêutico do MTX pode levar à neurotoxicidade (Quinn et al, 1997; Vezmar et al, 2003), sendo esta reação adversa bastante preocupante uma vez que pode levar ao agravamento do quadro clínico do paciente. Confusão, cefaléia, edema cerebral, convulsões, encefalopatia e problemas cognitivos são alguns dos sintomas que podem se manifestar durante a neurotoxicidade aguda, subaguda e crônica do MTX (Quinn et al, 1997; Lo Nigro et al, 2000; Vezmar et al, 2003, Kuker et al, 2005).

A neurotoxicidade do MTX tem sido muito estudada nos últimos tempos; entretanto, até o presente momento, não estão totalmente esclarecidos os mecanismos de toxicidade desse fármaco (Quinn et al, 1997). A depleção dos folatos (Bruce-Gregorios et al, 1991a), altos níveis de homocisteína e dos aminoácidos excitatórios sulfurados (Quinn et al., 1997; Drachtman et al., 2002), alterações na síntese de biopterinas e da adenosina (Millot et al., 1995; Bernini et al, 1995), são os mecanismos de ação propostos pela literatura como os responsáveis pela manifestação, tanto aguda quanto crônica, da neurotoxicidade do MTX.

Visando ampliar os conhecimentos a respeito dos efeitos do MTX no SNC, desenvolvemos nesta dissertação de Mestrado dois trabalhos com diferentes modelos em animais, nos quais foram estudados os possíveis mecanismos de ação tóxica desse fármaco, como também propusemos a utilização do marcador bioquímico S100B na detecção de injúrias cerebrais.

No primeiro capítulo desta Dissertação, desenvolvemos um modelo de intoxicação pelo MTX através da administração de uma dose diária intraperitonial de 1, 3 ou 10 mg/Kg de MTX em ratos adultos fêmeas, durante três dias consecutivos. Verificamos que a administração da dose de 10 mg/Kg de MTX levava ao aumento significativo da proteína S100B nas amostras de líquor, coletadas 24 horas após a última dose. Entretanto, quando analisamos as amostras de líquor, sangue e homogeneizado de hipocampo e córtex, não foi detectada a presença do MTX em nenhuma dessas amostras. Porém, quando a coleta das amostras foi realizada 3 horas após a última dose, tanto as amostras de sangue como as de homogeneizado de córtex apresentavam concentrações expressivas de MTX.

O aumento da S100B nas amostras de líquor comprova que o MTX de fato atingiu o SNC e induziu uma manifestação astrocitária perante o insulto tóxico. Este resultado está de acordo com trabalhos prévios nos quais foram detectadas alterações astrocitárias após o tratamento com MTX, sendo estas bastante proeminentes quando comparadas com as reações neuronais (Gregorios et al., 1989; Bruce-Gregórios et al., 1991b). Entretanto, a partir do resultado que obtivemos, não é possível afirmar se este aumento se deve a uma reação astrocitária ou se reflete a morte desse tipo celular.

O aumento das concentrações de S100B também sugere que este marcador bioquímico pode ser utilizado como uma importante ferramenta na detecção da neurotoxicidade do MTX, principalmente porque as suas concentrações permaneceram elevadas mesmo quando o MTX já não era detectável nas amostras cerebrais. Um trabalho anterior utilizou os marcadores neuronais Tau, fosfo-Tau e neuromodulina para investigar a ação tóxica do MTX em pacientes que utilizavam esse fármaco na terapia contra a leucemia lifoblástica aguda (Van Gool et al., 2004). Porém, o aumento desses marcadores neuronais

não somente refletiam a ação tóxica do fármaco no SNC, como também os danos ocasionados pela própria doença. Os marcadores neuronais e astrogliais, juntamente com as técnicas de diagnóstico por imagem e as manifestações clínicas, seriam de grande importância no monitoramento terapêutico deste fármaco, pois dessa forma os danos provenientes da neurotoxicidade poderiam ser minimizados.

