Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

ANA ELISA BÖHMER

Efeitos adversos do tratamento crônico com ciclosporina em ratos não transplantados.

Porto Alegre, maio de 2010

Ana Elisa Böhmer

Efeitos adversos do tratamento crônico com ciclosporina em ratos não transplantados.

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas – Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do título de doutor em Ciências Biológicas – Bioquímica.

Orientador: Prof. Dr. Diogo Onofre Souza

Porto Alegre, maio de 2010.

“ Toda escolha vem acompanhada de renúncias.”

Diogo e Marcelo, 2009.

AGRADECIMENTOS

Aos meus queridos mestres Diogo e Roska. Agradeço pela orientação e confiança depositada em mim. Mas principalmente por todos aqueles momentos em que rimos e choramos juntos... seja com um charuto cubano e rum 18 anos, ou uma(s) cerveja em tantas outras ocasiões. Obrigada... Mais do que mestres, vocês são amigos que levo pra toda a vida. Que a saudade nos aproxime, sempre. Amo vocês.

A Débora. Obrigada por todo o empenho e dedicação. Esta tese também é fruto do teu esforço. Além de agradecer, quero te parabenizar pela pessoa que és. Exemplo de determinação e luta! Desejo que a tua trajetória no mestrado seja repleta de boas conquistas! Estaremos juntas!

Ao Marcelo Ganzella, uma das mais maravilhosas surpresas que o doutorado me trouxe. Um grande e amado amigo! Obrigada pelo carinho e companheirismo.

A Gisele, que chegou de mansinho e conquistou seu espaço! E a Júlia, exemplo de trabalho e competência. Obrigada pela ajuda nos experimentos, pelas idéias, incentivos e disposição. E principalmente pela convivência e parceria.

A Denise, que está se tornando mestre em HPLC, obrigada pela grande ajuda no final desta tese.

Á Luisa, que esteve presente na maioria dos “mega-experimentos”, sempre de forma descontraída e bem humorada. Obrigada pela ajuda. Foi um prazer conviver contigo!

Ao João Américo, Letícia Rodrigues e Cléber pela fundamental participação na aquisição e análise de imagens por microscopia.

Ao André Mendes Ribeiro Corrêa (Dedé), agradeço pela paciência e disponibilidade de discutir e participar dos experimentos desta tese. Sua contribuição foi de fundamental importância para o andamento dos estudos.

Á Prof. Matilde Achaval, por compartilhar sua reconhecida experiência com astrócitos. Obrigada pela paciência e pela convivência. Foi um prazer te conhecer!

Ao André Schmidt, meu principal estímulo na busca da metodologia de quantificação de purinas no HPLC. Agradeço pela amizade e por ser um exemplo de pesquisador e médico.

Ao Victor, Alexandre, Roberto, Rafael, Christine, Fernanda, Jean, Clarissa, Catiele, Giana, Dietrich, Lucas, Mazzini, Felipe, Marcelo Costa, Vanessa, Dioguinho, Jussânia, Zimmer, Bina, Paulo, Giordano, Letícia e todos os amigos e colegas com quem tive o grande prazer de conviver.

Á Universidade Federal do Rio Grande do Sul, aos professores e funcionários do Departamento de Bioquímica, especialmente à Cléia.

Ao CNPq por ter financiado a minha bolsa de iniciação científica, mestrado e doutorado.

À minha família, pela formação dada até minha juventude que me proporcionou a continuidade nos estudos até a chegada a este doutorado. Agradeço por estar sempre presente e pelo estímulo para continuar.

Ao Lucas, por me fazer tão feliz...

APRESENTAÇÃO

Esta tese está organizada em 4 partes, cada uma sendo constituída dos seguintes ítens:

Parte I: Resumo, Introdução, Objetivos Geral e Específico;

Parte II: Resultados que estão apresentados na forma de Artigos Científicos. Cada artigo científico representa um Capítulo e são subdivididos em: Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas;

Parte III: Discussão, Conclusão, Perspectivas e Referências Bibliográficas citadas na Introdução da Parte I e Discussão da Parte III;

Parte IV: Anexos.

SUMÁRIO

PARTE I

RESUMO

A ciclosporina (CsA) é um agente imunossupressor freqüentemente utilizado na clínica para a prevenção da rejeição ao transplante e no tratamento de doenças auto-imunes. Apesar da ação no sistema imune, o tratamento com CsA apresenta efeitos adversos graves. Múltiplos fatores de confusão na prática clínica, incluindo fatores de risco prévios, diferenças genéticas e de resposta imune dos receptores do órgão dificultam a determinação dos efeitos adversos especificamente relacionados ao tratamento com CsA. A busca por protocolos de imunossupressão eficazes, que não afetem a qualidade de vida dos pacientes conduz a investigação do envolvimento da CsA em doenças vasculares. A doença vascular é uma das principais causas de morbidade e mortalidade entre pacientes transplantados e o tratamento com CsA parece estar envolvido neste evento. O objetivo desta tese foi investigar parâmetros bioquímicos sistêmicos envolvidos em distúrbios vasculares induzidos pelo tratamento crônico com CsA em ratos não-transplantados. Primeiramente, demonstramos que o nosso protocolo de administração de CsA foi capaz de induzir imunossupressão, com diminuição dos níveis séricos de citocinas que medeiam a resposta imunológica (IL-1α, IL-1β e IL-2), sem causar nefro e hepatotoxicidade. Além disso, verificamos um perfil bioquímico coerente com intolerância à glicose, incluindo a diminuição dos níveis séricos de insulina em jejum e alteração do teste de tolerância à glicose, sugerindo o desenvolvimento de um perfil de diabetes em ratos tratados com CsA 15 mg/kg. O tratamento com CsA também aumentou os níveis de homocisteína total (tHcy), um fator de risco para doenças cardiovasculares. Os níveis de fibrinogênio no plasma, o número de plaquetas, os níveis séricos de triglicérides, colesterol e VLDL também aumentaram com o tratamento de CsA 15 mg/kg. Além disso, demonstramos que a atividade ecto-nucleotidásica sobre nucleotídeos de adenina em soro foi inibida pelo tratamento com CsA. Ratos tratados com CsA apresentaram uma diminuição na hidrólise de ATP, ADP e AMP em soro, além de aumento dos níveis plasmáticos de ADP e redução dos níveis de adenosina (ADO). Enquanto os níveis de tHcy correlacionaram-se negativamente com a inibição da hidrólise de nucleotídeos e os níveis plasmáticos de ADO, esta correlação foi positiva para os níveis plasmáticos de ADP. Isto sugere que a inibição da atividade ecto-nucleotidásica pode estar relacionada ao aumento dos níveis séricos de tHcy. Além disso, a análise morfológica de ratos tratados com CsA 15 mg/kg apresentou lesão celular e resposta inflamatória envolvendo o endotélio e a camada íntima da artéria aorta. Em conclusão, as evidências obtidas nesta tese podem ser relevantes para futuros estudos que visam investigar os mecanismos de ação da CsA sobre os distúrbios vasculares que acompanham seu uso. Além disso, contribuímos para a elucidação de um método de quantificação das purinas derivadas da adenina e guanina em plasma e líquor por HPLC. Esta metodologia tem sido amplamente utilizada em amostras de humanos, ratos e camundongos, contribuindo para o esclarecimento dos mecanismos de ação das purinas.

ABSTRACT

Cyclosporine (CsA) is an immunosuppressive agent frequently used in the clinic for prevention of allograft rejection and for the treatment of autoimmune diseases. Despite its desired action on the immune system, CsA treatment may present serious adverse effects. It is difficult to distinguish, in the clinical setting, a direct effect of CsA treatment from other confounding variables, such as allograft rejection and effects due to other drug therapies. The search for effective immunosuppression protocols which does not affect the quality of life of patients is driving research to investigate the CsA involvement in vascular diseases. Vascular disease is a major cause of morbidity and mortality among transplanted recipients and CsA treatment has been consistently implicated in this event. Here, we investigate systemic biochemical parameters involved in vascular disturbances induced by chronic CsA treatment in non-transplanted rats. Firstly, we demonstrate that our CsA immunosuppressive protocol was able to induce immunosuppression by decreasing the serum levels of cytokines that mediate immunological responses (IL-1α, IL-1β and IL-2) without causing nephro and hepatotoxicity. Moreover, we demonstrate biochemical profile coherent with glucose intolerance, including decreased fasting serum insulin levels and altered glucose tolerance test, suggesting the development of a framework straight to diabetes on CsA 15 mg/kg treated group. CsA treatment also increased serum total homocysteine (tHcy) concentration, an important and independent risk factor for cardiovascular diseases. Plasma fibrinogen levels, platelet number, serum triglycerides, cholesterol and VLDL were also increased by CsA 15 mg/kg treatment. Also, we showed that ecto-nucleotidase activities on adenine nucleotides in serum were inhibited by CsA treatment. CsA treated rats showed a decrease in serum ATP, ADP and AMP hydrolyzes, and increased ADP and decreased ADO plasma levels. THcy levels correlated negatively with the inhibition of nucleotides hydrolyzes and ADO plasma levels, and positively with ADP plasma levels. These correlations suggest that the inhibition of serum ecto-nucleotidase activities could be related to the increase of serum tHcy levels. In addition, morphological evaluation of CsA 15 mg/kg treated rats exhibited cell injury and inflammatory response involving the endothelium and the intimal layer of aorta artery. In conclusion, the evidences obtained in this thesis may be relevant for future studies that aim to investigate the mechanisms of action of CsA on vascular disturbances that accompany patients under immunosuppressive therapy with CsA. In addition, we contribute to the elucidation of a method for quantification of adenine- and guanine-based purines in plasma and cerebrospinal fluid by HLPC. This methodology has been widely used in human, mice and rats samples, contributing to the elucidation of the mechanisms of action of purines.

LISTA DE ABREVIATURAS

ADP – adenosina 5’-difosfato

AMP – adenosina 5’-monofosfato

ATP – adenosina 5’-trifosfato

CD39 – antígeno de ativação celular linfóide

CsA –ciclosporina

GMP - guanosina monofosfato

GUO - guanosina

Hcy – homocisteína

HPLC - Cromatografia líquida de alta performance

IFN-γ - interferon-gama

IL – interleucina

MTHF – 5-metiltetraidrofolato

NFAT - fator nuclear de ativação de células T

NTPDase – ecto-nucleotídeo trifosfato difosfoidrolase

SAH – S-adenosilhomocisteína

SAM – S-adenosilmetionina

SNC - Sistema nervoso central

TGF - fator transformante de crescimento

tHcy –homocisteína total

1. Introdução

1.1. Ciclosporina

A ciclosporina (CsA) é um medicamento imunossupressor amplamente utilizado por pacientes transplantados e por portadores de doenças auto-imunes. Sua introdução na terapêutica, em 1979, revolucionou o tratamento de rejeição de órgãos e hoje é um dos principais componentes dos protocolos de imunossupressão na linha terapêutica (Serkova et al., 2004).

A CsA é um polipeptídeo cíclico de 11 aminoácidos, extraído do fungo *Tolypocladium inflatum gams*, sua estrutura química é altamente hidrofóbica por ser constituído de aminoácidos apolares e metilados. No sangue, a CsA liga-se eritrócitos e a lipoproteínas ricas em colesterol, sendo metabolizada pelo sistema enzimático citocromo P-450 no fígado. Seu mecanismo de ação ocorre através da modulação da função das células T (Fruman et al., 1992). Ao entrar na célula, a CsA liga-se ao seu receptor ciclofilina formando um complexo que bloqueia a ativação da calcineurina, uma enzima citosólica heterodimérica composta pelas subunidades A e B. O complexo interage com a subunidade B e com uma região do domínio catalítico da subunidade A, bloqueando sua atividade fosfatásica e inibindo uma via de sinalização Ca^{+2} dependente (Avramut and Achim, 2003; Serkova et al., 2004). Ao impedir a atividade fosfatásica da calcineurina, a CsA inibe a translocação do fator nuclear de ativação de células T (NFAT) do citoplasma para o núcleo de células T ativadas. O grupo NFAT está envolvido na ativação da transcrição dos genes que codificam interleucinas (IL) -2, IL-4, CD40L e interferon-gama (IFN-γ). Assim, a inibição da NFAT modulada pela CsA resulta em uma inibição específica de produção de interleucinas nas células T (Schreiber and Crabtree, 1992).

Sabe-se que mais de 200 genes são ativados e inativados em linfócitos T e uma grande parte do genoma pode se tornar transcricionalmente ativo quando estas células passam de um estado inativo para o estado imunologicamente ativo. A regulação da função imune é exercida através da secreção de citocinas e da expressão de moléculas de superfície celular que medeiam contato intercelular. As moléculas que ativam linfócitos B são produzidas quase exclusivamente por células T no estado ativado, e entre elas estão as interleucinas (IL) -2, -4, -5 e -6 e o fator transformante de crescimento (TGF) -β (Ho et al, 1996). A CsA inibe a transcrição dos genes envolvidos na produção destas citocinas resultando em uma diminuição da resposta imunológica (Serkova et al., 2004; Zhang et al., 2005).

1.1.1. Efeitos adversos do tratamento imunossupressor com CsA

Apesar de ser o medicamento de primeira escolha na linha terapêutica, o uso da CsA no tratamento imunossupressor é acompanhado por diversos efeitos adversos. Entre eles, os mais estudados são a nefrotoxicidade e hepatotoxicidade (Grub et al., 2000a, b; Vercauteren et al., 1998), distúrbios da homeostasia da glicose (Marchetti, 2004), hiperlipidemia pós-transplante (Markell et al., 1994; Subramanian and Trence, 2007) e neurotoxicidade (Klawitter et al., 2010; Klawitter et al.; Serkova et al., 2004; Sklar, 2006). Entre 10 e 60% dos pacientes sob tratamento crônico com CsA sofrem efeitos neurotóxicos relacionados à administração do imunossupressor, mesmo em doses terapêuticas (Bechstein, 2000; Serkova et al., 2004). Sintomas mais leves incluem dor de cabeça, insônia, tremores, ansiedade e amnésia. Entre os sintomas mais graves se incluem convulsões, alucinações, cegueira cortical, síndromes cerebelar e extrapiramidal (Sklar, 2006). Apesar do mecanismo de ação imunossupressor estar bem estabelecido, muitos aspectos bioquímicos relacionados aos efeitos da CsA no cérebro

permanecem desconhecidos (Klawitter et al., 2010; Klawitter et al.; Serkova et al., 2004). Sabe-se, no entanto, que o transporte de CsA através da barreira hemato-encefálica é limitada pela glicoproteína-P e também pelas *tight junctions* das células endoteliais dos capilares cerebrais (Dohgu et al., 2000; Dohgu et al., 2004; Kochi et al., 1999; Takata et al., 2007).

Estudos clínicos com tomografia computadorizada e imagens de ressonância magnética demonstraram uma correlação entre os sintomas clínicos da neurotoxicidade CsA-dependente e alterações morfológicas no cérebro, como hipodensidade de substância branca, edema cerebral, encefalopatia metabólica e danos relacionados a hipóxia (Bartynski et al., 1997; Deuse et al., 2008; Drachman et al., 1996; Jansen et al., 1996; Sheth et al., 1999).

Além disso, há evidências de que o uso prolongado de CsA esteja associado a doenças vasculares obstrutivas progressivas, importante causa de morbidade e mortalidade no período pós-transplante (Demirag et al., 1998; Serkova et al., 2004; Tellides and Pober, 2007). Entretanto, a presença do órgão transplantado e a co-administração de outros medicamentos dificultam o entendimento dos efeitos primários da CsA no sistema vascular. Além disso, múltiplos fatores de confusão na prática clínica, incluindo fatores de risco prévios, diferenças genéticas e de resposta imune dos receptores do órgão dificultam a determinação dos efeitos adversos especificamente relacionados ao tratamento com CsA.

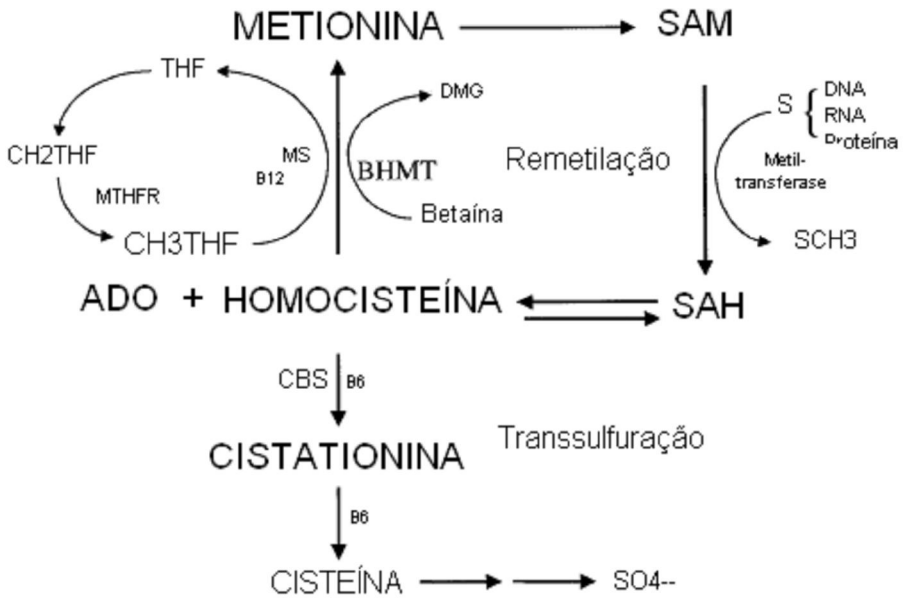
1.2 Homocisteína

Estudos demonstram que a administração de CsA está associada ao aumento das concentrações de homocisteína total em pacientes transplantados (Arnadottir et al., 1996; Cole et al., 1998; Herrero et al., 2000; Nouri-Majalan et al., 2009). A

homocisteína (Hcy) é um aminoácido não-essencial formado a partir da desmetilação de metionina, um aminoácido especialmente abundante em carnes e laticínios. A Hcy está presente no sangue sob a forma oxidada (homocistina e mistura de dissulfetos) e reduzida (tiol livre). Por isso, o termo homocisteína total (tHcy) tem sido usado para definir o somatório de Hcy reduzida e oxidada presentes no organismo (Welch and Loscalzo, 1998).

O metabolismo da Hcy implica em duas vias metabólicas intracelulares: remetilação e transsulfuração, como ilustrado na figura 1.

Figura 1:



Adaptado de Jacobsen, DW. Clinical Chemistry, 1998;44(8):1833-1843.

Metabolismo da homocisteína. Remetilação: a homocisteína é remetilada até metionina pela ação do metilenotetrahidrofolato redutase (MTHFR) e metionina sintase (MS), dependente de vitamina B12. A homocisteína também pode ser remetilada até metionina pela ação da betaína-homocisteína-metiltransferase (BHMT) no fígado e rins. A metionina é convertida em S-adenosilmetionina (SAM), que serve como substrato doador de metila para metiltransferases. Outro produto desta reação é a S-adenosilhomocisteína (SAH), que ao ser hidrolisado pela SAH-hidrolase produz homocisteína e adenosina (ADO). Transsulfuração: a primeira enzima deste processo é a cistationina beta-sintase (CBS), dependente de vitamina B6. Cistationina é convertida a cisteína, que por sua vez é catabolizada até sulfato inorgânico e excretada na urina.

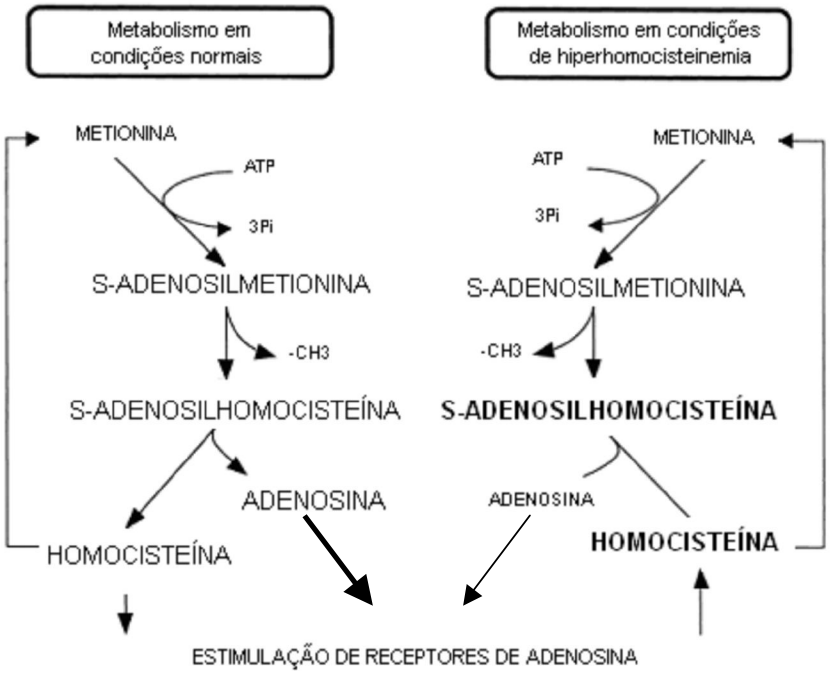
A Hcy intracelular é transportada para o sangue através de um mecanismo de saturação, que ajuda a manter o nível intracelular de Hcy baixo. Quando o metabolismo da Hcy está alterado, o mecanismo de exportação limita a toxicidade intracelular, porém, expõe o tecido vascular a altas concentrações de Hcy, ocasionando a chamada hiperhomocisteinemia (Christensen et al., 1991; Ueland, 1995).

Em 1969, McCully (McCully, 1969) descreveu os problemas vasculares periféricos em pacientes com homocistinúria, revelando a importância dos altos níveis de tHcy no desenvolvimento da aterosclerose e do tromboembolismo. Desde então, observou-se que a concentração de tHcy é consistentemente mais elevada em pacientes com problemas vasculares periféricos, coronarianos e cerebrais quando comparado a indivíduos saudáveis (Welch and Loscalzo, 1998). Outros estudos comprovaram que o aumento de tHcy é um fator de risco independente para aterosclerose, doenças vasculares periféricas, infarto do miocárdio e tromboembolismo (Antoniades et al., 2009; Boushey et al., 1995; Eikelboom et al., 1999; Herrero et al., 2000; Malinow, 1996; Mayer et al., 1996; Potter et al., 2008). A tHcy parece alterar as propriedades anticoagulantes das células endoteliais para um fenótipo procoagulante e, além disso, altera a morfologia vascular, estimula a inflamação, danifica o endotélio e estimula as vias de coagulação (Stanger et al., 2004; Upchurch et al., 1997).

O aumento da tHcy está relacionado a diversos efeitos aterotrombogênicos. A diminuição da disponibilidade de óxido nítrico (vasodilatador endógeno), como conseqüência do aumento do estresse oxidativo causado pela Hcy, é sugerida como uma possível causa de disfunções endoteliais (Faraci, 2003). Loscalzo, 1996 propôs que o dano endotelial causado pela Hcy seja mediado pelo peróxido de hidrogênio, que expõe a matriz do vaso e as células musculares lisas, fazendo-as proliferarem e promoverem a ativação plaquetária e leucocitária (Loscalzo, 1996). Além disso, a produção de

superóxido poderia ativar a peroxidação lipídica, da membrana do endotélio vascular e das lipoproteínas, propiciando condições favoráveis para a formação de placas ateromatosas (Huang et al., 2001). Riksen et al. também propõe que a diminuição de adenosina seria um dos mecanismos envolvidos no desenvolvimento das doenças cardiovasculares causado por elevados níveis de tHcy (como ilustrado na figura 2) (Riksen et al., 2005a; Riksen et al., 2003; Riksen et al., 2005b).

Figura 2:



Adaptado de Riksen et al. Cardiovascular Research, 2003;59:271-276.

Em condições normais, a adenosina é formada a partir da hidrólise da S-adenosilhomocisteína (SAH) (lado esquerdo do diagrama). A concentração extracelular contribui para a homeostasia do sistema vascular, através da estimulação de receptores específicos de adenosina. Em situações de altas concentrações de homocisteína, a reação catalisada pela SAH-hidrolase ocorrerá no sentido inverso (lado direito do diagrama).

1.3 O sistema purinérgico

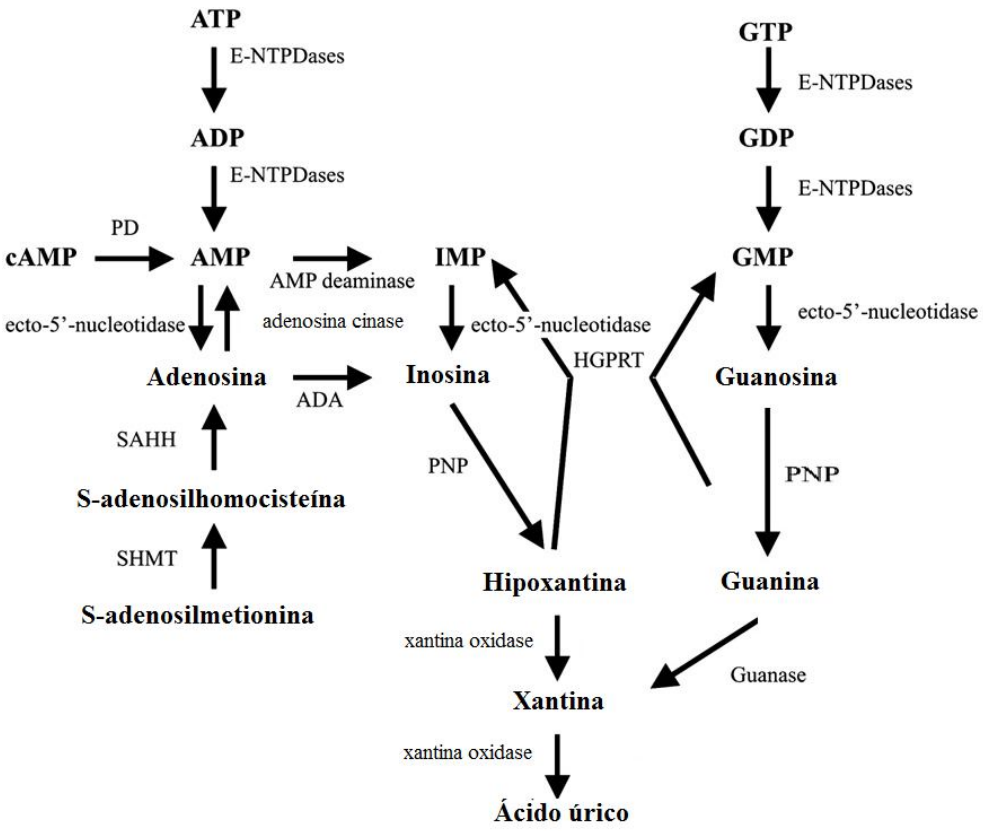
As bases purínicas, como adenina e guanina, e seus nucleotídeos e nucleosídeos são moléculas amplamente distribuídas dentro e fora das células de organismos vivos. Dentre suas diversas funções biológicas podemos enfatizar seu papel na construção do DNA e RNA (adenina e guanina), nas vias bioquímicas envolvidas no metabolismo energético celular (ATP) ou nos mecanismos intracelulares de transdução de sinal como mensageiros secundários (AMPc e GMPc) (Barnstable et al., 2004; Bourne et al., 1990). Entretanto, nos últimos anos diversos trabalhos demonstraram o papel fundamental destas moléculas no espaço extracelular sobre a homeostase vascular (Burnstock, 2007; Burnstock et al. 2010).