Uma vez que a literatura reporta que os astrócitos são bastante afetados pelo MTX e que este tipo celular é o principal responsável pela manutenção de baixas concentrações de glutamato na fenda sináptica (Danbolt, 2001), realizamos ensaios *in vitro* visando estudar os efeitos do MTX sobre a captação de glutamato. Para tal, utilizamos fatias de córtex de ratas adultas que não foram submetidas ao modelo de intoxicação. Durante o ensaio de captação de glutamato expomos estas fatias às doses de 0,1; 0,5; 2,5 e 10,0 mg/mL de MTX. Verificamos que as fatias de córtex expostas às doses de 0,5 e 10,0 mg/mL de MTX apresentavam diminuição significativa da captação de glutamato de aproximadamente 24 % e 49 %, respectivamente. Este resultado demonstra que o sistema glutamatérgico pode estar envolvido na neurotoxicidade do MTX. Entretanto, não podemos afirmar se a diminuição da captação foi decorrente da ação direta do MTX sobre os transportadores, ou se foi através de outras substâncias provenientes da ação do MTX. Especulamos que o mau funcionamento dos transportadores de glutamato poderia ocasionar o aumento das concentrações desse neurotransmissor na fenda sináptica, levando conseqüentemente à excitotoxicidade. Ainda, o aumento de S100B seria proveniente da resposta astrocitária a estes estímulos nocivos na tentativa de proteger os neurônios da toxicidade do glutamato, como previamente demonstrado em culturas de neurônios (Ahlemeyer et al, 2000).

No segundo capítulo desta Dissertação, desenvolvemos um modelo animal de convulsões induzidas pelo MTX para estudar o mecanismo de neurotoxicidade deste fármaco, assim como o mecanismo envolvido nas convulsões. A convulsão é um sintoma da neurotoxicidade aguda, subaguda e crônica do MTX, que pode afetar tanto adultos como crianças que utilizam esse fármaco para o tratamento de diversas doenças (Lo Nigro et al, 2000; Vezmar et al, 2003; Atra et al, 2004). Entretanto, este sintoma tem sido mais incidente naqueles pacientes que fazem a utilização de altas doses sistêmicas de MTX, como também a administração desse fármaco pela via intratecal (Mahoney et al, 1998; Lo Nigro et al, 2000; Atra et al, 2004).

No trabalho que desenvolvemos, a administração intracerebroventricular de 90 μg de MTX induziu convulsões tônico-clônicas em praticamente todos os camundongos. Porém, quando os camundongos eram submetidos a um pré-tratamento com os antagonistas de receptores ionotrópicos glutamatérgicos MK-801 e DNQX, e do neuromodulador adenosina, as convulsões foram prevenidas em 80 %, 62 % e 50 %, respectivamente. Além disso, o MK-801 e o DNQX também protegeram os camundongos da morte em 100 % e 94 %, respectivamente. Já o pré-tratamento com o fenobarbital não impediu as convulsões.

Estes resultados mostram que as convulsões induzidas pelo MTX envolvem o sistema glutamatérgico, pois com o bloqueio da ativação dos receptores ionotrópicos de glutamato NMDA, AMPA e cainato, existe a prevenção das convulsões. Trabalhos anteriores já haviam demonstrado que as convulsões induzidas pelo MTX estavam relacionadas com o aumento de homocisteína e os aminoácidos excitatórios sulfurados provenientes da metabolização deste aminoácido (Drachtman et al, 2002; Vezmar et al, 2003). Os aminoácidos excitatórios sulfurados são capazes de estimular os receptores

excitatórios do SNC, como o receptor de glutamato NMDA, assim como estimulam a liberação dos neurotransmissores glutamato e aspartato (Quinn et al, 1997; Drachtman et al., 2002; Vezmar et al., 2003).

A adenosina é conhecida como neuromoduladora do sistema glutamatérgico, pois através da ativação de seus receptores A_1 existe a inibição da liberação de glutamato dos terminais pré-sinápticos e hiperpolarização dos terminais pós-sinápticos (Cunha et al, 2001-2005). Acreditamos que as convulsões induzidas pelo MTX foram prevenidas através destas propriedades da adenosina, mesmo que de maneira sutil. Entretanto, não foi observado nenhum efeito da adenosina sobre o parâmetro de morte.

Após verificarmos que as convulsões induzidas pelo MTX estavam diretamente relacionadas com o sistema glutamatérgico, realizamos ensaios de captação de glutamato no intuito de verificar como estava este parâmetro neuroquímico após as convulsões induzidas pelo MTX, e também quando havia a prevenção das mesmas. Alguns trabalhos vêm relatando que prejuízos na atividade dos transportadores de glutamato estão relacionados com a patogênese da convulsão (Ingram et al, 2001; Ueda et al, 2001; Maragakis e Rothstein, 2004). Nosso grupo já demonstrou que convulsões induzidas pelo ácido quinolínico, um agonista de NMDA, leva à diminuição da captação de glutamato tanto em ratos jovens como em adultos (Oliveira et al., 2004; Vinadé et al., 2005). Neste trabalho, constatamos a diminuição significativa de 20 % a 30 % da captação de glutamato em fatias de córtex dos camundongos convulsionados pela ação do MTX. Não foi observada nenhuma alteração na captação de glutamato nas fatias de hipocampo dos mesmos animais. Entretanto, quando os animais foram pré-tratados com MK-801, DNQX e adenosina e não manifestaram as convulsões, a diminuição da captação foi prevenida.

Ainda, quando a adenosina não prevenia as convulsões induzidas pelo MTX, a captação de glutamato apresentava uma leve diminuição, porém não significativa. A partir dos resultados obtidos sugerimos que a diminuição da captação de glutamato é provavelmente uma conseqüência das convulsões ao invés de uma ação direta do MTX sobre os transportadores de glutamato.

Em conjunto, os trabalhos desenvolvidos nesta Dissertação de Mestrado demonstram que realmente o sistema glutamatérgico está envolvido na neurotoxicidade do MTX. Até o presente momento pouco havia sido descrito sobre o envolvimento desse sistema neutransmissor com a toxicidade do MTX ao SNC. A partir dos resultados obtidos nos experimentos de captação de glutamato *in vitro*, verificamos que o MTX, ou algum produto de sua ação, interfere na remoção do glutamato da fenda sináptica, podendo levar à excitotoxicidade. Também, o aumento da proteína S100B auxilia no entendimento dos mecanismos de ação do MTX, pois além de confirmar resultados anteriores que mostraram que os astrócitos são afetados quando expostos ao MTX, sugere que estas células estão respondendo a um insulto na tentativa de neuroproteção. Além disso, a S100B, aliada a outros marcadores neuroquímicos e técnicas de diagnóstico por imagem, seria muito importante no monitoramento terapêutico, pois poderia detectar alterações celulares sutis e ajudaria a prevenir a neurotoxicidade pelo MTX.

Os resultados que obtivemos nos experimentos de convulsões induzidas pelo MTX, mais uma vez demonstraram participação do sistema glutamatérgico na neurotoxicidade desse fármaco. Mais importante, evidenciamos o envolvimento dos receptores ionotrópicos glutamatérgicos na patogênese das convulsões. Porém, neste modelo experimental, a

captação de glutamato diminuiu em decorrência das convulsões e não por uma ação direta, ou indireta, do MTX.

O entendimento dos mecanismos de ação é muito importante para a clínica médica, pois permite que novas ferramentas sejam criadas no intuito de prevenir os danos tóxicos induzidos por fármacos. Assim, mais estudos devem ser realizados para tentar desvendar os mecanismos de neurotoxicidade do MTX, como também para descobrir potenciais marcadores bioquímicos que auxiliem no monitoramento terapêutico.

Perspectivas

O desenvolvimento deste trabalho proporcionou que surgissem novas perpectivas para futuros estudos. Algumas de nossas perspectivas são:

- Dosar as concentrações de glutamato e homocisteína em amostras de líquor de ratos intoxicados com MTX.
- Verificar o perfil de atividade das enzimas ecto-nucleotidásicas em amostras de líquor e sangue de ratos intoxicados pelo MTX.
- Verificar as concentrações de S100B, e de outros marcadores bioquímicos, como a enolase específica de neurônio e de lactato, no líquor de ratos em diferentes tempos durante a intoxicação pelo MTX.
- Desenvolver modelos de toxicidade em cultura de astrócitos.
- Avaliar se guanosina exerce um papel protetor contra a neurotoxicidade do MTX.

Referências Bibliográficas

Ahlemeyer B, Beier H, Semkova I, Schaper C, Krieglstein J. S-100beta protects cultured neurons against glutamate-and staurosporine-induced damage and is involved in the antiapoptotic action of the 5 HT(1A)-receptor agonist, Bay x 3702. Brain Res; 858: 121–128, 2000.

Atra A, Pinkerton CR, Bouffet E, Norton A, Hobson R, Imeson JD, Gerrard M. Acute neurotoxicity in children with advanced stage B-non-Hodgkin's lymphoma and B-acute lymphoblastic leukaemia treated with the United Kingdom children cancer study group 9002/9003 protocols. Eur J Cancer; 40: 1346-1350, 2004.