As purinas podem ser classificadas em derivados da adenina (ATP, ADP, AMP e adenosina) e derivados da guanina (GTP, GDP, GMP e guanosina). Ainda compõem as purinas os metabólitos diretos dos derivados da adenina e da guanina: inosina, xantina, hipoxantina e ácido úrico. Adicionalmente, as purinas são importantes moduladoras da atividade sináptica no sistema nervoso central, interagindo com vários sistemas, como glutamatérgico, dopaminérgico, serotoninérgico e colinérgico (Rathbone et al., 2008; Schmidt et al., 2007). No sistema cardiovascular as purinas atuam em processos de dilatação e contração vascular, agregação plaquetária, proliferação celular, resposta inflamatória e dor (Ralevic and Burnstock, 2003). A manutenção da sinalização purinérgica fisiológica depende da concentração destes nucleotídeos/nucleosídeo circulantes, e este controle ocorre principalmente através da atividade de enzimas nucleotidases.

Ecto-nucleotidases incluem membros da família de enzimas ecto-nucleotídeo-trifosfato difosfohidrolase (E-NTPDase) e ecto-5'-nucleotidase, entre outros. Quatro

entre as oito E-NTPDases conhecidas são expressas na membrana plasmática. Estas E-NTPDases possuem dois domínios transmembrana e hidrolisam nucleotídeos extracelulares como ATP e / ou ADP a AMP, mas também metabolizam outros nucleotídeos tri-e difosfatos. Já os nucleotídeos monofosfatados, como o AMP, são hidrolisados por enzimas da família das ecto-5´-nucleotidases (Yegutkin, 2008).

Figura 3:



Adaptado de Schmidt et al. Pharmacology & Therapeutics. 116 (2007) 401–416

Modelo esquemático das fontes extracelulares de adenina, guanina e derivados. E-NTPDases, ectonucleotide-trifosfato difosfoidrolase; ADA, adenosina deaminase; SHMT, serina hidroximetiltransferase; SAHH, S-adenosilhomocisteina hidrolase; PD, ectofosfodiesterase; PNP, fosforilase de nucleosídeos purínicos; HGPRT, hipoxantina-guanina fosforibosiltransferase.

1.3.1 Derivados da adenina no sistema cardiovascular

Os nucleotídeos ATP e ADP influenciam fortemente o sistema cardiovascular exercendo potentes efeitos em diversos processos fisiológicos e patológicos (Ralevic and Burnstock, 2003, 1991; Yegutkin, 2008). Múltiplos fatores são importantes para determinar a resposta provocada por estas purinas, entre eles destaca-se a natureza do subtipo de purinoreceptor envolvido e a sua localização (Ralevic and Burnstock, 1991). Todas as células do sistema cardiovascular expressam um ou mais subtipos de purinoreceptores (Ralevic and Burnstock, 2003), dependendo da célula e do receptor, essas purinas podem desempenhar numerosos efeitos vasculares.

O nucleotídeo ATP é capaz de atuar com efeitos opostos dependendo da concentração, da célula e do receptor. O ATP liberado como co-transmissor de nervos simpáticos é capaz de contrair a musculatura vascular lisa via receptores P2X, enquanto o ATP liberado das células endoteliais durante mudanças do fluxo sangüíneo (*shear stress*) ou durante hipóxia é capaz de agir em receptores P2Y nestas células e liberar óxido nítrico, resultando em relaxamento (Burnstock, 2002a; Kunapuli and Daniel, 1998). O ATP também pode exercer influência sobre o sistema vascular por interferir no processo de agregação plaquetária e promover proliferação de células musculares lisas e células endoteliais (Ralevic and Burnstock, 2003; Soslau and Youngprapakorn, 1997).

O nucleotídeo ADP, é uma molécula capaz de estimular a agregação plaquetária por meio da interação com os receptores P2Y das plaquetas (Gachet, 2006). Portanto, o controle dos níveis extracelulares de ADP é importante para a regulação de processos trombóticos e/ou hemorrágicos. O ADP pode atuar nos receptores P2Y das células endoteliais e musculares lisas, causando dilatação (Ralevic and Burnstock, 2003), sendo que esta também pode ocorrer pela liberação de óxido nítrico que o ADP é capaz de

estimular (Kunapuli and Daniel, 1998). O AMP, por sua vez, é um agente antiagregador plaquetário e de extrema importância como substrato para formação da adenosina.

A adenosina, um precursor ou um metabólito dos nucleotídeos da adenina, é uma molécula presente em todas as células do sistema biológico (Burnstock et al. 2010). A adenosina, além de ser um potente vasodilatador, agindo nos receptores A2 das células endoteliais (Ralevic and Burnstock, 2003), também inibe a agregação plaquetária via estimulação da adenilato ciclase pelos receptores A2A plaquetários (Cristalli et al., 1994; Kawashima et al., 2000); inibe a proliferação das células musculares lisas e endoteliais (Burnstock, 2002b; Dubey et al., 1997) e a adesão de neutrófilos ao endotélio vascular (Cronstein, 1996). Desta forma, a adenosina desempenha um papel protetor importante em processos de isquemia, hipóxia, hipertensão, aterosclerose, trombose e inflamação (Mubagwa et al., 1996; Ralevic and Burnstock, 2003).

A adenosina circulante tem duas origens distintas: liberação celular para a corrente sangüínea via transportadores bidirecionais de nucleosídeos ou então pode ser produzida pela degradação dos nucleotídeos extracelulares ATP, ADP e AMP (Dunwiddie and Masino, 2001; Latini and Pedata, 2001; Zimmermann, 1992). Os transportadores celulares de adenosina são bidirecionais e equilibram os níveis intracelulares e extracelulares de adenosina (Dunwiddie and Masino, 2001).

1.3.2 Derivados da guanina no sistema nervoso central

As purinas derivadas da guanina têm sido tradicionalmente estudadas como moduladoras de processos intracelulares, principalmente a atividade da proteína G. No entanto, elas também exercem diversos efeitos extracelulares não relacionados às proteínas G.

Recentemente, os derivados da guanina, incluindo os nucleotídeos GTP, GDP, e GMP e o nucleosídeo guanosina receberam atenção de pesquisadores. Estudos demonstram que estas purinas estão envolvidas em processos como: efeito inibitório sobre a atividade do sistema glutamatérgico em condições fisiológicas e patológicas; efeitos sobre a memória e o comportamento; e efeitos tróficos em células neurais (Schmidt et al., 2007).

Nucleotídeos derivados da guanina, quando administrados intracerebroventricularmente, previnem convulsões induzidas por ácido quinolínico, uma toxina que super estimula a neurotransmissão glutamatérgica (Baron et al., 1989; de Oliveira et al., 2004; Schmidt et al., 2005; Schmidt et al., 2000; Vinade et al., 2003; Vinade et al., 2005). Ainda, GMP e guanosina têm apresentado um perfil neuroprotetor em vários protocolos *in vivo* e *in vitro* (Lara et al., 2001; Schmidt et al., 2005; Schmidt et al., 2000; Vinade et al., 2003; Vinade et al., 2005). No entanto, a maioria dos efeitos neuroprotetores relacionados aos nucleotídeos de guanina (principalmente GMP) parece ser limitada à conversão destes nucleotídeos para a guanosina (Soares et al., 2004).

Estudos já demonstraram que a guanosina é capaz de prevenir convulsões (evento que envolve isquemia cerebral) em roedores (Schmidt et al., 2000; Schmidt et al., 2007), tem efeito analgésico em modelos glutamatérgicos de dor (Schmidt et al., 2009a; Schmidt et al., 2008; Schmidt et al., 2009b; Schmidt et al.) e também diminui o dano neural em modelo de isquemia focal (Chang et al., 2008). Além disto, ratos submetidos à isquemia *in vivo* apresentam uma diminuição da captação de glutamato (medida *in vitro*) e esta diminuição é evitada pela administração sistêmica de guanosina (Moretto et al., 2005; Moretto et al., 2009).

As purinas e seus metabolitos além das enzimas solúveis responsáveis pela sua hidrólise são detectados em diversas amostras biológicas. Nosso grupo de pesquisa

sempre buscou correlacionar os níveis de purinas com diferentes patologias e prognósticos, especialmente em amostras de sangue e líquor, tanto de humanos como em camundongos e ratos. Portanto, uma técnica de identificação e quantificação de purinas em diferentes tecidos biológicos sempre foi de grande interesse para de nossos estudos.

Além disso, os efeitos adversos associados ao tratamento crônico com CsA envolvem múltiplos mecanismos periféricos e centrais. Neste trabalho discutiremos as implicações do tratamento crônico com CsA no perfil metabólico e purinérgico de ratos não transplantados. Além disso, discutiremos o desenvolvimento de uma metodologia de quantificação de purinas por HPLC e os resultados obtidos até então sobre a neurotoxicidade do tratamento com CsA em ratos.

2. Objetivos gerais

Este trabalho tem como objetivo geral avaliar parâmetros que contribuam para a o entendimento e a elucidação dos mecanismos envolvidos nos efeitos adversos causados pela administração crônica de CsA em ratos Wistar não transplantados.

2.1. Objetivos específicos

1- Estabelecer um modelo animal de tratamento crônico com CsA em ratos Wistar não transplantados;

2- Avaliar o estado metabólico dos animais;

3- Avaliar parâmetros de coagulação dos animais;

4- Avaliar perfil de hidrólise de nucleotídeos de adenina no soro dos animais;

5- Quantificar os níveis de purinas derivadas da adenina no plasma;

6- Desenvolver e aperfeiçoar uma técnica de quantificação de purinas e derivados em plasma e líquor de humanos, camundongos e ratos.

7- Avaliar parâmetros comportamentais nos animais tratados com CsA;

8- Avaliar o efeito do tratamento com CsA sobre parâmetros morfológicos astrocitários.

9- Avaliar parâmetros de neurotoxicidade relacionados ao sistema glutamatérgico astrocitários.

PARTE II

CAPÍTULO I

Long-term cyclosporine treatment: Evaluation of serum biochemical parameters and histopathological alterations in Wistar rats.

Ana Elisa Böhmer, André Mendes Ribeiro Corrêa, Débora Guerini de Souza, Luisa Knorr, Gisele Hansel, Luís Gustavo Corbellini, David Driemeier, Luis Valmor Portela, Diogo Onofre Souza.

Artigo publicado no periódico Experimental and Toxicologic Pathology.

Experimental and Toxicologic Pathology ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.de/etp

Long-term cyclosporine treatment: Evaluation of serum biochemical parameters and histopathological alterations in Wistar rats

Ana Elisa Böhmer [a], André Mendes Ribeiro Corrêa [b], Débora Guerini de Souza [a], Luisa Knorr [a], Gisele Hansel [a], Luís Gustavo Corbellini [c], David Driemeier [b], Luis Valmor Portela [a], Diogo Onofre Souza [a,*]

[a] Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Avenida Ramiro Barcelos, 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil
[b] Department of Veterinary Clinic Pathology, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] Department of Preventive Veterinary Medicine, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 18 August 2009
Accepted 29 October 2009

Keywords:
Cyclosporine
Adverse effects
Interleukins
Biochemical alterations
Histopathological

ABSTRACT

The immunosuppressant agent cyclosporine (CsA) is currently used in transplanted patients and in the therapy of autoimmune disorders. CsA treatment has significant acute and chronic side effects on the liver and kidney. However, in the clinical setting, it is difficult to distinguish a direct effect of CsA treatment from other confounding variables, such as allograft rejection and effects due to other drug therapies. In the present study, we assessed for direct associations between CsA immunosuppressive therapy and cytokines levels, kidney and liver functionality, as well as lung histopathological status in rats submitted to chronic CsA treatment without undergoing any transplantation. Male Wistar rats were divided into three groups. The control group received vehicle (corn oil), and treated groups received CsA 5 or 15 mg/kg, by daily gastric gavage during 8 weeks. The results demonstrated that CsA treatment decreases blood levels of interleukins 1α (IL-1α), 1β (IL-1β) and interleukin 2 (IL-2), but does not alter interleukin 6 (IL-6) and IFN-γ levels. Serum biochemical markers of renal (creatinine) and hepatic (SGPT and SGOT) injury/dysfunction did not vary with CsA treatment, despite the presence of small histological alterations, suggesting that the function of these metabolic organs were preserved. Pulmonary histopathological lesions were observed in the CsA groups, and they were attributed to the activation of the local immunoresponse mechanisms by the normal microbiota in immunosuppressive CsA cases. These results suggest that the CsA concentrations administered in our experimental protocol were able to induce immunosuppression in rats without causing nephro and hepatotoxicity.

1. Introduction

Cyclosporine A (CsA) is widely utilized as an immunosuppressant in organ transplantation, as well as in the therapy of several immunodisorders (Borel et al., 1996). However, its clinical use is often limited by severe side effects. CsA inhibits the production of interleukins, gamma-interferon (IFN-γ) and other lymphokines (Ho et al., 1996; Zhang et al., 2005). These cytokines, which modulate the immune and inflammatory reactions, present diverse physiological roles regulating the innate and adaptative immunity, as well as stimulating the hematopoiesis. Thus, the blood levels of interleukins 1α (IL-1α) and 1β (IL-1β), interleukin 2 (IL-2), interleukin 6 (IL-6) and IFN-γ may be used as parameters for evaluating the CsA immunosuppressive effects.

CsA treatment has significant acute and chronic side effects on the liver and kidney (Grub et al., 2000a, b). *In vivo*, CsA increases lipoperoxidation in rat kidney and liver, depletes the hepatic and renal pool of glutathione (Wolf et al., 1997), and impairs antioxidant defense systems.

The histopathological changes occurring in the liver comprise sinusoidal dilatation, cytoplasmic vacuolization of hepatocytes, cell infiltration (especially in the periportal areas), parenchymal mitosis and moderate hepatocellular necrosis. The mechanisms underlying the hepatic side effects have not been explained despite extensive studies (Diao et al., 2002). Chronic CsA nephropathy is characterized by irreversible renal-striped interstitial fibrosis, inflammatory cell infiltrations and hyalinosis of the afferent glomerular arterioles (Bennett et al., 1996; Myers et al., 1984).

Since most of CsA studies have been done in transplanted recipients, the presence of multiple confounding factors in the clinical setting, such as allograft rejection and other drugs therapies, hinders the evaluation of specific effects of CsA on the

* Corresponding author. Tel.: +55 51 33085559/33085558; fax: +55 51 33085540/33085535.
E-mail address: diogo@ufrgs.br (D. Onofre Souza).

0940-2993/$ - see front matter © 2009 Elsevier GmbH. All rights reserved.
doi:10.1016/j.etp.2009.10.005

organs functions. Thus, in the present study, we aimed to determine direct associations between CsA immunosuppressive therapy and cytokines levels, kidney and liver functionality, as well as lung histopathological status in rats submitted to a chronic CsA treatment without undergoing any transplantation.

2. Materials and methods

2.1. Animals and treatments

Rats were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Adult male Wistar rats, 120 days old, weighing 300–350 g, were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.), at a room temperature of 22 ± 2 °C. The rats had free access to food (standard laboratory rat chow) and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

Rats were divided into three groups ($N=10$/group) according to the treatment schedule. The control group received vehicle (corn oil) and treated groups received CsA 5 mg/kg or CsA 15 mg/kg. The drug or vehicle administration was performed by a daily gastric gavage during 8 weeks. The animals were anesthetized 24 h after the last CsA or vehicle administration with sodium thiopental (40 mg/kg). Blood samples were collected by cardiac function and then animals were killed by decaptation, and organs were removed immediately.

2.2. Biochemical analysis

CsA concentration in whole blood was determined by enzyme multiplied immunoassay test (EMIT-Green Liquid, Dade-Behring on Cobas Mira, Roche Diagnostic Systems, USA). Serum IFN-γ and interleukins 1α (IL-1α), 1β (IL-1β), 2 (IL-2) and 6 (IL-6) levels were measured using commercially available ELISA kits (Quantikine, R&D Systems, USA).

Serum biochemical parameters as creatinine, glutamic oxalacetic transaminase (SGOT) and glutamic pyruvic transaminase (SGPT) were analyzed using commercial kits manufactured by Roche (USA) in automatized equipment (Cobas Integra 400, Roche, USA).

2.3. Morphological evaluation

Morphological data were collected from 30 treated animals. Kidney, liver and lung were removed and fixed with 10% buffered formalin at room temperature. Tissues were dehydrated in gradual alcohol from 50% to 100%, cleared by xylene and embedded in paraffin. Two to five micrometers-thick sections were stained with hematoxylin and eosin (H&E) to observe the general structure. Kidney sections were also stained with periodic acid-Schiff's reagent (PAS). A minimum of 10 fields at 100× and 400× magnifications from each slide were assessed and graded in each biopsy. Slides were evaluated by two blinded histologists under light microscopy.

Semi-quantitative scores were used to evaluate the extent of changes in tissues sections from each group (Shihab et al., 1996).

Kidney: For tubular injury the following score was used: 0=no tubular injury, 1= < 25% of tubules injured, 2=from 25% to 50% of tubules injured, 3=from 50% to 75% of tubules injured, 4=more than 75% of tubules injured. Interstitial damage was estimated semi-quantitatively using the following scores: 0=normal interstitium, 1= < 25% of areas injured, 2=from 25% to 50% of areas injured, 3=from 50% to 75% of areas injured, 4=more than 75% of areas injured. The hyalinosis, identified through the PAS staining, consisted of hyaline deposition within the tunica media of afferent arterioles and terminal portions of interlobular arteries. It was assessed in the afferent arterioles using the scores as follows: 0=no arterioles injured, 1= < 25% of arterioles injured, 2=from 25% to 50% of arterioles injured, 3=from 50% to 75% of arterioles injured, 4= > 75% of arterioles injured.

Liver: The hepatic changes examined consisted of sinusoidal dilation, cell infiltration and hepatocellular vacuolization according to Kurus et al. (2008). Sinusoidal dilatation was graded as follows: 0=normal sinusoids, 1=mild dilatation, 2=moderate dilatation and 3=severe dilatation. Cell infiltration was graded as follows: 0=normal parenchyma and portal areas, 1=mild infiltration especially in the periportal areas, 2=moderate infiltration and 3=widespread infiltration. Hepatocellular vacuolization as follows: 0=normal hepatocytes, 1=mild vacuolization, 2=moderate vacuolization and 3=severe vacuolization.

Lung: Pulmonary changes evaluation was focused on bronchiolar associated lymphoid tissue (BALT), lymphohistiocytic perivasculitis and bronchoalveolar infiltrates. BALT was graded as follows: 0=normal BALT; 1=mild BALT hyperplasia; 2=moderate BALT hyperplasia and 3=severe BALT hyperplasia. Lymphohistiocytic perivasculitis was graded as follows: 0=normal vessels; 1=mild perivasculitis; 2=moderate perivasculitis and 3=severe perivasculitis. Bronchoalveolar infiltrates was graded as follows: 0=no bronchoalveolar infiltrates; 1=mild bronchoalveolar infiltrate, 2=moderate bronchoalveolar infiltrate and 3=severe bronchoalveolar infiltrate.

2.4. Statistical analysis

Continuous data were analyzed using one-way ANOVA, followed by the Tukey's multiple range tests. $P \leq 0.05$ was considered to represent a statistically significant difference. Biochemical analyses were performed using the Statistical Package for Social Sciences (SPSS) software.

chi-square for trend was used to check the hypothesis of linear trend between the treatments and the presence of lesions. For this, the control group was used as dummy variable in order to compare the odds of lesion according to treatment protocol (i.e. 5 or 15 mg of CsA). This analysis was performed using the software EpiInfo 6.0.

3. Results

3.1. Cyclosporine serum levels

Blood CsA concentration 24 h after the last CsA administration is shown in Table 1. CsA 15 mg/kg group presented higher CsA

Table 1
Serum biochemical parameters in control and CsA-treated rats.

Variable	Control	CsA 5 mg/kg	CsA 15 mg/kg
CsA (ng/mL)	Not detectable	92.4 ± 30.1	$425.7 \pm 66.5^{**}$
IFN-gamma (pg/mL)	229.0 ± 18.0	223.0 ± 13.0	242 ± 21.7
Creatinine (mg/dL)	0.68 ± 0.05	0.66 ± 0.06	0.70 ± 0.08
SGOT (U/L)	69.0 ± 18.4	71.0 ± 20.5	58.0 ± 10.1
SGPT (U/L)	21.2 ± 5.5	23.4 ± 7.6	17.8 ± 6.7

Data are given as mean ± standard deviation. $^{**}P \leq 0.001$ compared with CsA 5 mg/kg group.

Please cite this article as: Böhmer AE, et al. Long-term cyclosporine treatment: Evaluation of serum biochemical parameters and histopathological alterations in Wistar rats. Exp Toxicol Pathol (2009), doi:10.1016/j.etp.2009.10.005

concentration compared with CsA 5 mg/kg. No detectable CsA level was found in the control group.

3.2. Inflammatory parameters

CsA (5 and 15 mg/kg) treatment decreased the serum levels of IL-1α (from 49.0 ± 6.9 to 35.0 ± 9.1 and 30.0 ± 3.6 pg/mL), IL-1β (from 711.0 ± 46.8 to 529.0 ± 58.1 and 403.0 ± 11.7 pg/mL) and IL-2 (from 358.0 ± 139.0 to 323.0 ± 14.5 and 224.0 ± 38.7 pg/mL) (Fig. 1). Despite a slight decrease of IL-6 concentration in CsA treated groups, no statistically significant differences were observed in IL-6 levels among CsA 5 mg/kg (504.0 ± 103.3 pg/mL) and CsA 15 mg/kg (514.0 ± 80.9 pg/mL) when compared with control group (629.0 ± 94.8 pg/mL) (Fig. 1). IFN-γ level did not differ among groups (Table 1).

3.3. Renal and hepatic function biochemical parameters

SGPT, SGOT (liver enzymes) and creatinine serum (renal function) levels did not differ among groups (Table 1).

3.4. Histopathologic findings

The most important histological lesion in the CsA treated groups was observed in the kidney, which was primarily characterized by arteriolar hyalinosis consisting of PAS positive hyaline deposition within the afferent arteriole wall causing thickening of the media (Fig. 2). This lesion occurred in almost the totality of the afferent arterioles in the CsA 15 mg/kg treated group, and was present in approximately a half-part of afferent arterioles in the CsA 5 mg/kg treated group. Although tubular injury and tubulointerstitial fibrosis were not prominent findings observed in all groups, they had a significant association with the CsA dose (Table 2).

Hepatocellular vacuolization, sinusoidal dilatation and cell infiltration were mild and not clearly differentiated among the groups (Fig. 3).

Pulmonary alterations such as inflammatory cellular infiltration, BALT hyperplasia and perivasculitis (Fig. 4) were most prominent in the CsA treated groups. CsA 15 mg/kg group showed a clear increase in the pulmonary reactivity compared with the CsA 5 mg/kg and control groups.

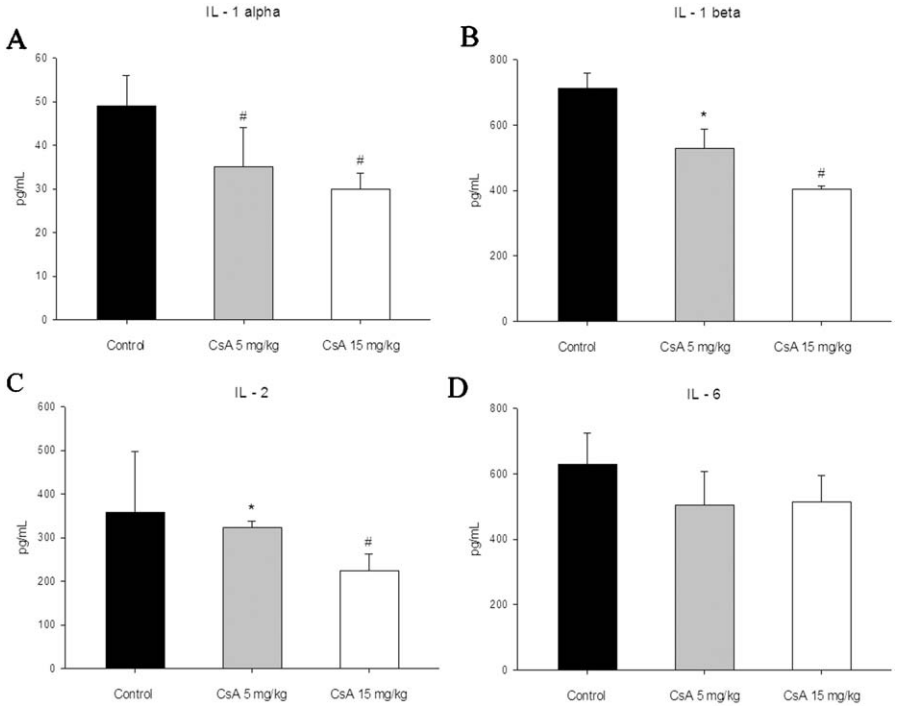


Fig. 1. Serum interleukins levels. CsA treatment decreased the levels of IL-1α (A), IL-1β (B) and IL-2 (C) in both CsA 5 and 15 mg/kg groups, compared with control group. IL-6 remained at basal levels. Values are presented as mean ± S.D. *$P < 0.05$ and **$P < 0.001$, compared with control.