Bernini JC, Fort DW, Griener JC, Kane BJ, Chappell WB, Kamen BA. Aminophylline for methotrexate-induced neurotoxicity. Lancet; 345: 544-547, 1995.

Bruce-Gregorios JH, Agarwal RP, Oracion A, Ramirez A, Lin L. Effects of methotrexate on RNA and purine syntesis of astrocytes in primary culture. J Neuropathol Exp Neurol; 50: 770-778, 1991. a.

Bruce-Gregorios, D.M. Soucy, M.G. Chen, M.D. Norenberg, Effect of methotrexate on glial fibrillary acid protein content of astrocytes in primary culture, J Neuropathol Exp Neurol; 50: 118-125, 1991. b.

Cai S, Horne DW. Transport of 5-formyltetrahydrofolate into primary cultured rat astrocytes. Arch Biochem Biophys; 410: 161-166, 2003.

Calabresi P, Chabner BA. Antineoplastic agents. In: Hardman JG, Limbird LE, Gilman GA, editors. Goodman & Gilman's the pharmacological basis of therapeutics. 10 ed. McGraw-Hill. North America. 2002. p. 1399-1404.

Chan ESL, Cronstein BN. Molecular action of methotrexate in inflammatory diseases. Arthritis Res; 4: 266-273, 2002.

Chen Y, Swanson, RA. Astrocytes and brain injury. J Cereb Blood Flow Metab; 23: 137-149, 2003.

Choi DW. Glutamate neurotoxicity and diseases of the nervous system. Neuron; 1: 623-634, 1988.

Correa DD, DeAngelis LM, Shi W, Thaler H, Glass A, Abrey LE. Cognitive functions in survivors of primary central nervous system lymphoma. Neurology; 62: 548-555, 2004.

Cunha RA. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem Int; 38: 107-125, 2001.

Cunha RA. Neuroprotection by adenosine in the brain: From A1 receptor activation to A2A receptor blockade. Purinergic Signal: 1: 111-134, 2005.

Danbolt NC. Glutamate uptake. Prog. Neurobiol: 65: 1-105, 2001.

Donato R. S100: a multigenic family of calcium-modulated proteins of the Ef-hard type with intracellular and extracellular functional roles. Int J Biochem Cell Biol; 33: 637-668, 2001.

Drachtman RA, Cole PD, Golden CB, James SJ, Melnyk S, Aisner J, Kamen BA. Dextromethorphan is effective in treatment of subacute methotrexate neurotoxicity. Pediatr Hematol and Oncol; 19: 319-327, 2002.

Frizzo JK, Tramontina F, Bortoli E, Gottfried C, Leal RB, Lengyel I, Donato R, Dunkley PR, Goncalves CA. S100B-mediated inhibition of the phosphorylation of GFAP is prevented by TRTK-12. Neurochem Res; 29: 735-740, 2004. a.

Frizzo JK, Tramontina AC, Tramontina F, Gottfried C, Leal RB, Donato R, Goncalves CA. Involvement of the S100B in cAMP-induced cytoskeleton remodeling in astrocytes: a study using TRTK-12 in digitonin-permeabilized cells. Cell Mol Neurobiol; 24: 833-840, 2004. b.

Gregorios, A.B. Gregorios, J. Mora, A. Marcillo, R.M. Fojaco, B. Gren, Morfologic alterations in rat brain following systemic & intraventricular methotrexate injection: light and electron microscopic studies, J Neuropathol Exp Neurol; 48: 33- 47, 1989.

Herrmann M, Curio N, Jost S, Wunderlich MT, Synowitz H, Wallesch CW. Protein S-100B and neuron specific enolase as early neurobiochemical markers of the severity of traumatic brain injury. Restor Neurol Neurosci; 14: 109–114, 1999.

Igarashy H, Inomata K, Tateno A. The Effect of Methotrexate on Development of Synapses in the Neonatal Rat hippocampus. Neuropediatrics; 20: 196-198, 1989.

Ingram EM, Wiseman, JW, Shoshi, T, Emson, PC. Reduction of glial glutamate transporters in the parietal cortex and hippocampus of EL mouse. J. Neurochem; 79: 564–575, 2001.

Izquierdo I, Medina JH. Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiol. Learn. Mem; 68: 285- 316, 1997.

Kishi S, Griener J, Cheng C, Das S, Cook EH, Pei D, Hudson M, Rubnitz J, Sandlund JT, Pui CH, Relling MV. Homocysteine, pharmacogenetics, and neurotoxicity in children with leukemia. J Clin Oncol; 21: 3084-3091, 2003.

Kleine TO, Benes L, Zöfel P. Studies of the brain specificity of S100B and neuron-specific enolase (NSE) in blood serum of acute care patients. Brain Res Bull; 61: 265-279, 2003.

Kuker W, Bader P, Herrlinger U, Heckl S, Nagele T. Transient encephalopathy after intrathecal methotrexate chemotherapy: diffusion-weighted MRI. J Neurooncol; 73: 47-49, 2005.

Langer T, Martus P, Ottensmeier H, Hertzberg H, Beck JD, Meier W. CNS late-effects after ALL therapy in childhood. Part III: neuropsychological performance in long-term survivors of childhood ALL: impairments of concentration, attention, and memory. Medical Pediatric Oncology; 38: 320-328, 2002.

Lara DR, Gama CS, Belmonte-de-Abreu P, Portela LV, Goncalves CA, Fonseca M, Hauck S, Souza DO. Increased serum S100B protein in schizophrenia: a study in medication-free patients. J Psychiatr Res; 35: 11-14. 2001.

Lesnik PG, Ciesielski KT, Hart BL, Benzel EC, Sanders JA. Evidence for cerebellar-frontal subsystem changes in children treated with intrathecal chemotherapy for leukemia. Arch Neurol; 55:1561-1568, 1998.

Linnebank M, Pels H, Kleczar N, Farmand S, Fliessbach K, Urbach H, Orlopp K, Klockgether T, Schmidt-Wolf IG, Schlegel U. MTX-induced white matter changes are associated with polymorphisms of methionine metabolism. Neurology 64: 912-913, 2005.

Lo Nigro L, Di Cataldo A, Schiliro G. Acute neurotoxicity in Children with b-lineage acute lymphoblastic leukemia (B-ALL) treated with intermediate risk protocols. Med. Pediatr. Oncol; 35: 449-455, 2000.

Madden T, Eaton V.E. Methotrexate. In: Schumacher EG, editor. Therapeutic Drug Monitoring. Norwalk: Appleton Lage; 1995.p. 527-553.

Madhyastha S, Somayaji SN, Rao MS, Nalini K, Bairy KL. Hippocampal brain amines in methotrexate-induced learning and memory deficit. Can. J. of Physiol. and Pharmacol; 80: 1076-1084, 2002.

Mahoney DH Jr, Shuster JJ, Nitschke R, Lauer SJ, Steuber CP, Winick N, Camitta B. Acute neurotoxicity in children with B-precursor acute lymphoid leukemia: an association with intermediate-dose intravenous methotrexate and intrathecal triple therapy- a Pediatric Oncology Group study. J Clin Oncol; 16: 1712-1722, 1998.

Maragakis, NJ, Rothstein JD. Glutamate transporters in neurologic disease. Arch. Neurol; 58: 365–370, 2001.

Maragakis NJ, Rothstein JD. Glutamate transporters: animal models to neurologic disease. Neurobiol Dis; 15: 461- 473, 2004.

Mattson MP, Chan SL, Duan W. Modification of brain aging and neurodegenerative disorders by genes, diet, and behavior. Phys. Rev; 82: 637–672, 2002

McGuire JJ. Anticancer antifolates: current status and future directions. Curr Pharm Des; 9: 2593- 2613, 2003.

Meldrum BS. The role of glutamate in epilepsy and other CNS disorders. Neurology; 44: S14– S23, 1994.

Meldrum BS. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J. Nutr; 130: 1007S– 1015S, 2000.

Millot F, Dhondt JL, Mazingue F, Mechinaud F, Ingrand P, Guilhot F. Changes of cerebral biopterin and biogenic amine metabolism in leukemic children receiving 5g/m2 intravenous methotrexate. Pediatr. Res; 37:151-154, 1995.

Mullenix PJ, Kernan WJ, Schunior A, Howes A, Waber DP, Sallan SE, Tarbell NJ. Interactions of steroid, methotrexate, and radiation determine neurotoxicity in an animal model to study therapy for childhood leukemia. Pediatr. Res; 35: 171-178, 1994.

Oliveira DL, Horn JF, Rodrigues JM, Frizzo MES, Moriguchi E, Souza DO, Wofchuk S. Quinolic acid promotes seizures and decreases glutamate uptake in young rats: Reversal by orally administered guanosine. Brain Res; 1018: 48-54, 2004.