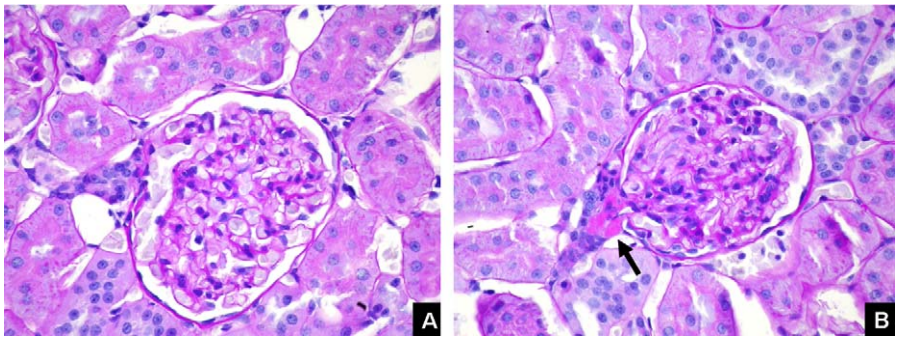


Fig. 2. Representative photomicrographs of glomerular histomorphologic aspects of control group (A) and CsA 15 mg/kg group (B). Hyaline deposition within the afferent arteriole wall causing thickening of the media (arrow) is observed in the CsA group (B). Periodic acid-Schiff's reagent (PAS), original magnification 400 ×.

Please cite this article as: Böhmer AE, et al. Long-term cyclosporine treatment: Evaluation of serum biochemical parameters and histopathological alterations in Wistar rats. Exp Toxicol Pathol (2009), doi:10.1016/j.etp.2009.10.005

Table 2
Histopathologic findings results of χ^2 for trend.

Treatment	Odds ratio			
	Tubular Injury	BALT hyperplasia	Lymphohistiocytic perivasculitis	Bronchoalveolar infiltrate
Control	1.00	1.00	1.00	1.00
CsA 5 mg	3.86	6.00	21.00	6.00
CsA 15 mg	21.00	15.00	23.00	40.00

Lesions with significant linear trend ($P \leq 0.05$), in which the odds of presence of lesion increased according to the treatment applied (control group used as dummy variable).

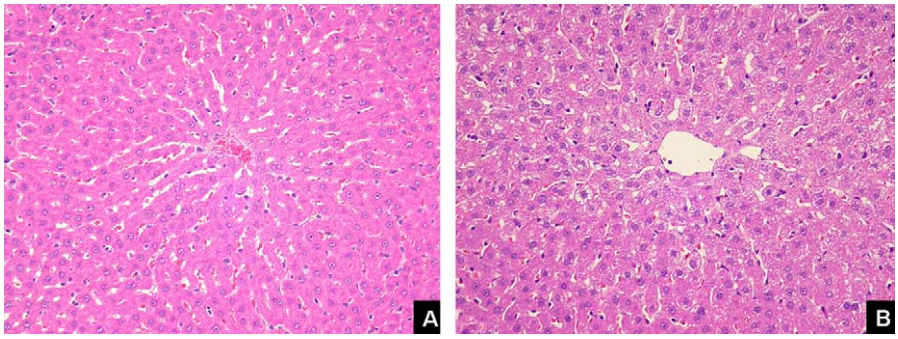


Fig. 3. Photomicrographs of hepatic histomorphologic aspects of control group (A) and a CsA 15 mg/kg rat (B). Mild hepatocellular tumefaction with a few scattered cells infiltration was observed in only 1 rat treated with CsA 15 mg/kg (B). All the other CsA 15 mg/kg photomicrographs resemble the control group (A). Hematoxylin and eosin (H&E), original magnification 200 × .

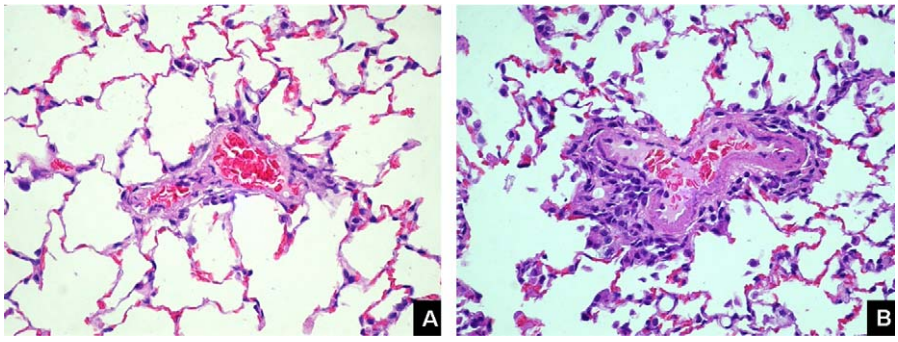


Fig. 4. Representative photomicrographs of pulmonary histomorphologic aspects of control group (A) and CsA 15 mg/kg group (B). Mild-to-moderate lympho-histiocytic perivasculitis with a few and scattered alveolar macrophages in the lumen is observed in the CsA 15 mg/kg group (B). Hematoxylin and eosin (H&E), original magnification 200 × .

There was a significant linear trend between the presence of tubular injury, BALT hyperplasia, lymphohistiocytic perivasculitis and bronchoalveolar inflammatory infiltrate and the treatment protocols (Table 2). For all these lesions, increased odds with greater concentration of CsA were found.

4. Discussion

The measurement of interleukins IL-1α, IL-1β, IL-2, IL-6 and IFN-γ may be used as a tool for evaluating CsA immune effects. In our study, we observed that rats submitted to CsA administration, without overstimulation of the immune system, present lower serum levels of IL-1α and IL-1β, which characterizes the action of CsA on inhibiting cytokines producing cells. The properties of IL-1 molecules (IL-1α and IL-1β) stem from their ability to induce the synthesis of cytokines, chemokines, proinflammatory molecules and the expression of adhesion molecules (Dinarello, 1996; Van Damme et al., 1987). The decreased level of IL-2, which acts as a cell growth, differentiator and regulatory factor in the immune system (Nelson, 2002), is in accordance to the results obtained with IL-1α and IL-1β.

The potential risk of CsA to induce nephrotoxicity (Linde et al., 1999; Vercauteren et al., 1998) and hepatoxicity (Taylor et al., 2005) is well known. Here, the serum biochemical markers of renal (creatinine) and hepatic (SGPT and SGOT) injury/dysfunction did not vary with the CsA treatment used, despite small alterations observed histologically, suggesting that the function of these metabolic organs were preserved.

By comparing the present study with previous observations in the literature (Blair et al., 1982; Ryffel et al., 1983; Thomson et al., 1984), we observed that the nephrotoxicity and hepatotoxicity depend on the protocol of CsA administration (especially on dose and administration time). Accordingly, impairment in renal and liver functions is observed in rats given CsA dose of ≥ 25 mg/kg/day for 3–15 weeks of administration (Blair et al., 1982; Thomson et al., 1981, 1984).

In this study, the lack of consistent renal tubular lesions and interstitial fibrosis are both indicative that the groups treated

Please cite this article as: Böhmer AE, et al. Long-term cyclosporine treatment: Evaluation of serum biochemical parameters and histopathological alterations in Wistar rats. Exp Toxicol Pathol (2009), doi:10.1016/j.etp.2009.10.005

with CsA did not present serious chronic lesions. However, in both groups degenerative hyaline changes in the afferent arteriole walls were observed. It is possible that sustained glomerular afferent arteriolar vasoconstriction eventually promotes structural alteration and occlusion of preglomerular vessels (Benigni et al., 1999). While prolonged vasoconstriction could contribute to chronic CsA nephropathy by producing chronic ischemia, this relationship has been difficult to demonstrate in experimental studies (Bennett et al., 1996). Usually this finding is associated with some degree of renal dysfunction (Cattaneo et al., 2004), but in the present study histological kidney lesions were mild with no significant tubulointertitial chronic lesions. The kidney has extensive reserve capacity and hence renal disease may present with or without any clinical signs or clinicopathological abnormalities (Sebastian et al., 2007).

There is commonly a predictable latent period between the time of exposure and clinical evidence of liver injury (Dahm and Jones, 1996). The absence of histopatological and clinicopathological alterations in the analyses of the livers in the present study could be explained by this fact. According to Kurus et al. (2008), the tissue damage caused by CsA is mild and reversible at the period when biochemical parameters are just starting to become abnormal. The normal levels of SGPT and SGOT together with the histopathological results corroborate a normal hepatic function in rats submitted to CsA 5 and 15 mg/kg treatment.

Little is known, however, about pulmonary pathological consequences of an immunosuppressive treatment in rats. The pulmonary histopathological lesions observed in the CsA groups can be attributed to an activation of the local immunoresponse mechanisms by the normal microbiota in immunosuppressive CsA cases. This might be due to opportunistic pathogens-induced macrophage activation, especially because immunosuppression by CsA has been described to play a key role in the impairment of airway mucociliary clearance in rats (Pazetti et al., 2008), which are especially vulnerable to respiratory pathogens. However, we cannot exclude a direct action of CsA in the etiology of vascular lesions.

Taken together, despite the well known ability of CsA to induce kidney and liver functional disturbances, the present results demonstrate that CsA treatments at 5 and 15 mg/kg during 8 weeks are not able to induce renal and hepatic dysfunction, as evaluated by histopathological and biochemical parameters. These findings suggest that the CsA concentrations obtained under our experimental protocol are able to induce immunosuppression in rats without causing nephro and hepatotoxicity. Thus, we can conclude that the systemic toxic effects of CsA depends on the protocol used and this observation may be useful for evaluating side effects observed in the therapeutic use of CsA.

Acknowledgements

This work was supported by INCT: Excitotoxicity and Neuroprotection, CNPq, and FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" # 01.06.0842-00.

References

Benigni A, Bruzzi I, Mister M, Azzollini N, Gaspari F, Remuzzi G. Nature and mediators of renal lesions in kidney transplant patients given cyclosporine for more than one year. Kidney Int 1999;55:674–ce:bold>685.

Bennett WM, DeMattos A, Meyer MM, Andoh T, Barry JM, et al. Chronic cyclosporine nephropathy: the Achilles' heel of immunosuppressive therapy. Kidney Int 1996;50:1089–100.

Blair JT, Thomson AW, Whiting PH, Davidson RJ, Simpson JG. Toxicity of the immune suppressant cyclosporin A in the rat. J Pathol 1982;138:163–78.

Borel JF, Baumann G, Chapman I, Donatsch P, Fahr A, Mueller EA, et al. In vivo pharmacological effects of ciclosporin and some analogues. Adv Pharmacol 1996;35:115–246.

Cattaneo D, Perico N, Remuzzi G. Functional loss of the transplanted kidney: immunological and non-immunological factors. G Ital Nefrol 2004;21(Suppl. 26):S28–33.

Dahm LJ, Jones DP. Textbook of liver disease: mechanisms of chemically induced liver disease. In: Zakim D, Boyer TD, editors, Hepatology. Philadelphia: WBS 1996, p. 875–90.

Diao TJ, Yuan TY, Li YL. Immunologic role of nitric oxide in acute rejection of golden hamster to rat liver xenotransplantation. World J Gastroenterol 2002;8:746–51.

Dinarello CA. Biologic basis for interleukin-1 in disease. Blood 1996;87:2095–147.

Grub S, Persohn E, Trommer WE, Wolf A. Induction of apoptosis by the *O*-hydroxyethyl-D(Ser)(8)-cyclosporine A derivative SDZ IMM 125 in rat hepatocytes. J Pharmacol Exp Ther 2000a;293:24–32.

Grub S, Persohn E, Trommer WE, Wolf A. Mechanisms of cyclosporine A-induced apoptosis in rat hepatocyte primary cultures. Toxicol Appl Pharmacol 2000b;163:209–20.

Ho S, Clipstone N, Timmermann L, Northrop J, Graef I, Fiorentino D, et al. The mechanism of action of cyclosporin A and FK506. Clin Immunol Immunopathol 1996;80:S40–5.

Kurus M, Esrefoglu M, Karabulut AB, Sogutlu G, Kaya M, Otlu A. Oral L-arginine protects against cyclosporine-induced hepatotoxicity in rats. Exp Toxicol Pathol 2008;60:411–9.

Linde T, Sandhagen B, Backman U, Fellstrom B. Altered flow properties of blood and increased plasma fibrinogen in cyclosporin-treated renal allograft recipients. Nephrol Dial Transplant 1999;14:1525–9.

Myers BD, Ross J, Newton L, Luetscher J, Perlroth M. Cyclosporine-associated chronic nephropathy. N Engl J Med 1984;311:699–705.

Nelson BH. Interleukin-2 signaling and the maintenance of self-tolerance. Curr Dir Autoimmun 2002;5:92–112.

Pazetti, R, Pego-Fernandes, PM, Lorenzi-Filho, G, Saldiva, PH, Moreira, LF, Jatene, FB. Effects of cyclosporine A and bronchial transection on mucociliary transport in rats. Ann Thorac Surg 2008;85:1925–9; [discussion 1929].

Ryffel B, Donatsch P, Madorin M, Matter BE, Ruttimann G, Schon H, et al. Toxicological evaluation of cyclosporin A. Arch Toxicol 1983;53:107–41.

Sebastian MM, Baskin SI, Czerwinski SE. Textbook of toxicology basic and clinical principles: renal toxicity. In: Gupta RC, editor, Veterinary Toxicology: Basic & Clinical Principles. New York, London: Academic, E, Press; 2007, p. 161–76.

Shihab FS, Andoh TF, Tanner AM, Noble NA, Border WA, Franceschini N, et al. Role of transforming growth factor-beta 1 in experimental chronic cyclosporine nephropathy. Kidney Int 1996;49:1141–51.

Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy. Crit Rev Oncol Hematol 2005;56:23–46.

Thomson AW, Whiting PH, Blair JT, Davidson RJ, Simpson JG. Pathological changes developing in the rat during a 3-week course of high dosage cyclosporin A and their reversal following drug withdrawal. Transplantation 1981;32:271–7.

Thomson AW, Whiting PH, Simpson JG. Cyclosporine: immunology, toxicity and pharmacology in experimental animals. Agents Actions 1984;15:306–27.

Van Damme J, Cayphas S, Van Snick J, Conings R, Put W, Lenaerts JP, et al. Purification and characterization of human fibroblast-derived hybridoma growth factor identical to T-cell-derived B-cell stimulatory factor-2 (interleukin-6). Eur J Biochem 1987;168:543–50.

Vercauteren SB, Bosmans JL, Elseviers MM, Verpooten GA, De Broe ME. A meta-analysis and morphological review of cyclosporine-induced nephrotoxicity in auto-immune diseases. Kidney Int 1998;54:536–45.

Wolf A, Trendelenburg CF, Diez-Fernandez C, Prieto P, Houy S, Trommer WE, et al. Cyclosporine A-induced oxidative stress in rat hepatocytes. J Pharmacol Exp Ther 1997;280:1328–34.

Zhang X, Chen BC, Xiang FL, Chang S, Zhou HM, Du DF, et al. Inhibition of MD-1 expression by immunosuppressants or antisense oligodeoxynucleotides on skin allograft survival in mice. Transplant Proc 2005;37:1965–7.

Please cite this article as: Böhmer AE, et al. Long-term cyclosporine treatment: Evaluation of serum biochemical parameters and histopathological alterations in Wistar rats. Exp Toxicol Pathol (2009), doi:10.1016/j.etp.2009.10.005

CAPÍTULO II

Long-term cyclosporine treatment in non transplanted rats and metabolic risk factors of vascular diseases.

Ana Elisa Böhmer, Débora Guerini de Souza, Gisele Hansel, Liz M. B. P. Brum, Luis Valmor Portela, Diogo Onofre Souza.

Artigo publicado no periódico Chemico-Biological Interactions.

Chemico-Biological Interactions 185 (2010) 53–58

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Long-term cyclosporine treatment in non-transplanted rats and metabolic risk factors of vascular diseases

Ana Elisa Böhmer[a], Débora Guerini Souza[a], Gisele Hansel[a], Liz M.B.P. Brum[b], Luis Valmor Portela[a], Diogo Onofre Souza[a,*]

[a] Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Cardiology Institute of Rio Grande do Sul, FUC, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 13 November 2009
Received in revised form 11 February 2010
Accepted 15 February 2010
Available online 24 February 2010

Keywords:
Cyclosporine
Vascular risk
Insulin
Homocysteine

ABSTRACT

Cyclosporine (CsA) is an immunosuppressive agent frequently used in the clinic for prevention of allograft rejection and for the treatment of autoimmune diseases. Despite its desired action on the immune system, CsA treatment may present serious adverse effects, which are masked by the concomitant use of other drugs. The search for effective immunosuppression protocols which does not affect the quality of life of patients is driving research to investigate the CsA involvement in vascular diseases, frequent in patients under immunosuppression. Thus, 45 non-transplanted Wistar rats were treated for 8 weeks with vehicle or 5 or 15 mg/kg CsA ($n = 15$/group) by gavage administration to evaluate the specific influence of cyclosporine on the levels of risk factors (metabolic and inflammatory) of vascular disease and its mechanism of action. Therefore, serum insulin levels, glucose tolerance test, serum lipids profile, total homocysteine and fibrinogen levels were assessed. The biochemical alterations reported here suggest the development of a framework straight to diabetes. Glucose homeostasis was affected as indicated by decreased insulin levels and altered glucose tolerance test in CsA 15 mg/kg group compared to other groups. Serum insulin and total homocysteine levels presented a significant negative correlation ($R = -0.76$, $P < 0.0001$). Fibrinogen and serum lipids profiles were significantly increased in CsA 15 mg/kg group compared to other groups and correlated positively with total homocysteine levels. Considering the well-established correlation among insulin resistance, lipid and total homocysteine levels, hypercoagulability and atherosclerosis, we can assume that this protocol of long-term CsA treatment in non-transplanted rats alter biochemical parameters related to cardiovascular and cerebrovascular risk, mainly in CsA 15 mg/kg group. Insulin and tHcy serum levels appear to be central in this process.

1. Introduction

Cyclosporine (CsA) is an immunosuppressive agent frequently used for prevention of allograft rejection and for the treatment of autoimmune diseases. The immunosuppressive mechanisms of action of CsA involve the inhibition of calcineurin activity by a complex originated from the binding of CsA with its cytosolic receptor, cyclophilin [1,2]. Despite the expected action on the immune system, CsA long-term treatment is associated with adverse effects that have deleterious outcome on the quality of life and survival of recipients [3–6].

Long-term transplanted patients experience an unusually accelerated form of vascular disease, which is an important cause of morbidity and mortality, surpassing even infection [5,7,8]. Because the arterial lesions are the key cause of graft ischemic injury and failure, some authors refer to this process as "graft arteriosclerosis", emphasizing the importance of arterial disease [8]. It is known that CsA directly inflict damage to endothelial vascular cells but the mechanisms underlying the CsA effects on metabolic, inflammatory and coagulatory disorders also may account for the atherosclerotic process [4,7,9–12].

New-onset diabetes after transplantation has been reported to reach about 15–50% of transplant recipients, which is partially attributable to immunosuppressant therapy [13–15], and it is increasingly recognized as a risk factor for cardiovascular diseases, reducing both graft and patient survival [16–18]. This metabolic abnormality also induces vascular disturbs that predisposes individuals to atherosclerosis and death due to cerebrovascular and cardiovascular diseases [19–21]. Simultaneously to the detrimental effect of CsA on glucose homeostasis and post-transplant hyperlipidemia [22,23], it has also been suggested that CsA increases the levels of total (the reduced and oxidized forms)

* Corresponding author at: Avenida Ramiro Barcelos, 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil. Tel.: +55 51 33085559/33085558; fax: +55 51 33085540/33085535.
E-mail address: diogo@ufrgs.br (D.O. Souza).

0009-2797/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2010.02.029

homocysteine (tHcy) [24,25], a sulfur-containing amino acid intermediate in methionine metabolism, which in turn is widely accepted as an independent risk factor for atherosclerosis and peripheral vascular disease [26–32].

Several consequences associated to allograft and immunosuppressive therapy can be readily assessed in tissues and body fluids, serving as clinical biomarkers to detect, monitor and predict undesirable effects on the patient's quality of life. However, considering the multiple confounding factors in the clinical setting including recipient previous risk factors, differences in the genetic background and immune responses of the recipient, it is difficult to determine in transplanted patients the adverse effects specifically related to CsA treatment. Other confounding variables, such as allograft rejection, impaired renal function and effects due to other drug therapies [4,29,33–36] mask the adverse effects related solely to cyclosporine treatment.

Since most of CsA studies have been done in transplanted recipients, the main objective of this work was to investigate in non-transplanted rats the effects of long-term treatment with CsA on metabolic and coagulatory parameters related to cardiovascular risk. Therefore, serum insulin levels, glucose tolerance test, serum lipids profile, tHcy and fibrinogen levels were assessed.

2. Materials and methods

2.1. Animals and treatments

Forty-five Wistar rats (adult male rats, 120 days old, weighting 300–350 g), were kept on a 12-h light/dark cycle (light on at 7:00 am) at temperatures of 22 ± 1 °C, housed in plastic cages (5 per cage) with tap water and commercial food *ad libitum*. Animals were obtained from the Central Animal House of the Biochemistry Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

Rats were randomly divided in three groups (n = 15/group) according to the treatment schedule. The control group received vehicle (corn oil), and CsA-treated groups received CsA 5 mg/kg or CsA 15 mg/kg diluted in corn oil. The drug or vehicle administration was performed by daily gastric gavage for 8 weeks. Animals were anesthetized by sodium thiopental (40 mg/kg, 1 mL/kg i.p.; supplemented as necessary) and blood samples were collected by cardiac punction, 24 h after the last CsA or vehicle administration. Serum or plasma was stored at −80 °C until analysis.

2.2. Biochemical analysis

2.2.1. Serum insulin levels and glucose tolerance test (GTT)

Serum insulin levels were assessed by radioimmunoassay (BioChem ImmunoSystems, Rome, Italy). Glucose tolerance test (GTT) was performed four days before sacrificing the animals. Glucose solution (50%) was injected into the animals (i.p, 2 g/kg) after 6 h of fasting. The blood was collected by tail bleeding immediately before (0 min) and 30, 60, and 120 min after the injection, accordingly to Muller et al. [37], and the area under the curve (AUC) was used to compare glucose tolerance among groups. Glucose levels were measured by a glucosimeter (AccuChek Active, Roche Diagnostics®, USA).

2.2.2. Total homocysteine (tHcy) serum levels and coagulatory parameters

Serum total Hcy (tHcy) levels were measured using a commercial MEIA kit (Abbott, USA).

Plasma fibrinogen levels and platelet count were assessed through a Fibritimer-II (Dade Behring®, Germany) and Pentra 60 (ABX®, France), respectively.

2.2.3. Serum lipids profile and leptin levels

Serum triglycerides, total cholesterol, VLDL, LDL and HDL were measured with commercially available kits manufactured by Roche Diagnostics® (USA). The measurements were carried out in automatized equipment (Cobas Integra 400, Roche Diagnostics®, USA).

Serum leptin levels were assessed by ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) and the final color was measured in Spectramax® M5 (Molecular Devices, USA).

2.3. Statistical analysis

The statistical analyses were performed using the GraphPad software (GraphPad software, San Diego, CA). Data, submitted to Kolmogorov–Smirnov testing for normality evaluation, indicated normal distribution for all data. Numerical variables were given as mean ± standard deviation. Data were analyzed using one-way ANOVA followed by the Tukey's multiple range tests, with the exception of data from GTT, which were analyzed using repeated-measures analysis of variance (ANOVA) followed by Tukey's multiple range tests. The area under the curve (AUC) for glucose blood levels was estimated using the trapezoid rule. Correlations among numerical variables were performed by Pearson's test. $P < 0.05$ was considered to represent a statistical significant difference.

3. Results

3.1. Serum CsA levels, systemic toxicity and body weight

In a previous study from our group we showed that 24 h after the last administration, the CsA blood levels increased in a dose dependent manner and that our experimental CsA treatment protocol induces a decrease in the serum levels of cytokines that mediate immunological responses (IL-1α, IL-1β and IL-2). Moreover, CsA does not cause evident histopathological alterations in kidney and liver, neither in their serum functions markers creatinine, urea, SGPT and SGOT [38].

Here, during treatment, rats from control group increased body weight (from 347 ± 30 to 394 ± 26 g, $P < 0.01$). CsA 5 mg/kg rats had a slight increase in body weight, with no statistical significance compared with the beginning of the treatment (from 356 ± 24 to 372 ± 28 g). CsA 15 mg/kg rats had a significant decrease in body weight compared with the beginning of the treatment (from 340 ± 21 to 301 ± 59 g, $P < 0.01$) and compared to other groups ($P < 0.001$, Fig. 1).

3.2. Serum insulin levels and glucose tolerance test (GTT)

As shown in Fig. 2A, serum insulin levels decreased in CsA 15 mg/kg group (33.7 ± 9.9 μIU/mL) compared with CsA 5 mg/kg (50.0 ± 17.0 μIU/mL, $P < 0.005$) and control groups (53.1 ± 12.8 μIU/mL, $P < 0.005$). Regarding GTT (Fig. 2B), at 0 min (baseline) control and both CsA groups had similar blood glucose levels. After glucose administration, blood glucose levels increased in all groups. However, at 30, 60 and 120 min, CsA 15 mg/kg group presented higher glucose levels when compared to control and CsA 5 mg/kg groups ($P < 0.001$). The area under the curve of glucose levels (Fig. 2C), m was significantly higher in CsA 15 mg/kg group, compared with control and CsA 5 mg/kg groups ($P < 0.001$).