Omuro AM, DeAngelis LM, Yahalom J, Abrey LE. Chemoradiotherapy for primary CNS lymphoma: an intent-to-treat analysis with complete follow-up.Neurology; 64: 69-74, 2005.

Ozawa S, Kamiya H, Tsuzuki K. Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol; 54: 581– 618, 1998.

Peckham VC, Meadows AT, Bartel N, Marrero O. Educational late effects in long-term survivors of childhood acute lynphocytic leukemia. Pediatrics; 81: 127-133, 1988.

Portela LV, Brenol JCT, Walz R, Bianchin M, Tort ABL, Canabarro UP, Beheregaray S, Marasca JA, Xavier RM, Neto EC, Gonçalves CA, Souza DO. Serum S100B levels in patients with lupus erythematosus: preliminary observation. Clinical and Diagnostic Laboratory Immunology; 9: 164-66, 2002.

Quinn CT, Griener JC, Bottiglieri T, Hyland K, Farrow A, Karmen BA. Elevation of homocysteine and excitatory amino acid neurotransmitters in CSF of children who receive methotrexate for the treatment of cancer. J Clin Oncol; 15: 2800- 2806, 1997.

Renard D, Westhovens R, Vandenbussche E, Vandenberghe R. Reversible posterior leucoencephalopathy during oral treatment with methotrexate. J Neurol; 251: 226-228, 2004.

Riva D, Giorgi C, Nichelli F, Bulghroni S, Massimino M, Cefalo G, Gandola L, Giannota M, Bagnasco I, Saletti V, Pantaleoni C. Intrathecal methotrexate affects cognitive function in children with medulloblastoma. Neurology; 59: 48-53, 2002.

Ruiz-Irastorza G, Khamashta MA, Castellino G, Hughes GR. Systemic lupus erythematosus. Lancet; 357: 1027-1032, 2001.

Sánchez-Carpintero R, Narbona J, López de Mesa R, Arbizu J, Sierrasesúmaga L. Transient posterior encephalopathy induced by chemotherapy in children. Pediatr Neurol; 24: 145-148, 2001.

Schroder O, Stein J. Low dose methotrexate in inflammatory bowel disease: current status and future directions. Am J Gastroenterol; 98: 530-537, 2003.

Segovia G, Porras A, Del Arco A, Mora F. Glutamatergic neurotransmission in aging: a critical perspective. Mech. Ageing Dev; 122: 1- 29, 2001.

Smolenska Z, Kaznowska Z, Zarówny D, Simmonds HA, Smolenski RT. Effect of methotrexate on blood purine and pirimidine levels in patients with rheumatoid arthritis. Rheumatology; 38: 997-1002, 1999.

Spinella MJ, Brigle KE, Freemantle SJ, Sierra EE, Goldman ID. Comparison of methotrexate poliglutamylation in L1210 leukemia cells when influx is mediated by the reduced folate carrier or the folate receptor. Biochem Pharmacol; 52: 703-712, 1996.

Takeda M, Khamadang S, Narikawa S, Kimura H, Hosoyamada M, Ho Cha S, Sekire T, Endou H. Characterization of methotrexate transport and its drug interactions with human organic anion transporters. J Pharmacol Exp Ther; 302: 666-671, 2002.

Ueda Y, Doi T, Tokumaru J, Yokoyama H, Nakajima A, Mitsuyama Y, Ohya-Nishiguchi H, Kamada H, Willmore LJ. Collapse of extracellular glutamate regulation during epileptogenesis: down-regulation and functional failure of glutamate transporter function in rats with chronic seizures induced by kainic acid. J. Neurochem; 76: 892–900, 2001.

Van Ede AE, Laan RFJM, De Abreu RA, Stegeman ABJ, van de Putte LBA. Purine enzymes in patients with rheumatoid arthritis treated with methotrexate. Ann Rheum Dis; 61: 1060-1064, 2002.

Van Eldik LJ, Wainwright MS. The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain, Restor Neurol Neurosci; 21: 97-108, 2003.

Van Gool SW, De Meyer G, Van de Voorde A, Vanmechelen E, Vanderstichele H. Neurotoxicity marker profiles in the CSF are not age-dependent but show variation

in children treated for acute lymphoblastic leukaemia. Neurotoxicology; 25: 471-480, 2004.