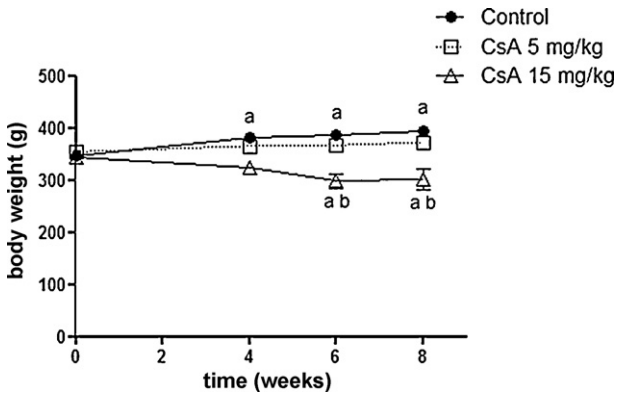


Fig. 1. The effects of CsA 5 and 15 mg/kg treatment on body weight during 8 weeks. Results are presented as mean ± standard deviation; $n = 15$/group. [a] $P < 0.01$ compared to the beginning of treatment; [b] $P < 0.001$ compared to control and CsA 5 mg/kg groups at the same time of treatment.

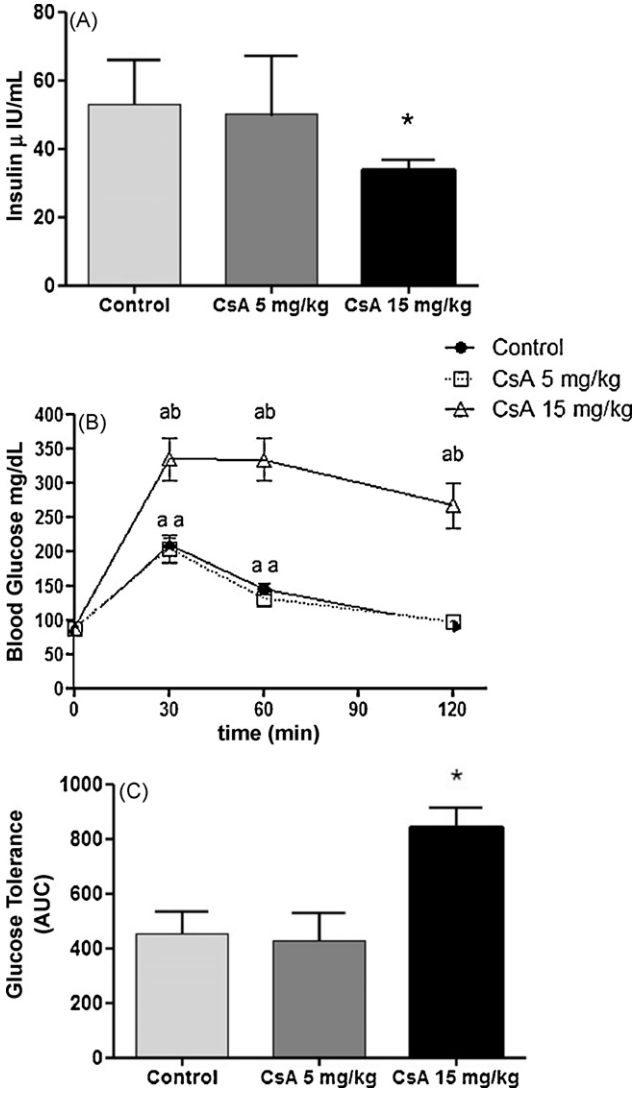


Fig. 2. (A) Insulin levels after 8 weeks of treatment with CsA (5 and 15 mg/kg) and vehicle (control), *$P < 0.001$ compared to control and CsA 5 mg/kg groups ($n = 7$/group). (B) Glucose tolerance test (GTT), [a] $P < 0.001$ compared to the beginning of glucose tolerance test; [b] $P < 0.001$ compared to control and CsA 5 mg/kg groups at the same time of treatment ($n = 15$/group). (C) Area under the curve (AUC) of glucose (mg/dL/min) was calculated and indicates glucose intolerance in CsA 15 mg/kg group, *$P < 0.001$ versus control and CsA 5 mg/kg. Results are presented as mean ± standard deviation.

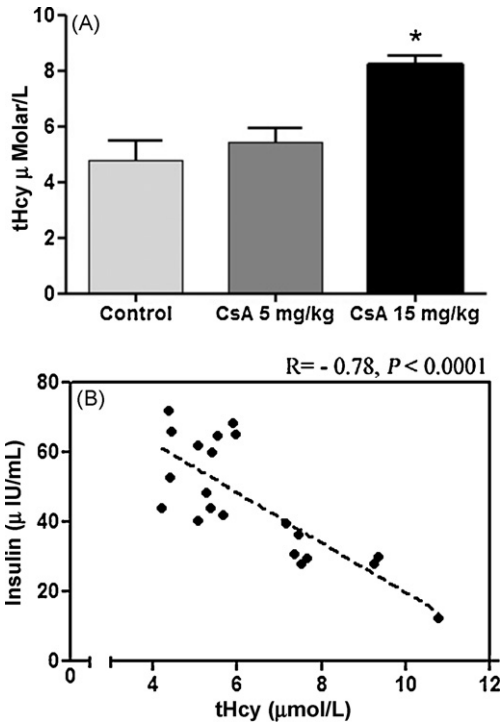


Fig. 3. (A) Serum tHcy levels. tHcy levels increased in CsA 15 mg/kg group (8.2 ± 1.2 µmol/L, $n = 14$), compared to CsA 5 mg/kg (5.4 ± 0.5, $n = 14$) and control groups (4.7 ± 0.7 µmol/L, $n = 14$). Values are presented as mean ± S.D. *$P < 0.001$. (B) Correlation between serum tHcy and insulin levels of all groups ($n = 7$/group).

3.3. Serum total homocysteine levels (tHcy)

Serum tHcy levels increased in CsA 15 mg/kg group (8.2 ± 1.2 µmol/L), compared to control (4.8 ± 0.7 µmol/L; $P < 0.001$) and CsA 5 mg/kg (5.4 ± 0.5 µmol/L; $P < 0.001$, Fig. 3A) groups. Serum tHcy and insulin levels presented a significant negative correlation ($R = -0.78$, $P < 0.0001$, Fig. 3B).

3.4. Coagulatory parameters

Plasma fibrinogen levels and platelet number significantly increased in CsA 15 mg/kg group compared to control and CsA 5 mg/kg groups ($P < 0.01$, Table 1). The increasing in fibrinogen and tHcy serum levels presented a positive correlation ($R = 0.43$, $P < 0.02$, Fig. 4A).

3.5. Serum lipids profile

Serum triglyceride, total cholesterol and VLDL levels were higher in CsA 15 mg/kg group, compared to control and CsA 5 mg/kg groups ($P < 0.005$, Table 1). Additionally, these parameters were positively correlated with serum tHcy levels (Fig. 4B–D). LDL and HDL serum levels did not differ among groups (Table 1).

3.6. Leptin serum levels

Leptin serum levels decreased by CsA 5 and 15 mg/kg treatments, compared to control group ($P < 0.01$, Table 1).

4. Discussion

CsA, widely used in the clinic as part of the protocol of immunosuppression [33,39], may present serious adverse effects, which is masked by the concomitant use of other drugs [4,29,33–36]. The

Table 1
Serum lipid and hemostatic parameters in control and CsA-treated rats.

Variable	Control	CsA 5 mg/kg	CsA 15 mg/kg
Leptin (μM)	134.7 ± 38.9	98.7 ± 7.1†	94.6 ± 16.7†
tHcy (μmol/L)	4.7 ± 0.7	5.4 ± 0.5	8.2 ± 1.2*
Fibrinogen (mg/dL)	362.7 ± 94.7	377.4 ± 132.1	596.0 ± 259.7*
Platelet number ($\times 10^4$/mm^3)	58.4 ± 3.8	64.4 ± 5.6	68.4 ± 9.3*
Triglycerides (mg/dL)	62.0 ± 12.4	72.0 ± 13.9	125.0 ± 24.0**
Total cholesterol (mg/dL)	58.0 ± 8.7	63.0 ± 9.0	73.0 ± 8.5**
VLDL (mg/dL)	12.0 ± 2.4	14.0 ± 2.8	25.0 ± 4.8**
LDL (mg/dL)	17.0 ± 6.0	16.6 ± 6.0	15.0 ± 6.0
HDL (mg/dL)	32.0 ± 8.0	32.7 ± 7.0	34.3 ± 9.0

Data are given as mean ± standard deviation; $n = 10$–15/group.
* $P < 0.01$ compared to control and CsA 5 mg/kg group.
** $P < 0.005$ compared to control and CsA 5 mg/kg group.
† $P < 0.01$ compared to control group.

search for effective immunosuppression protocols which does not affect the quality of life of patients is driving research to investigate the CsA involvement in vascular diseases, frequent in patients under immunosuppression [5,7,8,12].

The biochemical alterations reported here suggest the development of a framework straight to diabetes on CsA 15 mg/kg group. Accordingly, CsA 15 mg/kg treatment generated a biochemical profile coherent with glucose intolerance, including decreased fasting serum insulin levels and altered glucose tolerance test (GTT). The specific metabolic effects of calcineurin inhibitors as CsA in clinical studies are difficult to interpret, as the simultaneous administration of steroids, which frequently occurs, acts as a confounding factor [4]. Here we are indicating that CsA is involved in glucose homeostasis, and reinforcing reports that long-term CsA treatment affects glucose homeostasis, by decreasing serum insulin levels, increasing peripheral insulin resistance and serum glucose levels [14,15,19,20]. The effect on insulin levels may result from compromised insulin production either via β-cell toxicity or via inhibition of DNA synthesis [14,15,40–44].

Furthermore, we observed that non-transplanted rats under CsA 5 and 15 mg/kg treatment presented decreased serum leptin levels compared to control group. Together, during the treatment period, control group gained body weight, CsA 5 mg/kg remained with the same body weight and CsA 15 mg/kg group lost body weight. Leptin is mainly produced and secreted by fat tissue and, both insulin and leptin works as adiposity signals with numerous similarities in their physiological effects including glucose homeostasis [45]. Despite body fat mass was not assessed, we assume that, at least in part, the decrease in serum leptin levels could be related to a combination of decreased adipose tissue/body weight.

Although not yet included in the cluster of factors associated with the insulin resistance syndromes [46], serum tHcy levels are usually increased in conditions such as type 2 diabetes. A moderate elevation of tHcy is considered an independent risk factor for cardiovascular and cerebrovascular diseases [28,30–32,47,48]. Despite transplanted patients on CsA-based regimens have higher tHcy concentration associated with renal injury [29,32,35,36,49], in our experimental model, CsA 15 mg/kg increased serum tHcy concentrations in rats with no histopathological and functional kidney injury [38]. Thus, it is likely that tHcy serum concentrations are under influence of other mechanisms which does not involve kidney injury. Indeed, serum tHcy levels showed a nega-

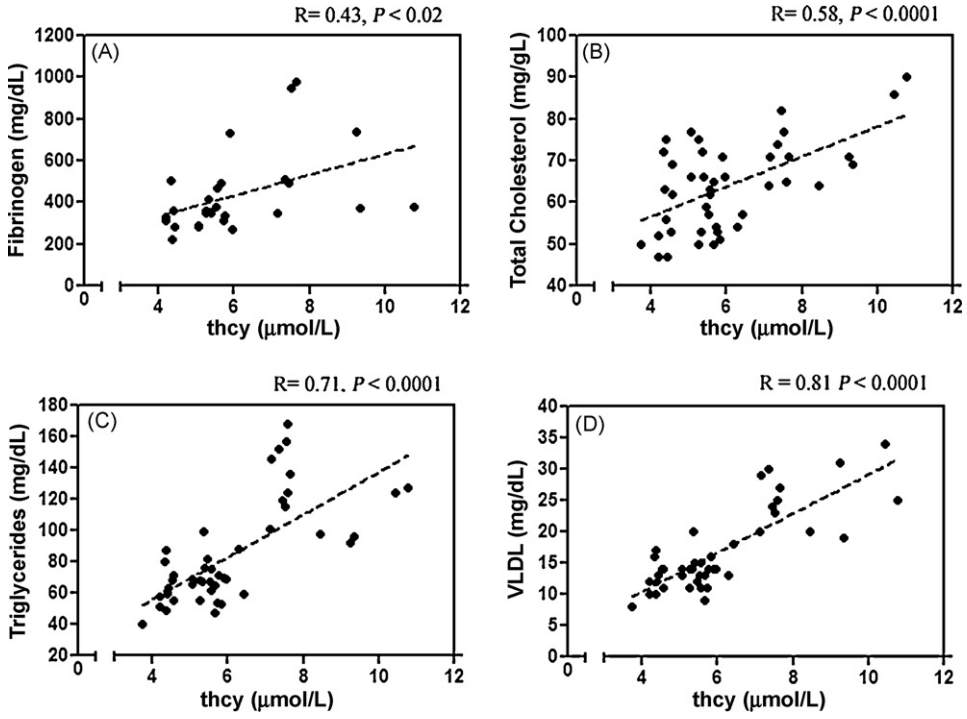


Fig. 4. Correlations among tHcy serum levels and plasma fibrinogen (A, $n = 26$), total cholesterol (B, $n = 45$), triglycerides (C, $n = 45$) and VLDL (D, $n = 45$) serum levels of all groups. There was a statistically significant correlation between tHcy levels and each parameter analyzed, by Pearson's correlation test.

tive correlation with insulin levels. Tessari et al. showed that insulin acutely increases homocysteine transmethylation, transsulfuration and clearance *in vivo*, and may thus prevent homocysteine accumulation in body fluids [50]. Here, we showed a significant negative correlation ($R = -0.78$, $P < 0.0001$) between serum tHcy and insulin levels of all groups. Albeit we did not establish a causal role, the modulation exerted by insulin on tHcy metabolism seems to be one relevant strategy to avoid adverse effects in CsA therapy.

Plasma fibrinogen levels and platelet number were also increased by CsA 15 mg/kg treatment. Fibrinogen participates in the two central processes of atherosclerosis (inflammation and thrombosis) and strongly affects serum viscosity, erythrocyte sedimentation rate [51] and increases platelet aggregability [52]. Increased plasma levels of fibrinogen have been associated with augmented risks for coronary disease and acute stroke [53]. Thus, an interaction toward an enhanced risk of thrombotic events might be expected in a scenario with high fibrinogen, platelets and tHcy levels, as observed in this study.

Clinical studies have already reported alteration in serum lipid levels in patients under CsA therapy. Here, serum triglycerides, cholesterol and VLDL were enhanced by CsA 15 mg/kg. It has been shown that CsA elicit a significant reduction in hepatic tissue cholesterol 7α-hydroxylase [3], a rate-limiting enzyme in cholesterol conversion to bile acid, and a marked down-regulation of lipoprotein lipase expression in adipose and skeletal muscle tissues of CsA-treated rats. Interestingly, enhanced triglyceride-rich lipoproteins have a considerable impact in patients with mild-to-moderate atherosclerotic lesions [54]. Once atherogenic lipoproteins VLDL and LDL deposit in the intima, they exert direct or indirect proinflammatory effects [55]. The high positive correlation among tHcy and lipid levels observed in our study suggests that tHcy, by increasing oxidant stress [56], could be altering lipid metabolism. Adverse effects including dyslipidemia and glucose intolerance are extremely common after transplantation and contribute significantly to cardiovascular morbidity and mortality. Considering the well-established correlation among insulin resistance, lipid and tHcy levels, hypercoagulability and atherosclerosis, we can assume that CsA therapy place individuals at increased risk for cardiovascular and cerebrovascular diseases [7,57].

In conclusion, our protocol of long-term CsA treatment in non-transplanted rats alter biochemical parameters related to cardiovascular and cerebrovascular risk, mainly in CsA 15 mg/kg group. Insulin, lipids, fibrinogen and tHcy serum levels appear to be central in this process.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by CNPq, FAPERGS and MCT-CNPq/MS-SCTIE-DECIT-DAF (No. 54/2005), IBNet, INCT for Excitotoxicity and Neuroprotection/CNPq. The authors thank Alexandre P. Müller and Marcos L. Perry for critical reading of the manuscript.

References

[1] S. Ho, N. Clipstone, L. Timmermann, J. Northrop, I. Graef, D. Fiorentino, J. Nourse, G.R. Crabtree, The mechanism of action of cyclosporin A and FK506, Clin. Immunol. Immunopathol. 80 (3 Pt 2) (1996) S40–45.
[2] R. Rezzani, Cyclosporine A and adverse effects on organs: histochemical studies, Prog. Histochem. Cytochem. 39 (2) (2004) 85–128.
[3] N.D. Vaziri, K. Liang, H. Azad, Effect of cyclosporine on HMG-CoA reductase, cholesterol 7alpha-hydroxylase, LDL receptor, HDL receptor, VLDL receptor, and lipoprotein lipase expressions, J. Pharmacol. Exp. Ther. 294 (2) (2000) 778–783.
[4] S. Subramanian, D.L. Trence, Immunosuppressive agents: effects on glucose and lipid metabolism, Endocrinol. Metab. Clin. North Am. 36 (4) (2007) 891–905, vii.
[5] N.J. Serkova, U. Christians, L.Z. Benet, Biochemical mechanisms of cyclosporine neurotoxicity, Mol. Interv. 4 (2) (2004) 97–107.
[6] A. Magnasco, A. Rossi, P. Catarsi, R. Gusmano, F. Ginevri, F. Perfumo, G.M. Ghiggeri, Cyclosporin and organ specific toxicity: clinical aspects, pharmacogenetics and perspectives, Curr. Clin. Pharmacol. 3 (3) (2008) 166–173.
[7] A Demirag, G. Moray, N. Ozdemir, N. Hizel, N. Bilgin, M. Haberal, Comparison of serum lipids, lipoproteins, and fibrinogen in patients with kidney transplantation and hemodialysis, Transplant. Proc. 30 (5) (1998) 2052–2053.
[8] G. Tellides, J.S. Pober, Interferon-gamma axis in graft arteriosclerosis, Circ. Res. 100 (5) (2007) 622–632.
[9] J. Malyszko, J.S. Malyszko, K. Pawlak, M. Mysliwiec, The coagulo-lytic system and endothelial function in cyclosporine-treated kidney allograft recipients, Transplantation 62 (6) (1996) 828–830.
[10] J.A. Kobashigawa, B.L. Kasiske, Hyperlipidemia in solid organ transplantation, Transplantation 63 (3) (1997) 331–338.
[11] F. Moien-Afshari, B.M. McManus, I. Laher, Immunosuppression and transplant vascular disease: benefits and adverse effects, Pharmacol. Ther. 100 (2) (2003) 141–156.
[12] J.M. Boots, M.H. Christiaans, J.P. van, Hooff, Effect of immunosuppressive agents on long-term survival of renal transplant recipients: focus on the cardiovascular risk, Drugs 64 (18) (2004) 2047–2073.
[13] G. Stallone, B. Infante, A. Schena, S. Di Paolo, G. Grandaliano, L. Gesualdo, F.P. Schena, Cardiovascular risk and renal transplantation, G. Ital. Nefrol. 21 (2) (2004) 144–155.
[14] J. Hjelmesaeth, A. Asberg, F. Muller, A. Hartmann, T. Jenssen, New-onset post-transplantation diabetes mellitus: insulin resistance or insulinopenia? Impact of immunosuppressive drugs, cytomegalovirus and hepatitis C virus infection, Curr. Diabetes Rev. 1 (1) (2005) 1–10.
[15] A Penfornis, S. Kury-Paulin, Immunosuppressive drug-induced diabetes, Diabetes Metab. 32 (5 Pt 2) (2006) 539–546.
[16] P. Marchetti, New-onset diabetes after transplantation, J. Heart Lung Transplant. 23 (5 Suppl.) (2004) S194–201.
[17] A.M. Miles, N. Sumrani, R. Horowitz, P. Homel, V. Maursky, M.S. Markell, D.A. Distant, J.H. Hong, B.G. Sommer, E.A. Friedman, Diabetes mellitus after renal transplantation: as deleterious as non-transplant-associated diabetes? Transplantation 65 (3) (1998) 380–384.
[18] S. Baid, A.B. Cosimi, M.L. Farrell, D.A. Schoenfeld, S. Feng, R.T. Chung, N. Tolkoff-Rubin, M. Pascual, Posttransplant diabetes mellitus in liver transplant recipients: risk factors, temporal relationship with hepatitis C virus allograft hepatitis, and impact on mortality, Transplantation 72 (6) (2001) 1066–1072.
[19] J Hjelmesaeth, A. Hartmann, J. Kofstad, T. Egeland, J. Stenstrom, P. Fauchald, Tapering off prednisolone and cyclosporin the first year after renal transplantation: the effect on glucose tolerance, Nephrol. Dial. Transplant. 16 (4) (2001) 829–835.
[20] J. Hjelmesaeth, K. Midtvedt, T. Jenssen, A. Hartmann, Insulin resistance after renal transplantation: impact of immunosuppressive and antihypertensive therapy, Diabetes Care 24 (12) (2001) 2121–2126.
[21] J.A. Beckman, M.A. Creager, P. Libby, Diabetes and atherosclerosis: epidemiology, pathophysiology, and management, JAMA 287 (19) (2002) 2570–2581.
[22] M.S Markell, V. Armenti, G. Danovitch, N. Sumrani, Hyperlipidemia and glucose intolerance in the post-renal transplant patient, J. Am. Soc. Nephrol. 4 (8 Suppl.) (1994) S37–47.
[23] K.C. Bilchick, C.A. Henrikson, D. Skojec, E.K. Kasper, R.S. Blumenthal, Treatment of hyperlipidemia in cardiac transplant recipients, Am. Heart J. 148 (2) (2004) 200–210.
[24] M. Arnadottir, B. Hultberg, V. Vladov, P. Nilsson-Ehle, H. Thysell, Hyperhomocysteinemia in cyclosporine-treated renal transplant recipients, Transplantation 61 (3) (1996) 509–512.
[25] N. Nouri-Majalan, R. Masoumi, R. Nafisi, H. Nogh, A. Ghafari, S. Moghaddasi, Relationship between serum homocysteine and other parameters in renal transplant patients, Transplant. Proc. 41 (7) (2009) 2826–2828.
[26] M.R. Malinow, Plasma homocyst(e)ine: a risk factor for arterial occlusive diseases, J. Nutr. 126 (4 Suppl) (1996) 1238S–1243S.
[27] E.L. Mayer, D.W. Jacobsen, K. Robinson, Homocysteine and coronary atherosclerosis, J. Am. Coll. Cardiol. 27 (3) (1996) 517–527.
[28] C.J. Boushey, S.A. Beresford, G.S. Omenn, A.G. Motulsky, A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes, JAMA 274 (13) (1995) 1049–1057.
[29] J.I. Herrero, J. Quiroga, B. Sangro, O. Beloqui, F. Pardo, J.A. Cienfuegos, J. Prieto, Hyperhomocysteinemia in liver transplant recipients: prevalence and multivariate analysis of predisposing factors, Liver Transplant. 6 (5) (2000) 614–618.
[30] J.W. Eikelboom, E. Lonn, J. Genest Jr., G. Hankey, S. Yusuf, Homocyst(e)ine and cardiovascular disease: a critical review of the epidemiologic evidence, Ann. Intern. Med. 131 (5) (1999) 363–375.
[31] C. Antoniades, A.S. Antonopoulos, D. Tousoulis, K. Marinou, C. Stefanadis, Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials, Eur. Heart J. 30 (1) (2009) 6–15.
[32] K. Potter, G.J. Hankey, D.J. Green, J. Eikelboom, K. Jamrozik, L.F. Arnolda, The effect of long-term homocysteine-lowering on carotid intima-media thickness and flow-mediated vasodilation in stroke patients: a randomized controlled trial and meta-analysis, BMC Cardiovasc. Disord. 8 (2008) 24.
[33] R. John, H.A. Rajasinghe, S. Itescu, S. Suratwala, K. Lietz, A.D. Weinberg, A. Kocher, D.M. Mancini, R.E. Drusin, M.C. Oz, C.R. Smith, E.A. Rose, N.M. Edwards,

Factors affecting long-term survival (>10 years) after cardiac transplantation in the cyclosporine era, J. Am. Coll. Cardiol. 37 (1) (2001) 189–194.
[34] J.C. Roussel, O. Baron, C. Perigaud, P. Bizouarn, S. Pattier, O. Habash, A. Mugniot, T. Petit, J.L. Michaud, M.F. Heymann, M. Treilhaud, J.N. Trochu, J.P. Gueffet, G. Lamirault, D. Duveau, P. Despins, Outcome of heart transplants 15 to 20 years ago: graft survival, post-transplant morbidity, and risk factors for mortality, J. Heart Lung Transplant. 27 (5) (2008) 486–493.
[35] M Arnadottir, B. Hultberg, J. Wahlberg, B. Fellstrom, E. Dimeny, Serum total homocysteine concentration before and after renal transplantation, Kidney Int. 54 (4) (1998) 1380–1384.
[36] M. Naesens, D.R. Kuypers, M. Sarwal, Calcineurin inhibitor nephrotoxicity, Clin. J. Am. Soc. Nephrol. 4 (2) (2009) 481–508.
[37] A.P. Muller, M. Cammarota, M.O. Dietrich, L.N. Rotta, L.V. Portela, D.O. Souza, I. Izquierdo, L.R. Bevilaqua, M.L. Perry, Different effect of high fat diet and physical exercise in the hippocampal signaling, Neurochem. Res. 33 (5) (2008) 880–885.
[38] A.E. Böhmer, A.M.R. Corrêa, D.G. Souza, L. Knorr, G. Hansel, L.G. Corbellini, D. Driemeier, L.V. Portela, D.O. Souza, Long-term cyclosporine treatment: evaluation of serum biochemical parameters and histopathological alterations in Wistar rats, Exp. Toxicol. Pathol. (2009), doi:10.1016/j.etp.2009.10.005.
[39] A.L. Taylor, C.J. Watson, J.A. Bradley, Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy, Crit. Rev. Oncol. Hematol. 56 (1) (2005) 23–46.
[40] H.J. Hahn, F. Laube, S. Lucke, I. Kloting, K.D. Kohnert, R. Warzock, Toxic effects of cyclosporine on the endocrine pancreas of Wistar rats, Transplantation 41 (1) (1986) 44–47.
[41] J.H. Nielsen, T. Mandrup-Poulsen, J. Nerup, Direct effects of cyclosporin A on human pancreatic beta-cells, Diabetes 35 (9) (1986) 1049–1052.
[42] H.J. Hahn, A. Dunger, F. Laube, W. Besch, E. Radloff, C. Kauert, G. Kotzke, Reversibility of the acute toxic effect of cyclosporin A on pancreatic B cells of Wistar rats, Diabetologia 29 (8) (1986) 489–494.
[43] M.D. Stegall, J. Chabot, C. Weber, K. Reemtsma, M.A. Hardy, Pancreatic islet transplantation in cynomolgus monkeys. Initial studies and evidence that cyclosporine impairs glucose tolerance in normal monkeys, Transplantation 48 (6) (1989) 944–950.
[44] J. Borlak, M. Niehof, HNF4alpha and HNF1alpha dysfunction as a molecular rational for cyclosporine induced posttransplantation diabetes mellitus, PLoS One 4 (3) (2009) e4662.
[45] L.A. Tartaglia, M. Dembski, X. Weng, N. Deng, J. Culpepper, R. Devos, G.J. Richards, L.A. Campfield, F.T. Clark, J. Deeds, C. Muir, S. Sanker, A. Moriarty, K.J. Moore, J.S. Smutko, G.G. Mays, E.A. Wool, C.A. Monroe, R.I. Tepper, Identification and expression cloning of a leptin receptor, OB-R, Cell 83 (7) (1995) 1263–1271.
[46] Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III) final report, Circulation 106 (25) (2002) 3143–3421.
[47] M. den Heijer, F.R. Rosendaal, H.J. Blom, W.B. Gerrits, G.M. Bos, Hyperhomocysteinemia and venous thrombosis: a meta-analysis, Thromb. Haemost. 80 (6) (1998) 874–877.
[48] A.E. Böhmer, D. Pochmann, J.J. Sarkis, In vitro effect of homocysteine on nucleotide hydrolysis by blood serum from adult rats, Chem. Biol. Interact. 160 (2) (2006) 159–164.
[49] D.E. Cole, H.J. Ross, J. Evrovski, L.J. Langman, S.E. Miner, P.A. Daly, P.Y. Wong, Correlation between total homocysteine and cyclosporine concentrations in cardiac transplant recipients, Clin. Chem. 44 (11) (1998) 2307–2312.
[50] P. Tessari, E. Kiwanuka, A. Coracina, M. Zaramella, M. Vettore, A. Valerio, G. Garibotto, Insulin in methionine and homocysteine kinetics in healthy humans: plasma vs. intracellular models, Am. J. Physiol. Endocrinol. Metab. 288 (6) (2005) E1270–1276.
[51] F. Van Lente, Markers of inflammation as predictors in cardiovascular disease, Clin. Chim. Acta 293 (1–2) (2000) 31–52.
[52] J. Lefkovits, E.F. Plow, E.J. Topol, Platelet glycoprotein IIb/IIIa receptors in cardiovascular medicine, N. Engl. J. Med. 332 (23) (1995) 1553–1559.
[53] W.H. Frishman, Biologic markers as predictors of cardiovascular disease, Am. J. Med. 104 (6A) (1998) 18S–27S.
[54] H.N. Hodis, W.J. Mack, S.P. Azen, P. Alaupovic, J.M. Pogoda, L. LaBree, L.C. Hemphill, D.M. Kramsch, D.H. Blankenhorn, Triglyceride- and cholesterol-rich lipoproteins have a differential effect on mild/moderate and severe lesion progression as assessed by quantitative coronary angiography in a controlled trial of lovastatin, Circulation 90 (1) (1994) 42–49.
[55] J. Fan, T. Watanabe, Inflammatory reactions in the pathogenesis of atherosclerosis, J. Atheroscler. Thromb. 10 (2) (2003) 63–71.
[56] J. Loscalzo, The oxidant stress of hyperhomocyst(e)inemia, J. Clin. Invest. 98 (1) (1996) 5–7.
[57] S.J. Bensinger, P. Tontonoz, Integration of metabolism and inflammation by lipid-activated nuclear receptors, Nature 454 (7203) (2008) 470–477.

CAPÍTULO III

Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and vascular disturbances in rats.

Ana Elisa Böhmer, Liz M. B. P. Brum, Débora Guerini de Souza, André Mendes Ribeiro Corrêa, Jean Pierre Oses, Giordano Gubert Viola, Paulo Jaconi Saraiva, David Driemeier, Luis Valmor Portela, Diogo Onofre Souza.

Artigo submetido ao periódico Chemico-Biological Interactions.

Elsevier Editorial System(tm) for Chemico-Biological Interactions
Manuscript Draft

Manuscript Number: CHEMBIOINT-D-10-00201

Title: Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and vascular disturbances in rats.

Article Type: Research Paper

Keywords: Adenosine, cyclosporine, homocysteine, ecto-nucleotidase, vascular disease.

Corresponding Author: Dr. D.O.G. Souza,

Corresponding Author's Institution: Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

First Author: Ana E Böhmer

Order of Authors: Ana E Böhmer; Liz M Brum; Débora G Souza; André M Corrêa; Jean P Oses; Giordano G Viola; Paulo J Saraiva; David Driemeier; Luis V Portela; D.O.G. Souza

Abstract: Vascular disease is a major cause of morbidity and mortality among transplanted recipients and cyclosporine (CsA) treatment has been consistently implicated in this event. In this study we assessed total blood homocysteine levels (tHcy), ecto-nucleotidase activities and adenine nucleotide/nucleoside levels searching for parameters related to the mechanisms of vascular damage induced by chronic CsA treatment in non-transplanted rats. Thirty male Wistar rats were divided in three groups: control group treated with corn oil, CsA 5 mg/Kg and CsA 15 mg/Kg, administered by daily gastric gavage during 8 weeks. CsA 15 mg/Kg treatment increased blood levels of tHcy. Both CsA treatments (5 and 15 mg/Kg) decreased adenine nucleotides hydrolysis by ecto-nucleotidases in serum, which negatively correlated with tHcy levels (r: - 0.74, r: - 0.63 and r: - 0.63, $p < 0.004$, for ATP, ADP and AMP, respectively). CsA 15 mg/Kg induced a statistically significant increase in ADP and decrease in adenosine (ADO) plasma levels compared to control group. THcy levels were positively correlated with plasma ADP levels and negatively correlated with ADO levels (r: 0.84, $p < 0.0001$ and r: - 0.68, $p < 0.0001$, respectively). Rats under CsA 15 mg/Kg treatment presented cell injury and inflammatory responses in the endothelium and intima layer of the aorta artery. In conclusion, blood ecto-nucleotidases activity, tHcy, and ADP and ADO levels may be implicated in vascular injury induced by CsA treatment.

Chemico-Biological Interactions

April 15th, 2010.

Dear Editor,

Please find enclosed a copy of the manuscript “Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and vascular disturbances in rats” by Böhmer et al. submitted as an original article to *Chemico-Biological Interactions*.

This manuscript is not submitted to another journal and the results presented are originals. This research was funded by the Brazilian funding agencies INCT: Excitotoxicity and Neuroprotection, CNPq, and FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)”; however, no author has a financial arrangement with any company or organization that might be a conflict of interest. Therefore, the authors declare that there are no conflicts of interest.

Moreover, we state that this research and all experimental animals’ procedures have been performed in accordance with the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and were approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

We greatly appreciate your consideration.

Respectfully,

(✉) Diogo Onofre Souza

Department of Biochemistry
Federal University of Rio Grande do Sul.
Avenida Ramiro Barcelos, 2600-Anexo
Zip Code 90035-003 Porto Alegre - RS – Brazil
Tel: (55-51) 33085559 / 33085558
Fax: (55-51) 33085540 / 33085535
E-mail: diogo@ufrgs.br

Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and vascular disturbances in rats

Ana Elisa Böhmer [a], Liz Marina P. Brum [b], Débora Guerini Souza [a], André Mendes Ribeiro Corrêa [c], Jean Pierre Oses [d], Giordano Gubert Viola [a], Paulo Jaconi Saraiva[e], David Driemeier [c], Luis Valmor Portela [a], Diogo Onofre Souza (✉) [a].

[a] Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

[b] Cardiology Institute of Rio Grande do Sul, FUC, Porto Alegre, RS, Brazil.

[c] Department of Preventive Veterinary Medicine, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

[d] Department of Psychology, Católica de Pelotas University, Pelotas, RS, Brazil.

[e] Department of Analysis, Pharmacy School, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

(✉) Diogo O. Souza

Avenida Ramiro Barcelos, 2600-Anexo

Zip Code: 90.035-003

Porto Alegre - RS - Brazil

Phone: (55-51) 33085559 / 33085558

Fax: (55-51) 33085540 / 33085535

E-mail: diogo@ufrgs.br

Abstract

Vascular disease is a major cause of morbidity and mortality among transplanted recipients and cyclosporine (CsA) treatment has been consistently implicated in this event. In this study we assessed total blood homocysteine levels (tHcy), ecto-nucleotidase activities and adenine nucleotide/nucleoside levels searching for parameters related to the mechanisms of vascular damage induced by chronic CsA treatment in non-transplanted rats. Thirty male Wistar rats were divided in three groups: control group treated with corn oil, CsA 5 mg/Kg and CsA 15 mg/Kg, administered by daily gastric gavage during 8 weeks. CsA 15 mg/Kg treatment increased blood levels of tHcy. Both CsA treatments (5 and 15 mg/Kg) decreased adenine nucleotides hydrolysis by ecto-nucleotidases in serum, which negatively correlated with tHcy levels (r: - 0.74, r: - 0.63 and r: - 0.63, $p < 0.004$, for ATP, ADP and AMP, respectively). CsA 15 mg/Kg induced a statistically significant increase in ADP and decrease in adenosine (ADO) plasma levels compared to control group. THcy levels were positively correlated with plasma ADP levels and negatively correlated with ADO levels (r: 0.84, $p < 0.0001$ and r: - 0.68, $p < 0.0001$, respectively). Rats under CsA 15 mg/Kg treatment presented cell injury and inflammatory responses in the endothelium and intima layer of the aorta artery. In conclusion, blood ecto-nucleotidases activity, tHcy, and ADP and ADO levels may be implicated in vascular injury induced by CsA treatment.

Key words: Adenosine, cyclosporine, homocysteine, ecto-nucleotidase, vascular disease.

1. Introduction

Organ transplantation may induce damage to the endothelium by triggering an inflammatory cascade that may evolve to a chronic process known as graft vasculopathy. Unfortunately, many immunosuppressant agents such as cyclosporine (CsA), extensively used to treat autoimmune diseases and to prevent graft rejection [1], contribute to the damage involved in this multifactorial process [2]. Vascular disease is the major cause of morbidity and mortality among transplant recipients, surpassing even infection [3-6] and there is evidence showing that CsA may exert direct action on vascular endothelial cells [2, 7]. Additionally, CsA increases total homocysteine (tHcy) blood concentrations [8], which is an amino acid widely accepted as an independent risk factor for atherosclerosis [9, 10] and peripheral vascular disease [11, 12]. Hcy-induced vascular damage appears to be due to increased platelet adhesiveness [13], increased oxidant stress [14] and decreased plasmatic adenosine (ADO) levels [15].

Extracellular adenine nucleotides and ADO have been implicated in a variety of physiological roles in cardiovascular system. The regulation of extracellular concentrations of these purines is pivotal to achieve their effects through specific receptors. The nucleoside ADO, which may derive from the hydrolysis of ATP, ADP and AMP, has important beneficial effects on the vascular system including vasodilatation and inhibition of platelet aggregation [16, 17]. Several authors have described the implication of relevant roles of ATP and ADP in haemostatic, thrombotic and inflammatory processes [16, 18-20]. Ecto-nucleotidases are ecto-enzymes that hydrolyze extracellular nucleotides to their respective nucleosides, thus controlling the concentration of purines in the blood. The ecto-nucleotidase chain, composed by NTPDases (which hydrolyze tri- and diphosphate

nucleosides) and ecto-5'-nucleotidase (which hydrolyze monophosphate nucleosides) [21], modulates the responses mediated by nucleotides/nucleosides within the vascular system and may potentially be altered in pathological states. Accordingly, NTPDases activities are substantively reduced in the vasculature of injured or rejected grafts [20].

In this study we assessed systemic biochemical parameters involved in vascular disturbances induced by chronic CsA treatment in non-transplanted rats. Ecto-nucleotidase activities on adenine nucleotides in serum, the levels of nucleotides in plasma and tHcy serum levels were evaluated. Morphological evaluation of aorta artery was performed to correlate with serum/plasma parameters.

2. Materials and methods

2.1 Animals and treatments

Thirty Wistar rats (adult male rats, 120 days old, weighting 300-350 g), were kept on a 12-hour light/dark cycle (light on at 7:00 am) at temperatures of 22 º ± 1 ºC, housed in plastic cages (5 per cage) with tap water and commercial food *ad libitum*. Animals were obtained from the Central Animal House of the Biochemistry Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

Rats were divided in three groups according to the treatment schedule. The control group received vehicle (corn oil), and CsA-treated groups received CsA 5 mg/kg or CsA 15 mg/kg diluted in corn oil. The drug or vehicle administration was performed by daily gastric gavage for 8 weeks. Animals were anesthetized by sodium thiopental (40 mg/kg, 1

mL/kg i.p.; supplemented as needed) and blood/plasma samples were collected by cardiac punction 24 hours after the last CsA or vehicle administration. All samples were centrifuged in plastic tubes at 5,000×g for 5 min at 4 °C and the serum or plasma were stored at −70 °C until analysis.

2.2 Reagents and biochemical measurements

CsA concentration in whole blood was determined by enzyme multiplied immunoassay test (EMIT-Green Liquid, Dade-Behring on Cobas Mira, Roche Diagnostic Systems, USA). Serum tHcy concentrations were measured using a commercial MEIA kit (Abbott, USA). Nucleotides and nucleoside were purchased from Sigma Chemical Co., St Louis, MO, USA. All others reagents were of analytical grade. Sodium thiopental was obtained from Cristália (São Paulo, Brazil).

2.3 Measurement of ATP and ADP hydrolysis in rat serum

ATP and ADP hydrolysis were evaluated as described [22]. The reaction medium containing 3.0 mM ADP or ATP as substrate, 1.0-1.5 mg serum protein and 112.5 mM Tris-HCl, pH 8.0, was incubated at 37 ^{o}C for 40 minutes in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of trichloroacetic acid 10% (TCA). All samples were centrifuged at 5,000 g for 5 min and the supernatants were used for measuring the amount of inorganic phosphate (Pi) released through a colorimetric assay [23]. Incubation time and protein concentrations were chosen to ensure the linearity of the reaction (results not shown). To correct for non-enzymatic hydrolysis, controls were performed by adding serum after TCA. All samples were assayed in duplicate. Enzyme activities were expressed as nmol of Pi released per minute per milligram of protein.

2.4 Measurement of AMP hydrolysis in rat serum

To evaluate AMP hydrolysis the reaction medium, containing 3.0 mM AMP as substrate in 100 mM Tris-HCl, pH 7.5, was incubated with 1.0–1.5 mg of serum protein at 37 ^{o}C for 40 minutes in a final volume of 0.2 mL. All other procedures were the same as described above for ATP and ADP hydrolysis.

2.5 Protein determination

Protein was measured by the Coomassie Blue method [24], using bovine serum albumin as standard.

2.6 HPLC procedure

High-performance liquid chromatography (HPLC) was performed with plasma. Samples were deproteinized with trifluoroacetic acid 7% and cell-free supernatants aliquots were used for determination of purines concentration, according to the method used by Schmidt et al., 2009 [25]. Plasma concentrations of the following purines were determined: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine (ADO). Analyses were performed with Shimadzu Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 μL loop, and an UV detector. Separations were achieved on a Supelco C18 250 mm × 4.6 mm, 5 μm particle size column. The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24 °C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile

in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 50 μL were injected into the injection valve loop.

2.7 Morphological Evaluation

Morphological data were obtained from the aorta artery of five animals of each experimental group (Control, CsA 5 and CsA 15 mg/Kg). After exsanguination, the aorta were removed, gently washed and fixed with 10% buffered formalin. Tissues were dehydrated in gradual alcohol from 50% to 100%, cleared by xylene and embedded in paraffin. Two to five micrometers-thick sections were stained with hematoxylin and eosin (H&E) to observe the general structure. Sections were also processed for immunohistochemical (IHC) staining using a monoclonal antibody against von Willebrand factor (the former designation for von Willebrand factor was Factor VIII-related antigen - Dako M0616) at 1:200 dilution in the streptavidin–biotin–immunoperoxidase (Dako) technique, with diaminobenzidine (Dako) as chromogen to evaluate the pattern of endothelial cells. Slides were evaluated with light microscopy by two pathologists blinded to groups treatments.

2.8 Data analysis

The statistical analyses were performed using the GraphPad software (GraphPad software, San Diego, CA). Data were analyzed using one-way ANOVA followed by the Tukey's multiple range tests. Correlations among numerical variables were performed by

Pearson's test. Data are expressed as mean ± S.E.M. $P < 0.05$ was considered to represent a statistical significant difference.

3. Results

3.1 Serum total homocysteine

Serum tHcy levels were significantly higher in CsA 15 mg/kg group (8.72 ± 0.33 µmol/L) compared to CsA 5 mg/kg and control groups (5.56 ± 0.10 and 4.72 ± 0.15 µmol/L, respectively, $p < 0.0001$, n = 9/group), with no statistical significant difference between the lowest dose and controls.

3.2 ATP, ADP and AMP hydrolysis

CsA treatment inhibited serum hydrolysis of nucleotides (Figure 1). CsA 5 mg/kg and 15 mg/kg groups inhibited ATP hydrolysis respectively by 27% and 37%, compared to control group (0.70 ± 0.05 nmol Pi/min/mg). ADP hydrolysis by 36% and 46% and AMP hydrolysis by 34% in both CsA treated groups compared to control groups (0.83 ± 0.07 and 1.36 ± 0.07 nmol Pi/min/mg, respectively). $P < 0.05$.

There were inverse correlations of serum tHcy levels with serum ATP, ADP and AMP hydrolysis (Figure 2).

3.3 Plasma levels of adenosine and nucleotides

As shown in Figure 3, administration of CsA 15 mg/Kg produced a 3 fold increase in plasma ADP levels when compared to control group (0.048 ± 0.008 µM and 0.015 ± 0.003, respectively). Simultaneously, CsA 15 mg/kg produced a significant decrease in

ADO plasma levels compared to control group (0.010 ± 0.002 µM and 0.025 ± 0.004 µM, respectively). Neither ATP nor AMP plasma levels were affected by CsA treatments.

Serum tHcy levels were significantly correlated with ADP plasma levels (Figure 4A) and negatively correlated with ADO plasma levels (Figure 4B).

3.4 Morphological evaluation of aorta artery

The control and CsA 5 mg/kg groups showed a similar pattern of aorta artery morphology, with normal spindle-shaped endothelial cells in the H&E sections, with flat and almost imperceptible endothelial cells and intimal layer, as shown in histological sections of a representative aorta from control group (Figure 5A). However, CsA 15 mg/Kg treated group presented hypertrophy of the endothelial cells with roughening of the intimal surface. Endothelial cells were plump with nuclear enlargement (Figure 5B). Control and CsA 5 mg/kg groups showed normal spindle-shaped endothelial cells (Figure 5C), whereas the CsA 15 mg/kg group showed thickening of the endothelial layers cells filled with floccular or granular debris (Figure 5D).

4. Discussion

Cardiovascular disease refers to disorders, especially of atherogenic nature, which involve the heart, brain, and/or lower limbs. Among specific cardiovascular risk factors specific for transplantation, immunosuppression plays a relevant role. CsA administration has been the basis for most immunosuppressive protocols for more than two decades [1]. Unfortunately, a direct action of CsA on the vasculature has been evidenced as a possible mechanism involved in vascular disease [2, 7]. However, after transplantation the presence of multiple confounding factors related to the clinical setting make it difficult to establish a

causal effect between CsA treatment and adverse effects. For this reason, we investigated blood parameters that could be involved in vascular adverse effects induced by chronic CsA administration to non-transplanted rats.

Previous study from our group [26] showed that the same protocol of CsA administration increased CsA blood levels in a dose dependent manner and induces a decrease in the serum levels of cytokines that mediate immunological responses (IL-1α, IL-1β and IL-2). Moreover, such treatment with CsA does not cause evident histopathological alterations in kidney and liver, neither in serum markers of kidney and liver functions as creatinine, urea, SGPT and SGOT [26]. In the present study, CsA 15 mg/kg treatment increased serum tHcy concentrations, an important and independent risk factor for cardiovascular diseases [27, 28]. Increased serum tHcy levels in transplant recipients have been proposed as an event implicated in the processes of accelerated allograft vasculopathy [8, 10, 12, 29-32]. Moderate elevation in plasma tHcy levels may alter vascular morphology and stimulate inflammation and blood-clotting cascades, causing damage to endothelium and inhibiting fibrinolysis [33].

Furthermore, CsA treated rats showed a decrease in serum ATP, ADP and AMP hydrolysis, an effect probably related to the observed reduction in plasma ADO levels. The inverse correlation between tHcy levels and adenine nucleotides hydrolysis and the inhibition of nucleotide hydrolysis by homocysteine [34] suggest that the inhibition of ecto-nucleotidase activities could be related to the increase of serum tHcy levels.

Recently, Hcy has been postulated to interfere with the metabolism of ADO [35-37]. The main source of ADO is intracellular and extracellular hydrolysis of AMP by endo and ecto-5'-nucleotidases [38], but an alternative source of ADO is the intracellular hydrolysis

of S-adenosylhomocysteine. CsA administration may impair both sources of ADO production: i) ATP, ADP and AMP degradation cascade is inhibited and ii) tHcy levels are increased. Elevated concentrations of tHcy shift the reaction equilibrium towards synthesis of S-adenosylhomocysteine. Consequently, intracellular ADO concentration decreases and also its facilitated diffusion to the extracellular milieu [39], reducing extracellular ADO levels. The strong correlation between serum tHcy and plasma ADP (positively) and ADO (negatively) levels observed in this study reinforce this hypothesis.

The regulation of extracellular ADO and ADP concentration is critical due to their physiological effects. ADO is a molecule with a variety of cardiovascular protective effects such as vasodilatation and inhibition of platelet aggregation, whereas ADP is known to induce changes in platelet shape and aggregation [16]. Previous results of our group showed that CsA 15 mg/kg treated rats had increased platelet number and fibrinogen levels in comparison to control group [40]. This effect associated with an increase in ADP plasma levels and a decrease in ADO plasma levels may produce a favorable scenario for the development of vascular diseases. The observed effects on endothelial morphology contribute to this hypothesis. Moreover, CsA 15 mg/kg treatment induced cell injury and inflammatory response involving the endothelium and the intimal layer of aorta artery.

The biological effects of nucleotides on the cardiovascular system are mainly determined by their release rate to the extracellular medium, ecto-nucleotidase activities and their binding affinity to their specific receptors. Ecto-nucleotidases may prolong the beneficial or detrimental effects of nucleotides [41, 42]. Although vascular disorders can be associated to an imbalance of the ratio of nucleotides/nucleosides in the circulation, the effects of CsA on adenine nucleotides hydrolysis has received little attention. Even though soluble NTPDase and 5’nucleotidase account for a small part of vascular nucleotides

hydrolysis [43], the inhibition of these enzymes may be representative of the bulk of anchored and soluble vascular nucleotidases activities responses. This conjecture brings into light the potential role of monitoring blood purinergic parameters during the course of CsA therapy.

In conclusion, non-transplanted rats under long-term treatment with CsA 15 mg/Kg presented cell injury and inflammatory responses of the endothelium and the intima layer of the aorta artery. The assessment of blood tHcy, ADP and ADO levels and ecto-nucleotidase activities may have a potential role in vascular disturbances induced by CsA long-term treatment and their assessment in patients on CsA treatment may be useful clinically.

Acknowledgments

This work was supported by CNPq, FAPERGS and MCT-CNPq/MS-SCTIE-DECIT-DAF (Nº 54/2005), IBNet, INCT for Excitotoxicity and Neuroprotection/CNPq. The authors are grateful to Diogo R. Lara for helpful comments during the preparation of this paper and also thank Carolina G. de Souza and Mariana Streit for their help in carrying out some of the experiments.

Conflict of Interest

The authors declare that there are no conflicts of interest.

References

[1] B.D. Kahan, Forty years of publication of transplantation proceedings--the second decade: the cyclosporine revolution, Transplant Proc 41(5) (2009) 1423-1437.

[2] T. Nickel, C.L. Schlichting, M. Weis, Drugs modulating endothelial function after transplantation, Transplantation 82(1 Suppl) (2006) S41-46.

[3] M. Weis, W. von Scheidt, Cardiac allograft vasculopathy: a review, Circulation 96(6) (1997) 2069-2077.

[4] E. Kendrick, Cardiovascular disease and the renal transplant recipient, Am J Kidney Dis 38(6 Suppl 6) (2001) S36-43.

[5] N.J. Serkova, U. Christians, L.Z. Benet, Biochemical mechanisms of cyclosporine neurotoxicity, Mol Interv 4(2) (2004) 97-107.