Vezmar S, Becker A, Bode U, Jaehde U. Biochemical and clinical aspects of methotrexate neurotoxicity. Chemotherapy; 49: 92-104, 2003.

Vinade ER, Schmidt AP, Frizzo ME, Portela LV, Soares FA, Schwalm FD, Elisabetsky E, Izquierdo I, Souza DO. Effects of chronic administered guanosine on behavioral parameters and brain glutamate uptake in rats. J Neurosci Res; 79: 248-253, 2005.

Waber DP, Tarbell NJ, Fairclough, Atmore K, Castro R, Isquith P, Lussier F, Romer I, Carpenter PJ, Schiller M, Sallan SE. Cognitive sequelae of treatment in childhood acute lymphoblastic leukemia: cranial radiation requires accomplice. J Clin Oncol; 13: 2490-2496, 1995.

Walz R, Portela LV, Tort AB, Neto EC, Fernandes LN, Goncalves CA, Souza DO. Serum S100B levels in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis. Neurology; 54: 2021-2022, 2000.

Zeng H, Chen ZS, Belinsky MG, Rea PA, Kruh GD.Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. Cancer Res; 61: 7225-7232, 2001.

Ziegler DR, Innocente CE, Leal RB, Rodnight R, Goncalves CA. The S100B protein inhibits phosphorylation of GFAP and vimentin in a cytoskeletal fraction from immature rat hippocampus. Neurochem Res; 23: 1259-1263, 1998.

Zimmer DB, Cornwall EH, Landar A, Song W. The S100 protein family: history, function and expression. Brain Res Bull; 37: 417-429, 1995.

Anexos

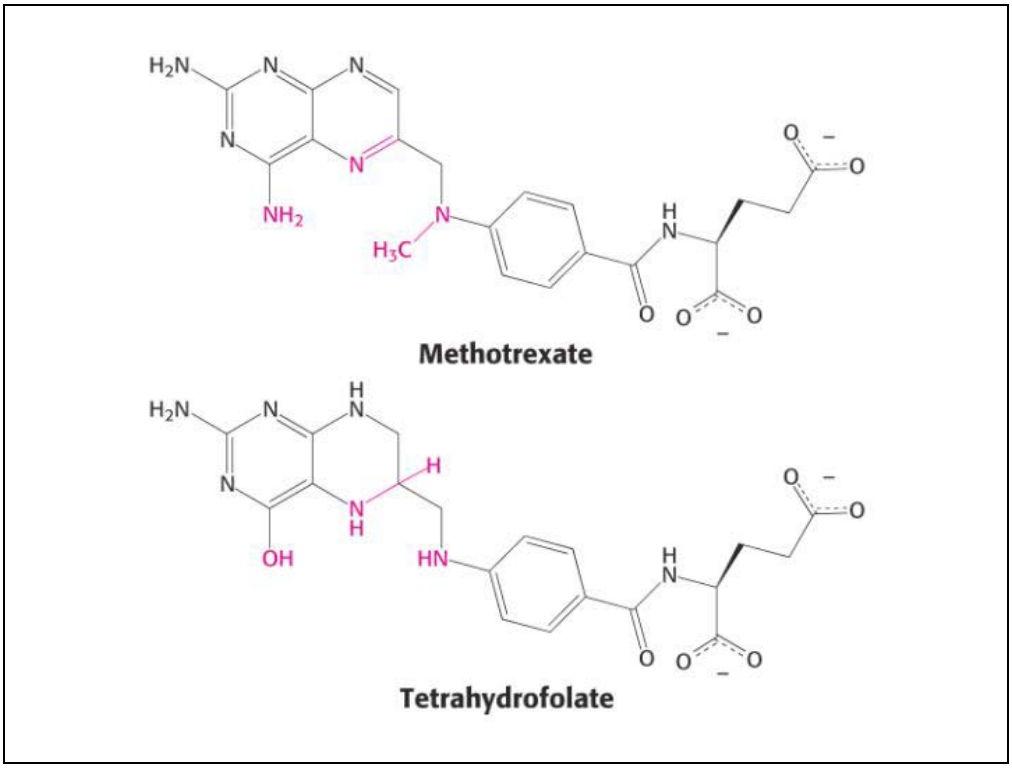

Figura 1: Estrutura molecular do tetrahidrofolato e do metotrexato.