[6] J.A. Belperio, A. Ardehali, Chemokines and transplant vasculopathy, Circ Res 103(5) (2008) 454-466.

[7] F. Moien-Afshari, B.M. McManus, I. Laher, Immunosuppression and transplant vascular disease: benefits and adverse effects, Pharmacol Ther 100(2) (2003) 141-156.

[8] M. Arnadottir, B. Hultberg, V. Vladov, P. Nilsson-Ehle, H. Thysell, Hyperhomocysteinemia in cyclosporine-treated renal transplant recipients, Transplantation 61(3) (1996) 509-512.

[9] M.R. Malinow, Plasma homocyst(e)ine: a risk factor for arterial occlusive diseases, J Nutr 126(4 Suppl) (1996) 1238S-1243S.

[10] C. Antoniades, A.S. Antonopoulos, D. Tousoulis, K. Marinou, C. Stefanadis, Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials, Eur Heart J 30(1) (2009) 6-15.

[11] E.L. Mayer, D.W. Jacobsen, K. Robinson, Homocysteine and coronary atherosclerosis, J Am Coll Cardiol 27(3) (1996) 517-527.

[12] K. Potter, G.J. Hankey, D.J. Green, J. Eikelboom, K. Jamrozik, L.F. Arnolda, The effect of long-term homocysteine-lowering on carotid intima-media thickness and flow-mediated vasodilation in stroke patients: a randomized controlled trial and meta-analysis, BMC Cardiovasc Disord 8 (2008) 24.

[13] S.H. Mudd, Levy, H.L., Skovby, F., Disorders of Transulfuration., in: A.L.B. C.R. Scriver, W.S. Sly, D. Valle (Ed.), The Metabolic and Molecular Basis of Inherited Disease, McGraw-Hill, New York, 2001. pp. 1279-1327.

[14] J. Loscalzo, The oxidant stress of hyperhomocyst(e)inemia, J Clin Invest 98(1) (1996) 5-7.

[15] N.P. Riksen, G.A. Rongen, D. Yellon, P. Smits, Human in vivo research on the vascular effects of adenosine, Eur J Pharmacol 585(2-3) (2008) 220-227.

[16] V. Ralevic, G. Burnstock, Involvement of purinergic signaling in cardiovascular diseases, Drug News Perspect 16(3) (2003) 133-140.

[17] G. Cristalli, S. Vittori, R.D. Thompson, W.L. Padgett, D. Shi, J.W. Daly, R.A. Olsson, Inhibition of platelet aggregation by adenosine receptor agonists, Naunyn Schmiedebergs Arch Pharmacol 349(6) (1994) 644-650.

[18] G.V. Born, Aggregation of blood platelets by adenosine diphosphate and its reversal, Nature 194 (1962) 927-929.

[19] S.B. Coade, J.D. Pearson, Metabolism of adenine nucleotides in human blood, Circ Res 65(3) (1989) 531-537.

[20] S.C. Robson, Y. Wu, X. Sun, C. Knosalla, K. Dwyer, K. Enjyoji, Ectonucleotidases of CD39 family modulate vascular inflammation and thrombosis in transplantation, Semin Thromb Hemost 31(2) (2005) 217-233.

[21] S.K. Mishra, N. Braun, V. Shukla, M. Fullgrabe, C. Schomerus, H.W. Korf, C. Gachet, Y. Ikehara, J. Sevigny, S.C. Robson, H. Zimmermann, Extracellular nucleotide signaling in adult neural stem cells: synergism with growth factor-mediated cellular proliferation, Development 133(4) (2006) 675-684.

[22] J.P. Oses, C.M. Cardoso, R.A. Germano, I.B. Kirst, B. Rucker, C.R. Furstenau, M.R. Wink, C.D. Bonan, A.M. Battastini, J.J. Sarkis, Soluble NTPDase: An additional system of nucleotide hydrolysis in rat blood serum, Life Sci 74(26) (2004) 3275-3284.

[23] K.M. Chan, D. Delfert, K.D. Junger, A direct colorimetric assay for Ca2+ - stimulated ATPase activity, Anal Biochem 157(2) (1986) 375-380.

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

[25] A.P. Schmidt, A.E. Bohmer, C. Antunes, C. Schallenberger, L.O. Porciuncula, E. Elisabetsky, D.R. Lara, D.O. Souza, Anti-nociceptive properties of the xanthine oxidase inhibitor allopurinol in mice: role of A1 adenosine receptors, Br J Pharmacol 156(1) (2009) 163-172.

[26] A.E. Bohmer, A.M.R. Correa, D.G. Souza, L. Knorr, G. Hansel, L.G. Corbellini, D. Driemeier, L.V. Portela, D.O. Souza, Long-term cyclosporine treatment: Evaluation of serum biochemical parameters and histopathological alterations in Wistar rats, Exp Toxicol Pathol. - *in press.* doi:10.1016/j.etp.2009.10.005

[27] C.J. Boushey, S.A. Beresford, G.S. Omenn, A.G. Motulsky, A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes, JAMA 274(13) (1995) 1049-1057.

[28] S.E. Miner, J. Evrovski, D.E. Cole, Clinical chemistry and molecular biology of homocysteine metabolism: an update, Clin Biochem 30(3) (1997) 189-201.

[29] D.E. Wilcken, V.J. Gupta, A.K. Betts, Homocysteine in the plasma of renal transplant recipients: effects of cofactors for methionine metabolism, Clin Sci (Lond) 61(6) (1981) 743-749.

[30] P.B. Berger, J.D. Jones, L.J. Olson, B.S. Edwards, R.P. Frantz, R.J. Rodeheffer, B.A. Kottke, R.C. Daly, C.G. McGregor, Increase in total plasma homocysteine concentration after cardiac transplantation, Mayo Clin Proc 70(2) (1995) 125-131.

[31] A.G. Bostom, R.Y. Gohh, M.Y. Tsai, B.J. Hopkins-Garcia, M.R. Nadeau, L.A. Bianchi, P.F. Jacques, I.H. Rosenberg, J. Selhub, Excess prevalence of fasting and postmethionine-loading hyperhomocysteinemia in stable renal transplant recipients, Arterioscler Thromb Vasc Biol 17(10) (1997) 1894-1900.

[32] D.E. Cole, H.J. Ross, J. Evrovski, L.J. Langman, S.E. Miner, P.A. Daly, P.Y. Wong, Correlation between total homocysteine and cyclosporine concentrations in cardiac transplant recipients, Clin Chem 44(11) (1998) 2307-2312.

[33] O. Stanger, W. Herrmann, K. Pietrzik, B. Fowler, J. Geisel, J. Dierkes, M. Weger, Clinical use and rational management of homocysteine, folic acid, and B vitamins in cardiovascular and thrombotic diseases, Z Kardiol 93(6) (2004) 439-453.

[34] A.E. Bohmer, D. Pochmann, J.J. Sarkis, In vitro effect of homocysteine on nucleotide hydrolysis by blood serum from adult rats, Chem Biol Interact 160(2) (2006) 159-164.

[35] N.P. Riksen, G.A. Rongen, H.J. Blom, G.H. Boers, P. Smits, Reduced adenosine receptor stimulation as a pathogenic factor in hyperhomocysteinemia, Clin Chem Lab Med 43(10) (2005) 1001-1006.

[36] N.P. Riksen, G.A. Rongen, G.H. Boers, H.J. Blom, P.H. van den Broek, P. Smits, Enhanced cellular adenosine uptake limits adenosine receptor stimulation in patients with hyperhomocysteinemia, Arterioscler Thromb Vasc Biol 25(1) (2005) 109-114.

[37] Y.F. Chen, P.L. Li, A.P. Zou, Effect of hyperhomocysteinemia on plasma or tissue adenosine levels and renal function, Circulation 106(10) (2002) 1275-1281.

[38] A. Deussen, Metabolic flux rates of adenosine in the heart, Naunyn Schmiedebergs Arch Pharmacol 362(4-5) (2000) 351-363.

[39] N.P. Riksen, G.A. Rongen, H.J. Blom, F.G. Russel, G.H. Boers, P. Smits, Potential role for adenosine in the pathogenesis of the vascular complications of hyperhomocysteinemia, Cardiovasc Res 59(2) (2003) 271-276.

[40] A.E. Böhmer, D.G. Souza, G. Hansel, L.M.B.P. Brum, D.O. Souza, Long-term cyclosporine treatment in non transplanted rats and metabolic risk factors of vascular diseases, Chemico-Biological Interactions 185(1) (2010) 53-58.

[41] M. Imai, K. Takigami, O. Guckelberger, K. Enjyoji, R.N. Smith, Y. Lin, E. Csizmadia, J. Sevigny, R.D. Rosenberg, F.H. Bach, S.C. Robson, Modulation of nucleoside [correction of nucleotide] triphosphate diphosphohydrolase-1 (NTPDase-1)cd39 in xenograft rejection, Mol Med 5(11) (1999) 743-752.

[42] F.P. Gendron, O. Benrezzak, B.W. Krugh, Q. Kong, G.A. Weisman, A.R. Beaudoin, Purine signaling and potential new therapeutic approach: possible outcomes of NTPDase inhibition, Curr Drug Targets 3(3) (2002) 229-245.

[43] G.G. Yegutkin, Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade, Biochim Biophys Acta 1783(5) (2008) 673-694.

Legends

Figure 1. Effect of CsA administration on ATP, ADP and AMP hydrolysis by rat serum. Values are expressed as mean ± S.E.M. of 7 (ATP and ADP) or 6 (AMP) independent experiments. Results are expressed as specific activity (nmol of Pi/min/mg). Values for ATP, ADP and AMP hydrolysis, respectively: Control - 0.70 ± 0.12, 0.83 ± 0.18 and 1.36 ± 0.17; CsA 5 mg/kg - 0.49 ± 0.16, 0.53 ± 0.20 and 0.90 ± 0.20; CsA 15 mg/kg groups - 0.43 ± 0.06, 0.45 ± 0.09 and 0.90 ± 0.18. * $p < 0.05$, ** $p < 0.005$ and $^{\#}$ $p < 0.0005$ compared to respective Control (one-way ANOVA, followed by the Tukey's multiple range test).

Figure 2. Correlation between ATP (A), ADP (B) or AMP (C) hydrolysis by rat serum and tHcy serum levels of all groups together (control, CsA 5 and 15 mg/Kg, Pearson's correlation test).

Figure 3. Effect of CsA administration on ATP, ADP, AMP and ADO plasma levels of rats. Values are expressed as mean ± S.E.M. ** $p < 0.005$, compared to Control. n = 9/group.

Figure 4. Correlation between ADP (A) or ADO plasma levels and tHcy serum levels of all groups together (control, CsA 5 and 15 mg/Kg, n = 9/ each group, Pearson's correlation test).

Figure 5. A - Representative photomicrographs (from 10 animals) of the endothelial cell layer in the intima of the aorta of control and CsA 5 mg/kg groups. The endothelial cells

display a normal spindle-shaped morphology. Hematoxylin and eosin (H&E), original magnification 400x (representative of five animals). B - Representative photomicrographs (from 5 animals) of the endothelial cell layer in the intima of the aorta of CsA 15mg/kg group. There is thickening of the endothelial layers cells with irregular surface. Endothelial cells are plump and contain prominent basophilic nucleus. Hematoxylin and eosin (H&E), original magnification 400x (representative of five animals). C - Representative photomicrographs (from 10 animals) of the endothelial cell layer immunohistochemical surface aspects of control and CsA 5 mg/kg groups. A normal spindle-shaped endothelial cells pattern. Factor VIII related antigen immunohistochemistry, diaminobenzidine chromogen, original magnification 400x (representative of five animals). D - Representative photomicrographs (from 5 animals) of the endothelial cell layer immunohistochemical surface aspects of CsA 15mg/kg group. The endothelial cell was thickened and filled with floccular or granular debris. Original magnification 400x.

Figure 1

Click here to download high resolution image

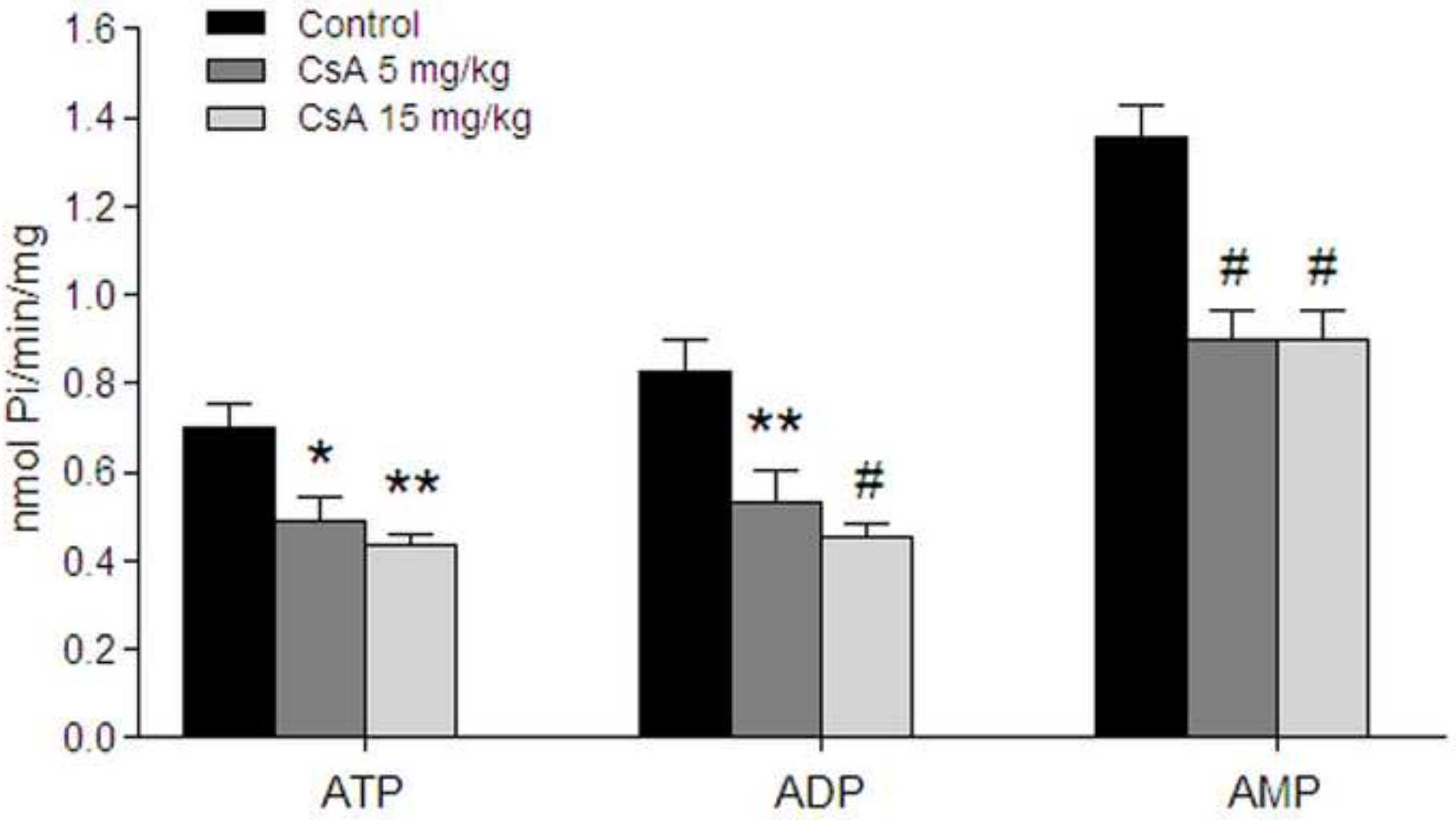


Figure 2

Click here to download high resolution image

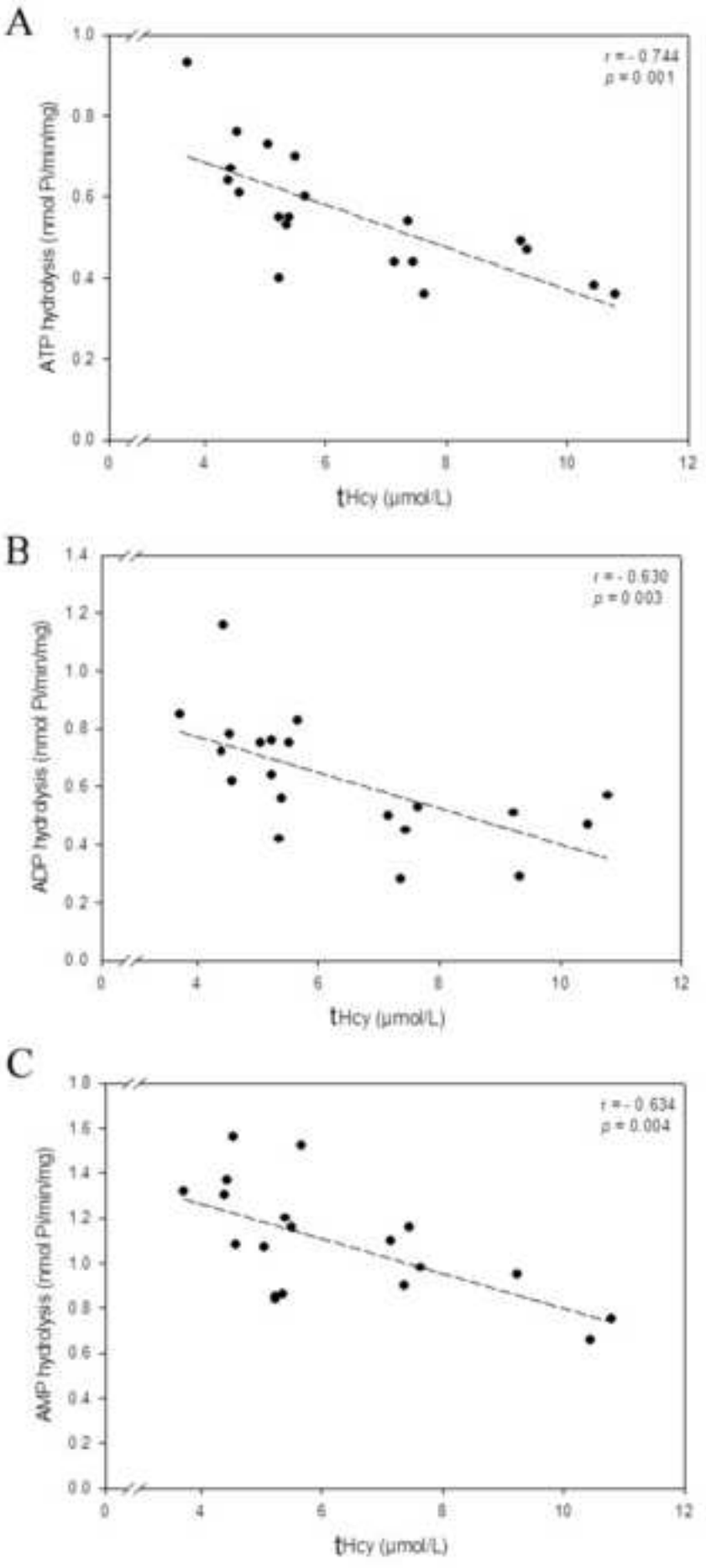


Figure 3

Click here to download high resolution image

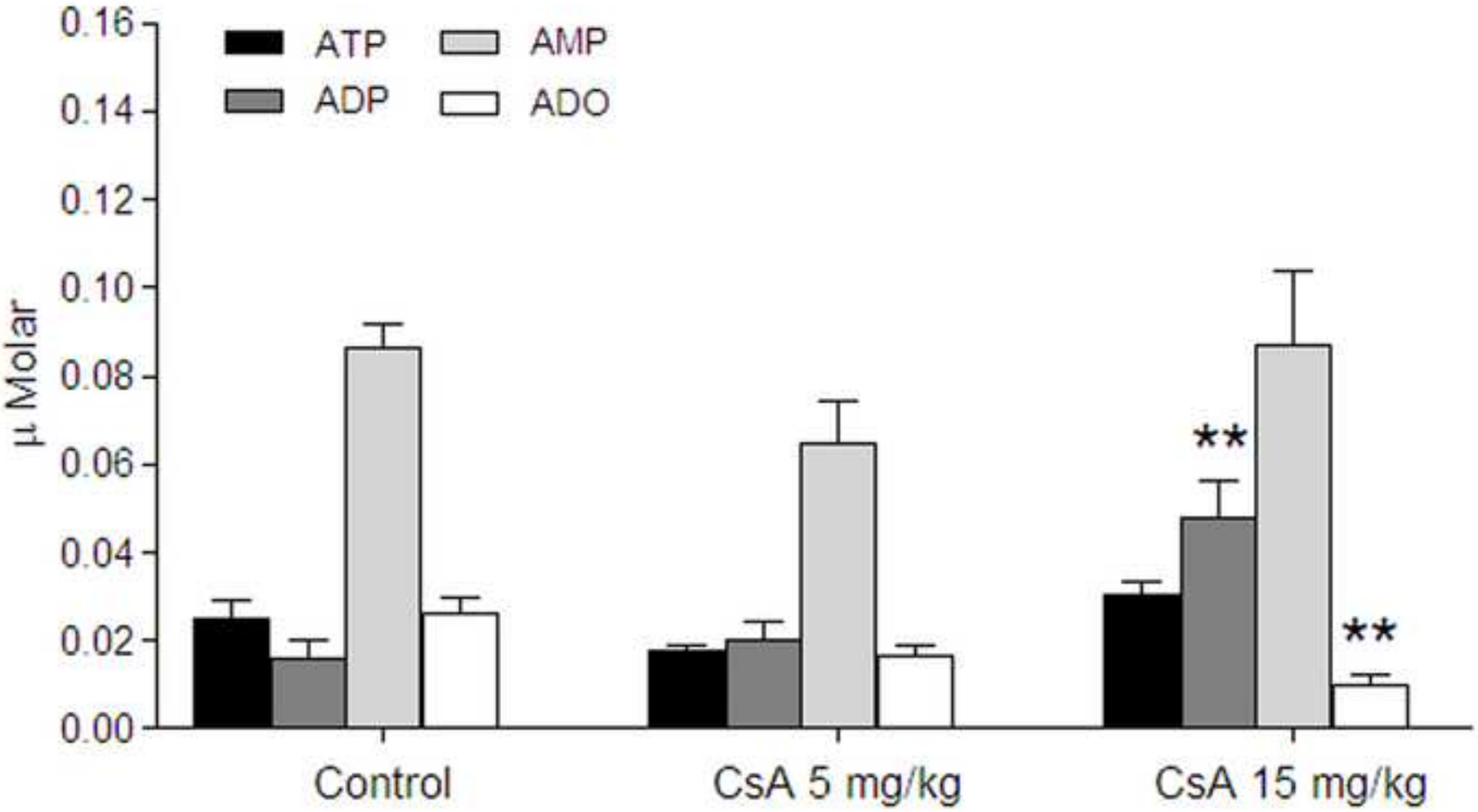


Figure 4

Click here to download high resolution image

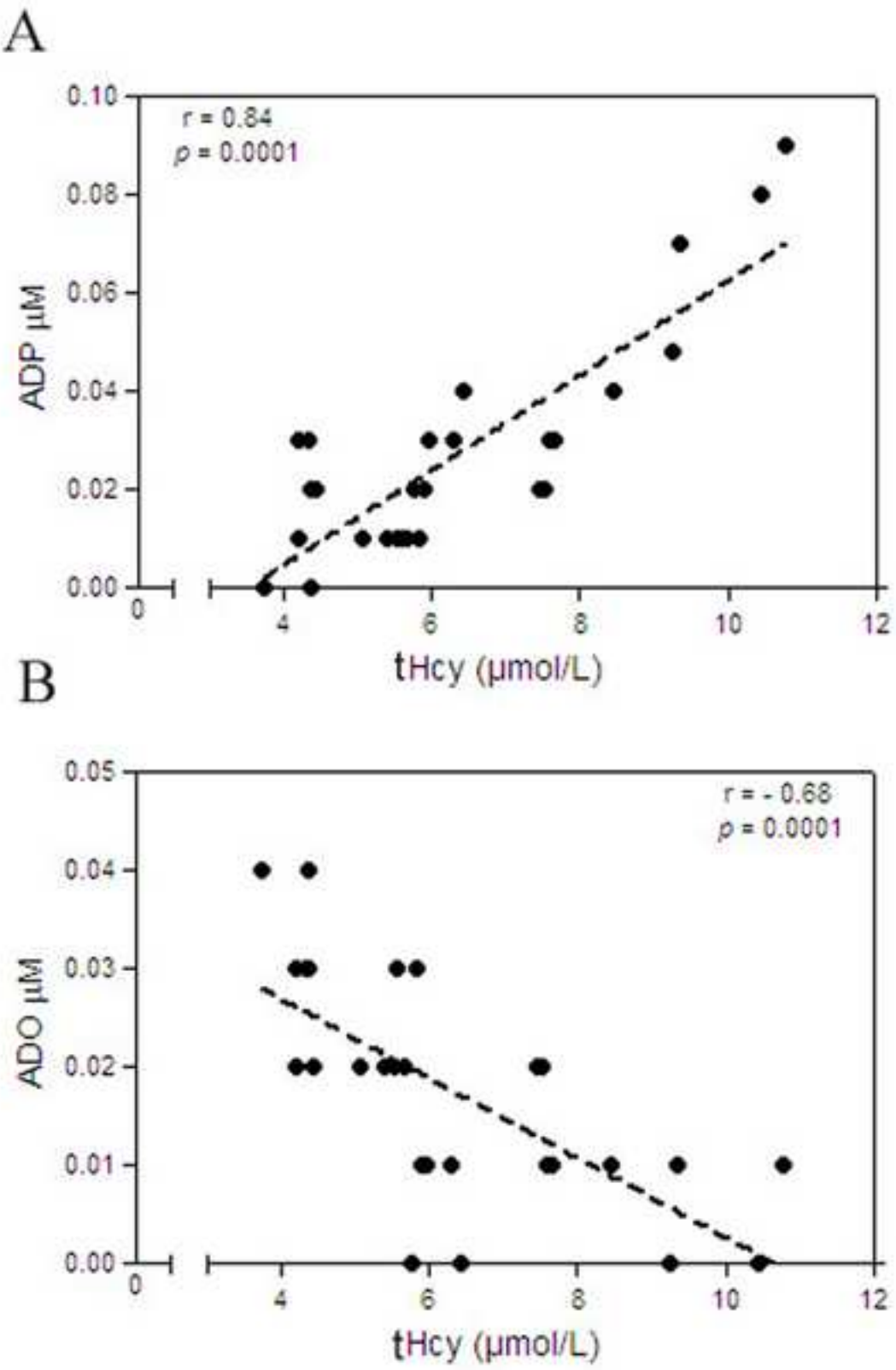
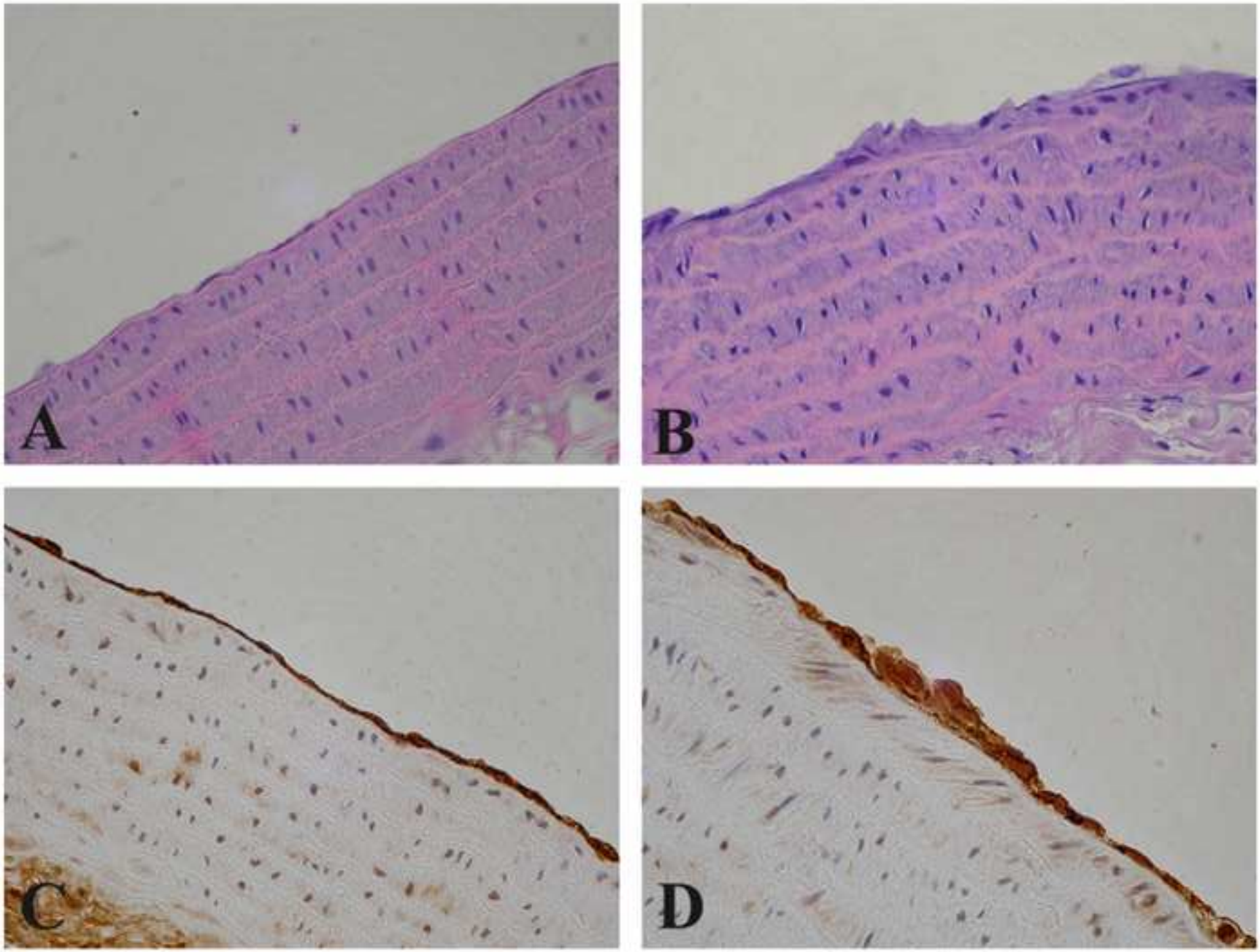


Figure 5

Click here to download high resolution image



Chemico-Biological Interactions

Manuscript title: Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and vascular disturbances in rats.

Dear editor,

Thank you for your useful comments and suggestions on the language of our manuscript. We have modified the manuscript accordingly.

1) In its current state, the level of English throughout your manuscript does not meet the journal's desired standard. There are a number of grammatical errors and instances of badly worded/constructed sentences. These editorial issues only served to detract from the overall scientific merit of your paper. Please check the manuscript and refine the language carefully. Also, we would strongly suggest that you solicit the input of one of your colleagues who is more familiar with the English language and who can carefully proof your paper prior to resubmission.

We revised the whole manuscript carefully to avoid language errors. In addition, we asked several colleagues who are skilled authors to check the English. We believe that the language is now acceptable for the review process.

The manuscript has been resubmitted to your journal. We look forward to your positive response. Respectfully,

(✉) Diogo Onofre Souza

Department of Biochemistry

Federal University of Rio Grande do Sul.

Avenida Ramiro Barcelos, 2600-Anexo

Zip Code 90035-003 Porto Alegre - RS – Brazil

Tel: (55-51) 33085559 / 33085558

Fax: (55-51) 33085540 / 33085535

E-mail: diogo@ufrgs.br

Submission Checklist

Corresponding Author:

• E-mail address
• Full postal address
• Telephone and fax numbers

All necessary files have been uploaded

• Keywords
• All figure captions
• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been "spellchecked" and "grammar-checked"
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Web)
• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print or to be reproduced in color on the Web (free of charge) and in black-and-white in print
• If only color on the Web is required, black and white versions of the figures are also supplied for printing purposes.

*Conflict of Interest Statement

Click here to download high resolution image

ELSEVIER

Chemico-Biological Interactions

Conflict of Interest Policy

Article Title:
Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and

Author name:
Ana Elisa Böhmer

Declarations

Chemico-Biological Interactions requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The author declares that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

The author declares that there are no sources of funding for this research.

Signature (a scanned signature is acceptable, but each author must sign)

Ana Elisa Böhmer

Print name

Ana Elisa Böhmer

*Conflict of Interest Statement

Click here to download high resolution image

ELSEVIER

Chemico-Biological Interactions

Conflict of Interest Policy

Article Title:
Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and

Author name:
André Mendes Ribeiro Corrêa

Declarations

Chemico-Biological Interactions requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The author declares that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

The author declares that there are no sources of funding for this research.

Signature (a scanned signature is acceptable, but each author must sign)

André Corrêa

Print name

André Mendes Ribeiro Corrêa

*Conflict of Interest Statement

Click here to download high resolution image

ELSEVIER

Chemico-Biological Interactions

Conflict of Interest Policy

Article Title:
Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and

Author name:
Débora Guerini Souza

Declarations

Chemico-Biological Interactions requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The author declares that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

The author declares that there are no sources of funding for this research.

Signature (a scanned signature is acceptable, but each author must sign)

Débora Souza

Print name

Débora Guerini Souza

*Conflict of Interest Statement

Click here to download high resolution image

Chemico-Biological Interactions

Conflict of Interest Policy

Article Title:
Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and

Author name:
David Driemeier

Declarations

Chemico-Biological Interactions requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The author declares that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

The author declares that there are no sources of funding for this research.

Signature (a scanned signature is acceptable, but each author must sign)

David Driemeier

Print name

David Driemeier

*Conflict of Interest Statement

Click here to download high resolution image

ELSEVIER

Chemico-Biological Interactions

Conflict of Interest Policy

Article Title:
Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and

Author name:
Diogo Onofre Souza

Declarations

Chemico-Biological Interactions requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The author declares that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

The author declares that there are no sources of funding for this research.

Signature (a scanned signature is acceptable, but each author must sign)

Print name

Diogo Onofre Souza

*Conflict of Interest Statement

Click here to download high resolution image

ELSEVIER

Chemico-Biological Interactions

Conflict of Interest Policy

Article Title:
Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and

Author name:
Giordano Gubert Viola

Declarations

Chemico-Biological Interactions requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The author declares that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

The author declares that there are no sources of funding for this research.

Signature (a scanned signature is acceptable, but each author must sign)

Giordano Viola

Print name

Giordano Gubert Viola

*Conflict of Interest Statement

Click here to download high resolution image

ELSEVIER

Chemico-Biological Interactions

Conflict of Interest Policy

Article Title:
Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and

Author name:
Jean Pierre Oses

Declarations

Chemico-Biological Interactions requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The author declares that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

The author declares that there are no sources of funding for this research.

Signature (a scanned signature is acceptable, but each author must sign)

Jean Oses

Print name

Jean Pierre Oses

*Conflict of Interest Statement

Click here to download high resolution image

ELSEVIER

Chemico-Biological Interactions

Conflict of Interest Policy

Article Title:
Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and

Author name:
Liz Marina P. Brum

Declarations

Chemico-Biological Interactions requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The author declares that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

The author declares that there are no sources of funding for this research.

Signature (a scanned signature is acceptable, but each author must sign)

Print name

Liz Marina P. Brum

*Conflict of Interest Statement

Click here to download high resolution image

Chemico-Biological Interactions

Conflict of Interest Policy

Article Title:
Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and

Author name:
Paulo Jaconi Saraiva

Declarations

Chemico-Biological Interactions requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The author declares that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

The author declares that there are no sources of funding for this research.

Signature (a scanned signature is acceptable, but each author must sign)

Print name

Paulo Jaconi Saraiva

*Conflict of Interest Statement

Click here to download high resolution image

Chemico-Biological Interactions

Conflict of Interest Policy

Article Title:
Chronic treatment with cyclosporine affects systemic purinergic parameters, homocysteine levels and

Author name:
Luis Valmor Portela

Declarations

Chemico-Biological Interactions requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The author declares that there are no conflicts of interest.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

The author declares that there are no sources of funding for this research.

Signature (a scanned signature is acceptable; but each author must sign)

Print name

Luis Valmor Portela

CAPÍTULO IV

HPLC method for the quantification of adenine and guanine based purines.

Ana Elisa Böhmer (✉)[a], Gisele Hansel [a], Denise Barbosa Ramos [a], Luis Valmor Portela [a], Diogo Onofre Souza [a].

Artigo submetido ao periódico Journal of Chromatography A

Elsevier Editorial System(tm) for Journal of Chromatography A
Manuscript Draft

Manuscript Number:

Title: Quantification of adenine and guanine based purines in a single HPLC chromatographic run.

Article Type: Full Length Article

Keywords: HPLC, purines, method, cerebrospinal fluid, plasma

Corresponding Author: PhD Ana Elisa Böhmer,

Corresponding Author's Institution: Federal University of Rio Grande do Sul

First Author: Ana E Böhmer

Order of Authors: Ana E Böhmer; Gisele Hansel; Denise B Ramos; Luis V Portela; Diogo O Souza

Journal of Chromatography A

April 22th, 2010.

Dear Editor,

Please find enclosed a copy of the manuscript "Quantification of adenine and guanine based purines in a single HPLC chromatographic run" by Böhmer et al. submitted as an original article to *Journal of Chromatography A.*

This manuscript is not submitted to another journal and the results presented are originals. This research was funded by the Brazilian funding agencies INCT: Excitotoxicity and Neuroprotection, CNPq, and FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)"; however, no author has a financial arrangement with any company or organization that might be a conflict of interest. Therefore, the authors declare that there are no conflicts of interest.

Moreover, we state that Federal University of Rio Grande do Sul, Brazil agrees to the submission of this paper to the journal.

We greatly appreciate your consideration.

Respectfully,

(✉) Ana Elisa Böhmer

Department of Biochemistry
Federal University of Rio Grande do Sul.
Avenida Ramiro Barcelos, 2600-Anexo
Zip Code 90035-003 Porto Alegre - RS – Brazil
Tel: (55-51) 33085559 / 33085558
Fax: (55-51) 33085540 / 33085535
E-mail: anaelisab@gmail.com

***Reviewer Suggestions**

Manuel Castro-Gago

Adress: Servicio de Neuropediatria, Departamento de Pediatria,

Hospital Clínico Universitario, La Choupana s/n, Facultad de Medicina,

Universidad de Santiago de Compostela, 15706 Santiago de Compostela, Spain

E-mail address: pdcastro@usc.es

***Reviewer Suggestions**

Attila Kofalvi

Adress: Center for Neuroscience and Cell Biology of Coimbra,

Department of Zoology, Faculty of Medicine,

University of Coimbra,

1 Rua Larga, 3004-504 Coimbra, Portugal

E-mail: akofalvi@gmail.com

***Reviewer Suggestions**

João Batista Teixeira da Rocha
Adress: Centro de Ciências Naturais e Exatas - Prédio 18
Universidade Federal de Santa Maria-UFSM
Faixa de Camobi, Km 09 - Campus Universitário
Santa Maria, RS- Brazil
Zip Code: 97105-900
E-mail: jbtrocha@yahoo.com.br

Quantification of adenine and guanine based purines in a single HPLC chromatographic run.

Ana Elisa Böhmer (✉)[a], Gisele Hansel[a], Denise Barbosa Ramos[a], Luis Valmor Portela[a], Diogo Onofre Souza[a].

[a] Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

(✉) Ana Elisa Böhmer
Departamento de Bioquímica, ICBS, UFRGS
Avenida Ramiro Barcelos, 2600-Anexo
Zip Code: 90.035-003
Porto Alegre - RS - Brazil
Phone: (55-51) 3308-5559 / 3308-5558
Fax: (55-51) 3308-5540 / 3308-5535
E-mail: anaelisab@gmail.com

Abstract

Purines act as important signaling molecules that induce a multiplicity of effects in various biological systems and play important roles in physiological and pathological conditions. The purpose of this study was to improve and validate a single, rapid and sensitive high performance liquid chromatography (HPLC) method to analyze all adenine- and guanine-based purines at cerebrospinal fluid (CSF) and plasma of human, mice and rat. HPLC was performed to measure adenosine 5′-triphosphate, adenosine 5′-diphosphate, adenosine 5′-monophoshate, adenosine, guanosine 5′-triphosphate, guanosine 5′-diphosphate, guanosine 5′-monophosphate, guanosine, inosine 5′-monophoshate, inosine, hypoxanthine, xanthine and uric acid. Two phosphate buffers pH 6.0 were used, one being with acetonitrila 15%. The mobile phase flowed at a rate of 1.2 mL/min during 17 minutes and the C18 column temperature was 22 °C. An UV detector (254 nm) was used. The analytical method was validated through the analysis of accuracy, precision, limit quantification, linearity, and stability. Standard and biological samples remained stable for up to 6 months at -20º C. Linearity range was observed up to 2000 ρmol for all analyzed purines. The precision (0.05 % to 3.9 % for intra-day and 1.39 % to 4.92 % for inter-day) and accuracy test (89.3 % to 105.2% for intra-day and 86.1 % to 111.8 % for inter-day) suggest that the applied method have high precision and accuracy for all analyzed purines. This method proved to be simple, rapid, stable, sensitive, specific, and accurate, and allows measuring all these purines in a single running. The usefulness of this method is demonstrated by successful application for human, mouse and rat CSF and plasma samples.

Keywords: HPLC, purines, method, cerebrospinal fluid, plasma.

1. Introduction

The purinergic signaling system, which utilizes purines as extracellular messengers is one of the most widespread intercellular signaling system in the living tissues [1]. Despite the purinergic system usually relates to the adenine-based purines, it is also composed by the guanine-based purines, just as their pyrimidine counterparts are related to uracil, thymidine and cytidine- based compounds. The metabolites xanthine, hypoxanthine and uric acid can also be considered part of the purinergic system [2].

Purines act as important signaling molecules that induce a multiplicity of effects in various biological systems. Initially, studies on the physiological actions of purines showed that adenosine 5′-monophoshate and adenosine induced alterations in heart rate and arterial dilation [3]. Four decades later, studies have demonstrated the role of adenosine 5′-triphosphate as a neurotransmitter [1,4]. In peripheral system, several studies have recognized the role of adenine-based purines in heart [5], hepatic and gastrointestinal functions [6,7], regulation of blood flow [8], immunity system and inflammation [9,10], platelet aggregation [11], cellular proliferation [12], atherosclerotic plaque formation and vascular disturbances [8,13]. In central nervous system (CNS), there are an increasing number of studies indicating the involvement of adenine based purines on neurotransmission [4], neuron-glia interaction [14-16], ischemia and epilepsy [17], pain [18], inflammation [19], traumatic brain injury [20] and others.

Regarding guanine-based purines, traditionally they have been studied as modulators of intracellular processes, especially concerning the activity of G proteins for signal transduction. However, current evidence shows that these purines exert many extracellular effects not related to G proteins [2,21]. At CNS, guanine-based purines modulate glutamatergic parameters, such as glutamate uptake by astrocytes and synaptic vesicles, seizures induced by glutamatergic agents and response to ischemia and

excitotoxicity [22-25]. Also, they are able to affect learning, memory and anxiety behavior [26,27], have important trophic functions affecting the development, structure, or maintenance of neural cells [21,28,29], and are also involved in pain [30,31] and brain hypoperfusion [32,33].

The nucleotide inosine 5′-monophoshate, the nucleoside inosine, and metabolites like xanthine, hypoxanthine and uric acid have also received increasing interest by researchers. Recent studies demonstrated that increasing of inosine and hypoxanthine in human plasma samples are involved in acute cardiac ischemia [34]. Inosine has been shown to increase cell viability in stroke models [35] and uric acid is involved in many pathologies such as gout and auto inflammation [36,37].

Purines, their metabolites, and soluble enzymes responsible for their hydrolysis are detected in the human and animal cerebrospinal fluid (CSF) and blood [38-42] and the potential role of purines in innumerous biological processes lead to the development of analytical methods for purines analysis and quantification in various biological samples [34,43-47]. However, to our knowledge no developed method is able to separate and quantify all adenine- and guanine-based purines and their metabolites in a single and rapid HPLC technique applied for different biological samples.

Therefore, the purpose of this study was to improve and validate a simple, rapid and sensitive high performance liquid chromatography (HPLC) method to analyze all adenine- and guanine-based purines, as well inosine 5′-monophoshate, inosine, xanthine, hypoxanthine and uric acid at CSF and plasma of human, mice and rat samples.

1. Experimental procedure

1.1. Chemical reagents

All the purines and HPLC-grade acetonitrile were purchased from Sigma Aldrich (St Louis, MO, USA). HPLC-grade potassium phosphate dibasic ($K_2H\ PO_4$) and HPLC-grade trifluoroacetic acid were from Fluka-Sigma Aldrich (St Louis, MO, USA). HPLC-grade potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4) were purchased from Riedel-de Haën (Germany). Deionized water was supplied in house by a Purelab-ultra System (ELGA, High Wycombe, U.K.). All others reagents were of HPLC grade.

1.2. Analytical method

HPLC analyses were performed with the Shimadzu Series 20A chromatography system, consisting in a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL- 10AF auto injector valve with 50 µL loop and a SPD-20 AV UV detector (Shimadzu, Kyoto, Japan). Separations were achieved on a Supelcosil^{TM} LC-18, 5 µm – 250 mm x 4.6 mm column (Supelco, St Louis, MO).

The mobile phase flowed at a rate of 1.2 mL/min during 17 minutes and the column temperature was 22° C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L of KCl; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 minutes, 2% at 0.05 minutes, 7% at 2.45 minutes, 100% at 9.00 minutes, 100% at 11.00 minutes, and 2% at 12.40 minutes, stop running at 17 minutes. Absorbance was read at 254 nm.

1.3. Standard preparation

The stock solution with the 13 standards: adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophoshate (AMP), adenosine (ADO), guanosine 5′-triphosphate (GTP), guanosine 5′-diphosphate (GDP), guanosine 5′-monophosphate (GMP), guanosine (GUO), inosine 5′-monophoshate (IMP), inosine (INO), xanthine (XANT), hypoxanthine (HYPOX) and uric acid (UA) was prepared with deionized water, artificial cerebrospinal fluid (ACSF) or phosphate buffer (utilized at HPLC chromatographic run - buffer A). Standard samples were prepared by serial dilutions of the stock solution at the following concentrations: 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 10, 25, 50 and 100 µM.

1.4. Samples preparation of control subjects

1.4.1. Mouse and rat cerebrospinal fluid (CSF)

Mice and rats were anaesthetized with sodium thiopental (60 mg/kg, 10 ml/kg, i.p and 40 mg/kg, 1 mL/kg i.p, respectively), placed in a stereotaxic apparatus, and CSF samples (10–20 µl per mouse and 40– 60 µL per rat) were drawn by direct puncture of the cisterna magna with an insulin syringe (27 gauge×1/2 in length). In order to obtain cell-free supernatants, all samples were centrifuged at 10,000 g at 4° C in an Eppendorf centrifuge during 10 min and supernatant was aliquoted and frozen (−20° C) until being thawed for use. Immediately before analyses, CSF samples were filtered through a syringe driven filter unit (Millex-GV, 0.22 µm, Millipore - Bedford, MA).

1.4.2. Human CSF

CSF was collected by experienced anesthesiologists. The CSF samples were inspected visually and discarded if blood contamination was present. A total of 0.5 mL of CSF was collected from the patients after successful subarachnoid puncture and

before the intrathecal injection of anesthetics or analgesics. All further procedures were the same as for animals.

1.4.3. Mouse and rat plasma collection

Animal blood samples were collected by cardiac puncture in tubes containing EDTA and then immediately centrifuged for 10 min at 1500 x g, to collect the plasma, which samples were stored at -20^{o} C until being thawed for use.

1.4.4. Human plasma collection

Human blood samples were collected by vein puncture in tubes containing EDTA and then immediately centrifuged for 10 min at 1,500 x g, to collect the plasma. The plasma samples were stored at -20^{o} C until being thawed for use.

1.4.5. Plasma deproteinization

To analyze plasma samples at HPLC, samples have to be firstly deproteinized. This procedure should keep intact all purines levels, avoiding their degradation, and should not be harmful to HPLC column. For this reason, 3 different methods were tested: deproteinization by boiling the samples, and by adding methanol or TFA to the samples. Deproteinization by boiling and by adding methanol to the sample was not successful. Despite being cheaper, both methods resulted in hydrolysis of purines, mainly ATP (data not shown). TFA addition was the most effective, since it was able to deproteinize the samples without degradation of purines. TFA presents a combination of convenient properties: volatility, solubility in organic solvents, and its acid strength. However, as it is a strong acid, after being lyophilized with TFA the samples have to be washed (by lyophilization) twice with deionized water to remove all TFA. Accordingly, the same

amount of plasma (200 µL) and TFA 14% were mixed, lyophilized at UNIPAVO 100 H Vacuum concentrations (Montreal Biothec Inc., Montreal, Canada) and washed twice with deionized water. Samples were resuspended in 200 µL buffer A - pH 6.0 and filtered. It is important to note that purines standards were also submitted to the deproteinization procedure, to avoid methodology bias.

1.5. Statistic analysis

All results were presented as means ± SD. Linearity was analyzed by linear regression; accuracy was calculated as the percentage of recovery (RE%) and precision was calculated and expressed as relative standard deviation (RSD%); variation of injection volume were analyzed by Student t test and variation of different standard diluents were analyzed and expressed as relative standard deviation (RSD%). Analyses were performed with the 18 Statistical Package for the Social Sciences (SPSS 15.0) software and Shimadzu LC solution software. Differences were considered statistically significant if $p < 0.05$.

2. Results and discussion

2.1. Analytical method validation

The method was validated according to USP category I requirements [48]. The following validation characteristics for purines measurement were addressed: the stability of the solutions, linearity, range, accuracy, precision, and robustness.

2.1.1. Stability of the solutions

Standard and biological (CSF and plasma) samples stability was evaluated for fresh and stored aliquots. Samples stored at -20º C remained stable for up to 6 months after collection (data not shown).

2.1.2. Detection range

The limit of quantification (LOQ) is determined by the lowest concentration of standard whose accuracy and precision are within the acceptable value, according to USP category I requirements [48]. The limit of detection (LOD) is the lowest concentration that the analyte can be detected, determined by standard dilution from the concentration found for LOQ. Linear quantities are not necessary. Table 1 shows purines LOQ and LOD. LOQ was determined as 0.5 ρmol for AMP, GTP, GDP, GMP, IMP and UA, 1 ρmol for ATP, ADO, GUO, INO, HIPOX and XANT and 5 ρmols for ADP.

2.1.3. Linearity and range

The range of linearity was checked by injections of 8-10 concentrations of each purine standard (in quadruplicates) below and above the expected concentration of the biological samples. Linearity was obtained by linear regression of peak area x concentration. AMP, GTP, GDP, GMP and IMP linearity range 0.5 ρmol up to 2000 ρmol. ATP, ADO, GUO, INO, HYPOX and XANT linearity range 1 ρmol up to 2000 ρmol. ADP linearity ranges 5 ρmol up to 2000 ρmol. UA linearity presented a different profile from the other purines: the linearity ranging from 0.5 ρmol to 100 ρmol has a different linearity profile from 100 ρmol to 1000 ρmol. Table 2 shows linear range, mcf, slope, r^2 and Y-intercept values.