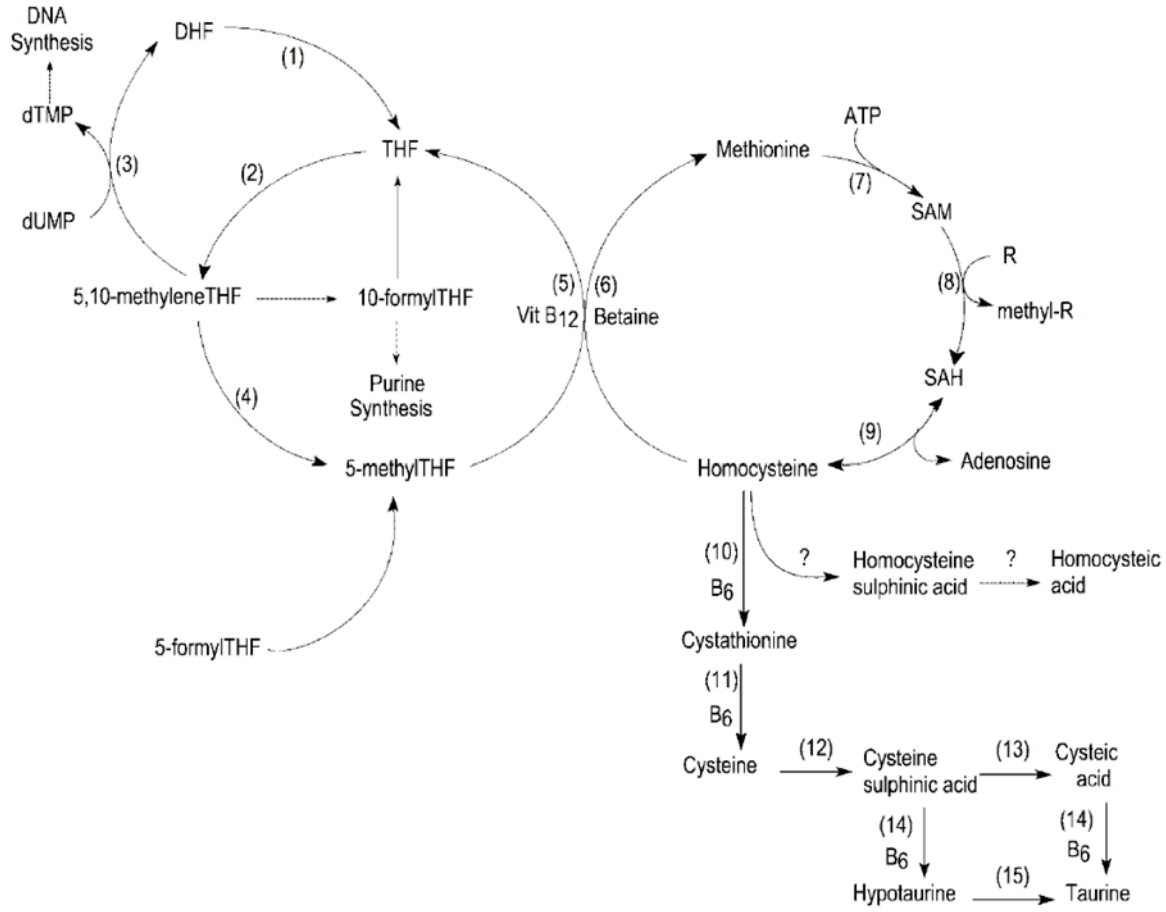

Fig. 2: Metabolismo dos folatos e homosisteína. ATP = Adenosina trifosfato; DHF= dihidrofolato; SAH =S-adenosilhomocisteina; SAM = S-adenosilmetionina; THF = tetrahidrofolate; dTMP = timidina monofosfato; dUMP = uridina monofosfato; 1 = dihidrofolate redutase; 2 = serina-hidroximetiltransferase; 3 = timidilato sintase; 4 = 5,10-metilenotetrahidrofolate redutase; 5 = metionina sintase; 6 = betaina- homocisteina metiltransferase; 7 = metionina adenosiltransferase; 8 = R-metiltransferase; 9 = SAH hidrolase; 10 = cistationina- ß-sintase; 11 = cistationase; 12 = cisteina dioxigenase; 13 = cisteina sulfídrica acida dehidrogenase; 14 = cisteina sulfídrica acida decarboxilase; 15 = hipotaurina desidrogenase. Figura obtida de Vezmat et al, 2003.