2.1.4. Precision and accuracy

Precision and accuracy results are shown in Table 3 and were calculated from three different standards: 5, 20 and 200 ρmol, to evaluate intra and inter-day assay. The precision of a measurement system, also called reproducibility or repeatability, is the degree to which repeated measurements under unchanged conditions show the same results. Precision describes the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample and calculated as relative standard deviation (RSD%), which cannot exceed the limit of 5%. In this study, precision ranged from 0.05 % to 3.9 % intra-day and 1.39 % to 4.92 % inter-day for all purines analyzed. The accuracy of a measurement system is the degree of closeness of measurements of a quantity to its actual (true) value. Accuracy ranged from 89.3 % to 105.2% intra-day and 86.1 % to 111.8 % inter-day for all purines analyzed. These data suggest that the applied methods have high precision and accuracy.

2.1.5. Robustness

Robustness is the ability of coping well with variations in operating environment with minimal damage, alteration or loss of functionality. Here, robustness was evaluated by system suitability standard analyze. Six different injection volumes were tested (1, 2, 5, 10, 20 and 50 µL), each of them with the same amount of purines. Table 4 shows the results for two different injection volumes (1 and 10 µL) with the same amount of purines. Peak areas remained the same, independently of the volume injection.

2.1.6. Calibration factor

The calibration factor was calculated by dividing the amount of each standard applied to the HPLC by the area of the respective peak. The mean calibration factor (mcf) was the mean value of all calibration factors of each curve. Table 2 shows parameters related to the calibration curve.

2.1.7. Standard diluents

This methodology was applied for analyzing the purines concentration in biological systems with distinct compositions, as plasma and CSF of mice, rats and humans. Thereby, we evaluated the effect of three different standard diluents (water, ACSF and buffer A) over the area and retention time of purines standards (RSD%). According to USP category I requirements, area and retention time must not vary more than (RSD%) < 2% and (RSD%) < 0.5%, respectively. Table 5 shows the results of chromatographic runs test. The use of different diluents did not interfere on area and retention time profile. CSF and plasma were assessed by comparing the peak areas obtained from multiple analyses of spiked samples with the peak areas from standard solution of all analytes in a solution.

2.2. Applications of this methodology to experimental and humans studies

This methodology demonstrated stability, specificity, linearity, range, accuracy and precision adequate to measure plasma and CFS samples from human, mouse and rat. Figure 1 shows two superimposed chromatographic runs of all purines standards and of a representative spiked CSF sample of control rats.

2.2.1. Plasma samples from rats

With this methodology we previously demonstrated that cyclosporine treated rats presented a significant increase in ADP and decrease in ADO plasma levels compared to control group. This data corroborate with the observed decrease in ATP, ADP and AMP hydrolysis in serum and the vascular damage observed in those animals. Thus, this HPLC method was of great importance to improve our study [49].

2.2.2. CFS samples

Human studies. A number of studies have already been published, mainly by our group, with the present HPLC methodology, specially related to CSF purines concentration in acute and/or chronic pain syndromes versus control subjects [39].. These studies strongly point the antinociceptive role of GUO in experimental and human studies. The CSF levels of IMP, INO, GUO and UA acid were significantly higher in the chronic pain group and correlated with pain severity. In the acute pain group, only INO and UA levels were significantly increased. This study suggests that purines, in special INO, GUO and UA are associated to spinal nociception mechanisms [39].

Experimental studies with rodents. The first study published with CSF mice sample using the present HPLC methodology showed that an intracerebroventricular injection of GUO and GMP had antinociceptive effect in pain models [31]. Another study using our HPLC methodology showed that intrathecal administration of GUO increases this purine at CSF preventing the increase of glutamate uptake at spinal cord induced by capsaicin. This study provided new evidence on the mechanism of action of GUO on antinociceptive effects at spinal sites [30]. Additionally, this method contributed to show that GUO produces dose-dependent antinociceptive effects on pain models when administered orally and intraperitonially [50]. In a study with allopurinol, a potent

inhibitor of the enzyme xanthine oxidase, our method was important to demonstrate that mice treated with allopurinol presented an increasing ADO and GUO CSF levels, and a decreasing UA levels compared to control animals, and that the anti-nociception effects of allopurinol may be related to ADO accumulation [51].

Regarding rat CSF samples we observed that intraperitoneal administration of GUO on a rat peripheral mononeuropathy model increases GUO CSF levels and modulates chronic pain. This study provides a new role for GUO on chronic pain modulation [52].

3. Conclusion

The present method of purines quantification by HPLC proved to be reproducible and validated, fulfilling the analytical validation characteristics. This method proved to be simple, rapid, stable, linear, sensitive, specific, and accurate, and allowed to measure all these purines: ATP, ADP, AMP, ADO, GTP, GDP, GMP, GUO, IMP, INO, XANT, HYPOX and UA, in a single chromatographic run. The usefulness of this method was demonstrated by successful application for mouse, rat and human CSF and plasma samples.

Acknowledgments

This work was supported by grants from CNPq, CAPES/Brazil, FAPERGS, IBN-Net: 01.06.0842-00 (FINEP) and INCT for Excitotoxicity and Neuroprotection/CNPq. The authors are grateful to Krzysztof Safranow for helpful explanation during the preparation of this technique.

References

[1] G. Burnstock, B.B. Fredholm, R.A. North, A. Verkhratsky, Acta Physiol (Oxf) (2010).

[2] A.P. Schmidt, D.R. Lara, D.O. Souza, Pharmacol Ther 116 (2007) 401.

[3] A.N. Drury, A. Szent-Gyorgyi, J Physiol 68 (1929) 213.

[4] G. Burnstock, Trends Pharmacol Sci 27 (2006) 166.

[5] D. Pochmann, A.M. Innocente, G. Cotomacci, M.L. Barreto-Chaves, J.J. Sarkis, Biosci Rep 28 (2008) 267.

[6] G.M. Braunstein, R.M. Roman, J.P. Clancy, B.A. Kudlow, A.L. Taylor, V.G. Shylonsky, B. Jovov, K. Peter, T. Jilling, Ismailov, II, D.J. Benos, L.M. Schwiebert, J.G. Fitz, E.M. Schwiebert, J Biol Chem 276 (2001) 6621.

[7] A.P. Feranchak, J.G. Fitz, R.M. Roman, J Hepatol 33 (2000) 174.

[8] G. Burnstock, Auton Autacoid Pharmacol 29 (2009) 63.

[9] M.R. Blackburn, C.O. Vance, E. Morschl, C.N. Wilson, Handb Exp Pharmacol (2009) 215.

[10] M.J. Bours, E.L. Swennen, F. Di Virgilio, B.N. Cronstein, P.C. Dagnelie, Pharmacol Ther 112 (2006) 358.

[11] C. Gachet, Annu Rev Pharmacol Toxicol 46 (2006) 277.

[12] G. Burnstock, Pharmacol Rev 58 (2006) 58.

[13] A. Solini, P. Chiozzi, A. Morelli, E. Adinolfi, R. Rizzo, O.R. Baricordi, F. Di Virgilio, Arterioscler Thromb Vasc Biol 24 (2004) 1240.

[14] M.P. Abbracchio, G. Burnstock, A. Verkhratsky, H. Zimmermann, Trends Neurosci 32 (2009) 19.

[15] A. Verkhratsky, O.A. Krishtal, G. Burnstock, Mol Neurobiol 39 (2009) 190.

[16] V. Ralevic, G. Burnstock, Pharmacol Rev 50 (1998) 413.

[17] N. Dale, B.G. Frenguelli, Curr Neuropharmacol 7 (2009) 160.

[18] G. Burnstock, Curr Pharm Des 15 (2009) 1717.

[19] F. Di Virgilio, S. Ceruti, P. Bramanti, M.P. Abbracchio, Trends Neurosci 32 (2009) 79.

[20] T.A. Lusardi, Curr Neuropharmacol 7 (2009) 228.

[21] R. Ciccarelli, P. Ballerini, G. Sabatino, M.P. Rathbone, M. D'Onofrio, F. Caciagli, P. Di Iorio, Int J Dev Neurosci 19 (2001) 395.

[22] R. Chang, A. Algird, C. Bau, M.P. Rathbone, S. Jiang, Neurosci Lett 431 (2008) 101.

[23] A.P. Schmidt, D.R. Lara, J. de Faria Maraschin, A. da Silveira Perla, D. Onofre Souza, Brain Res 864 (2000) 40.

[24] D.R. Lara, A.P. Schmidt, M.E. Frizzo, J.S. Burgos, G. Ramirez, D.O. Souza, Brain Res 912 (2001) 176.

[25] R.G. Tavares, A.P. Schmidt, J. Abud, C.I. Tasca, D.O. Souza, Neurochem Res 30 (2005) 439.

[26] R. Roesler, M.R. Vianna, D.R. Lara, I. Izquierdo, A.P. Schmidt, D.O. Souza, Neuroreport 11 (2000) 2537.

[27] E.R. Vinade, A.P. Schmidt, M.E. Frizzo, L.V. Portela, F.A. Soares, F.D. Schwalm, E. Elisabetsky, I. Izquierdo, D.O. Souza, J Neurosci Res 79 (2005) 248.

[28] M. Rathbone, L. Pilutti, F. Caciagli, S. Jiang, Nucleosides Nucleotides Nucleic Acids 27 (2008) 666.

[29] C. Su, N. Elfeki, P. Ballerini, I. D'Alimonte, C. Bau, R. Ciccarelli, F. Caciagli, J. Gabriele, S. Jiang, J Neurosci Res 87 (2009) 617.

[30] A.P. Schmidt, A.E. Bohmer, C. Schallenberger, C. Antunes, M.S. Pereira, R. Leke, S.T. Wofchuk, E. Elisabetsky, D.O. Souza, Eur J Pharmacol 613 (2009) 46.

[31] A.P. Schmidt, A.E. Bohmer, R. Leke, C. Schallenberger, C. Antunes, M.S. Pereira, S.T. Wofchuk, E. Elisabetsky, D.O. Souza, Brain Res 1234 (2008) 50.

[32] M.B. Moretto, B. Boff, D. Lavinsky, C.A. Netto, J.B. Rocha, D.O. Souza, S.T. Wofchuk, J Mol Neurosci 38 (2009) 216.

[33] A.P. Thomazi, B. Boff, T.D. Pires, G. Godinho, C.E. Battu, C. Gottfried, D.O. Souza, C. Salbego, S.T. Wofchuk, Brain Res 1188 (2008) 233.

[34] D. Farthing, D. Sica, T. Gehr, B. Wilson, I. Fakhry, T. Larus, C. Farthing, H.T. Karnes, J Chromatogr B Analyt Technol Biomed Life Sci 854 (2007) 158.

[35] C.R. Zamzow, R. Bose, F.E. Parkinson, Can J Physiol Pharmacol 87 (2009) 850.

[36] F. Martinon, Immunol Rev 233 (2010) 218.

[37] N. Conway, S. Schwartz, Med Health R I 92 (2009) 356.

[38] M. Castro-Gago, F. Camina, S. Lojo, S. Rodriguez-Segade, A. Rodriguez-Nunez, Eur J Clin Chem Clin Biochem 30 (1992) 761.

[39] A.P. Schmidt, A.E. Bohmer, F.A. Soares, I.P. Posso, S.B. Machado, F. Mendes, L.V. Portela, D.O. Souza, Neurosci Lett (2010).

[40] A.E. Bohmer, C.R. Furstenau, I.L. Torres, L. Crema, A.M. Battastini, C. Dalmaz, M.B. Ferreira, J.J. Sarkis, Pharmacol Biochem Behav 75 (2003) 467.

[41] L.V. Cruz Portela, J.P. Oses, A.L. Silveira, A.P. Schmidt, D.R. Lara, A.M. Oliveira Battastini, G. Ramirez, L. Vinade, J.J. Freitas Sarkis, D.O. Souza, Brain Res 950 (2002) 74.

[42] A. Regner, R.E. Crestana, F.J. Silveira, G. Friedman, I. Chemale, D. Souza, Neuroreport 8 (1997) 3771.

[43] M.E. Frizzo, F.A. Antunes Soares, L.P. Dall'Onder, D.R. Lara, R.A. Swanson, D.O. Souza, Brain Res 972 (2003) 84.

[44] N. Cooper, R. Khosravan, C. Erdmann, J. Fiene, J.W. Lee, J Chromatogr B Analyt Technol Biomed Life Sci 837 (2006) 1.

[45] R.T. Smolenski, D.R. Lachno, S.J. Ledingham, M.H. Yacoub, J Chromatogr 527 (1990) 414.

[46] L. Domanski, T. Sulikowski, K. Safranow, A. Pawlik, M. Olszewska, D. Chlubek, E. Urasinska, K. Ciechanowski, Eur J Pharm Sci 27 (2006) 320.

[47] A. Pinto-Duarte, J.E. Coelho, R.A. Cunha, J.A. Ribeiro, A.M. Sebastiao, J Neurochem 93 (2005) 595.

[48] U.N. ONLINE, (2008).

[49] A.E. Bohmer, L.M. Brum, D.G. Souza, A.M. Corrêa, J.P. Oses, G.G. Viola, P.J. Saraiva, D. Driemeier, L.V. Portela, D.O.G. Souza., Chemico-Biological Interactions (under submission).

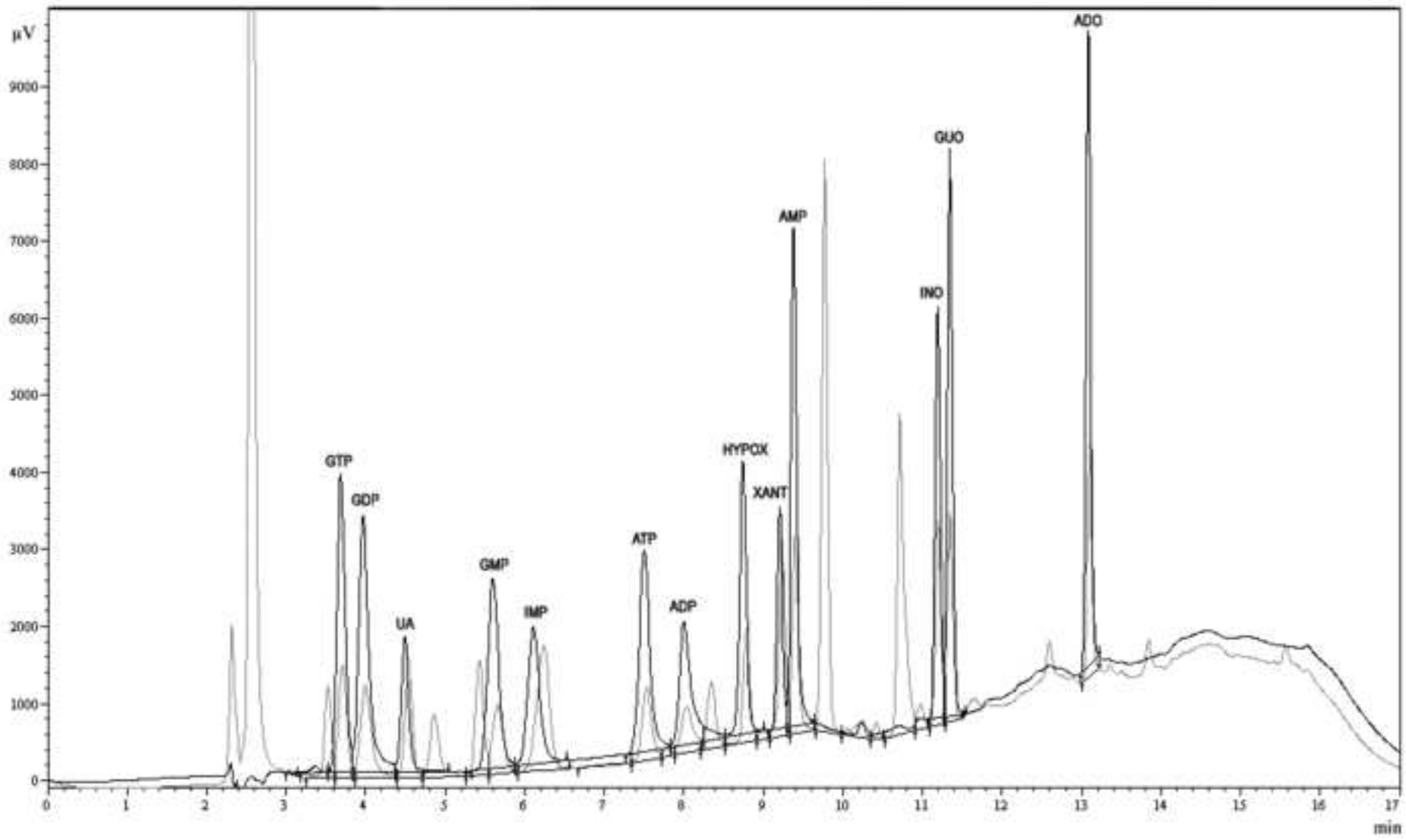
[50] A. Schmidt, A. Bohmer, C. Schallenberger, C. Antunes, R. Tavares, S. Wofchuk, E. Elisabetsky, D. Souza, Br J Pharmacol (2010).

[51] A.P. Schmidt, A.E. Bohmer, C. Antunes, C. Schallenberger, L.O. Porciuncula, E. Elisabetsky, D.R. Lara, D.O. Souza, Br J Pharmacol 156 (2009) 163.

[52] A.P. Schmidt, L. Paniz, C. Schallenberger, A.E. Bohmer, S.T. Wofchuk, E. Elisabetsky, L.V. Portela, D.O. Souza, J Pain 11 (2010) 131.

Figure

Click here to download high resolution image



Tables

Table 1
Limit of detection (LOD) and limit of quantification (LOQ)

Compound	LOD (ρmol)	LOQ (ρmol)
ATP	0.5 ± 0.00	1.0 ± 0.09
ADP	2.0 ± 0.03	5.0 ± 0.31
AMP	0.2 ± 0.01	0.5 ± 0.07
ADO	0.2 ± 0.02	1.0 ± 0.05
GTP	0.2 ± 0.05	0.5 ± 0.06
GDP	0.2 ± 0.02	0.5 ± 0.05
GMP	0.2 ± 0.00	0.5 ± 0.06
GUO	0.2 ± 0.01	1.0 ± 0.27
IMP	0.2 ± 0.00	0.5 ± 0.07
INO	0.2 ± 0.07	1.0 ± 0.22
HYPOX	0.5 ± 0.00	1.0 ± 0.23
XANT	0.5 ± 0.00	1.0 ± 0.41
UA	0.2 ± 0.04	0.5 ± 0.11

Mean ± S.D., n = 4.

Table 2
Parameters of calibration curve (n=4)

Coumpound	Linear range (ρmol)	r^2 value	mcf	slope	Y-intercept
ATP					
	1-5	0.98	0.0026	377.6 ± 15.76	-85.67 ± 49.85
	5-20	0.99	0.0023	438.5 ± 15.13	-417.8 ± 200.2
	20-200	0.99	0.0020	494.3 ± 5.750	-1407 ± 774.9
	200-2000	0.99	0.0020	499.0 ± 12.31	-12640 ± 14150
ADP					
	5-20	0.99	0.0040	252.6 ± 5.622	-467.0 ± 74.38
	20-200	0.99	0.0030	367.3 ± 4.088	-2933 ± 550.8
	200-2000	0.99	0.0025	407.3 ± 10.42	-19920 ± 11990
AMP					
	0.5-5	0.98	0.0016	611.5 ± 23.03	116.9 ± 63.32
	5-20	0.99	0.0017	580.5 ± 13.34	302.8 ± 176.4
	20-200	0.99	0.0015	646.9 ± 9.534	-859.9 ± 1285
	200-2000	0.99	0.0016	638.0 ± 17.34	-18820 ± 19950
ADO					
	1-5	0.99	0.0016	618.2 ± 21.91	199.7 ± 69.27
	5-20	0.99	0.0016	630.2 ± 16.23	130.1 ± 214.6
	20-200	0.99	0.0015	678.5 ± 6.736	-459.0 ± 907.7
	200-2000	0.99	0.0015	663.2 ± 14.51	-7483 ± 16680
GTP					
	0.5-5	0.99	0.0022	447.6 ± 9.534	-12.42 ± 26.22
	5-20	0.99	0.0020	503.4 ± 14.63	-294.9 ± 193.5
	20-200	0.99	0.0018	543.6 ± 7.313	-923.7 ± 985.5
	200-2000	0.99	0.0019	536.4 ± 13.26	-9458 ± 15250
GDP					
	0.5-5	0.99	0.0019	503.0 ± 21.13	-119.8 ± 58.12
	5-20	0.99	0.0019	538.4 ± 14.45	-225.0 ± 191.1
	20-200	0.99	0.0017	588.2 ± 11.47	-749.3 ± 1545
	200-2000	0.99	0.0016	606.2 ± 16.41	-11350 ± 18870
GMP					
	0.5-5	0.99	0.0023	428.7 ± 9.297	-47.82 ± 25.57
	5-20	0.99	0.0022	455.3 ± 12.84	-146.5 ± 169.9
	20-200	0.99	0.0020	503.2 ± 8.898	-1081 ± 1199
	200-2000	0.99	0.0020	496.2 ± 12.74	-8202 ± 14660
GUO					
	1-5	0.94	0.0016	633.0 ± 50.20	62.92 ± 158.8
	5-20	0.99	0.0016	639.5 ± 18.13	88.25 ± 239.9
	20-200	0.99	0.0015	685.5 ± 8.849	-478.8 ± 1192
	200-2000	0.99	0.0015	677.1 ± 13.55	-7716 ± 15580
IMP					
	0.5-5	0.99	0.0033	299.1 ± 6.642	-8.742 ± 18.27
	5-20	0.99	0.0030	331.4 ± 6.381	-146.4 ± 84.42
	20-200	0.99	0.0026	381.0 ± 5.417	-1080 ± 729.9
	200-2000	0.99	0.0027	376.1 ± 9.367	-6344 ± 10770
INO					
	1-5	0.97	0.0021	448.7 ± 25.38	78.17 ± 80.27
	5-20	0.99	0.0022	444.2 ± 14.39	110.0 ± 190.4
	20-200	0.99	0.0021	481.7 ± 3.887	-421.5 ± 523.8
	200-2000	0.99	0.0021	466.6 ± 10.57	-5116 ± 12160
HYPOX					
	1-5	0.98	0.0024	421.7 ± 18.23	-59.12 ± 57.64
	5-20	0.98	0.0023	431.5 ± 18.55	-52.00 ± 245.4

	20-200	0.99	0.0021	475.1 ± 15.87	-1301 ± 2139
	200-2000	0.99	0.0021	483.6 ± 14.91	-8168 ± 17150
XANT					
	1-5	0.89	0.0030	334.7 ± 36.71	168.2 ± 116.1
	5-20	0.98	0.0031	318.4 ± 13.80	281.0 ± 182.6
	20-200	0.99	0.0029	344.7 ± 5.578	-209.9 ± 751.7
	200-2000	0.99	0.0030	337.9 ± 8.222	-3112 ± 9455
UA					
	0.5-5	0.96	0.0057	175.8 ± 9.169	107.5 ± 25.21
	5-100	0.99	0.0055	182.8 ± 2.686	53.11 ± 106.7
	200-2000	0.97	0.0204	48.86 ± 2.772	11530 ± 2854

r^2: coefficient of determination; mcf: mean calibration factor.

Tables

Table 3

Quality control: precision (RSD%) and accuracy (RE%). Intra-day (n=4) and inter-day (n=8)

Compoud (ρmol)	RSD %		RE%	
	intra-day	inter day	intra-day	inter day
ATP				
5	2.05	2.48	98.0 ± 102.5	89.3 ± 101.6
20	2.07	2.01	98.9 ± 101.8	97.2 ± 109.8
200	1.46	2.18	99.2 ± 101.3	99.5 ± 102.3
ADP				
5	1.75	3.26	99.0 ± 101.5	95.2 ± 110.9
20	1.72	4.55	96.0 ± 103.3	96.8 ± 108.5
200	1.13	1.85	99.3 ± 100.9	99.3 ± 101.7
AMP				
5	2.33	4.74	93.0 ± 105.2	92.9 ± 110.2
20	1.71	4.00	94.6 ± 103.9	97.4 ± 109.9
200	0.96	3.00	98.0 ± 101.7	96.2 ± 103.0
ADO				
5	2.88	3.34	97.7 ± 101.8	92.7 ± 109.6
20	1.93	4.59	93.9 ± 104.5	94.6 ± 109.6
200	0.52	1.81	99.1 ± 100.8	97.5 ± 101.7
GTP				
5	1.15	4.13	97.5 ± 102.1	96.9 ± 106.8
20	0.52	1.83	98.3 ± 101.2	95.7 ± 103.6
200	1.93	2.69	98.4 ± 102.2	96.8 ± 103.3
GDP				
5	2.17	4.86	97.7 ± 102.7	92.7 ± 111.6
20	2.26	4.92	97.7 ± 102.7	96.9 ± 108.0
200	2.52	2.52	92.5 ± 105.6	96.8 ± 103.3
GMP				
5	1.94	3.51	95.5 ± 103.7	95.7 ± 110.7
20	1.50	2.17	98.8 ± 101.0	97.3 ± 107.2
200	0.96	3.66	97.2 ± 102.1	95.5 ± 103.6
GUO				
5	3.79	3.57	96.5 ± 101.8	91.7 ± 103.8
20	1.58	3.69	99.4 ± 101.4	95.4 ± 107.6
200	1.39	2.51	99.1 ± 101.4	96.6 ± 102.6
IMP				
5	0.65	2.66	96.9 ± 106.2	93.6 ± 109.1
20	1.58	2.02	95,3 ± 103.3	96.3 ± 105.8
200	0.05	4.73	98.2 ± 11.9	96.2 ± 103.2
INO				
5	2.79	3.14	97.9 ± 103.0	86.1 ± 109.8
20	1.60	3.44	91.7 ± 105.3	93.3 ± 108.4
200	1.08	1.39	99.4 ± 101.0	98.2 ± 101.6
HYPOX				
5	0.65	2.66	93.9 ± 103.5	95.2 ± 109.8
20	1.58	2.02	89.3 ±106.5	92.0 ± 111.8
200	0.05	4.73	93.4 ± 103.3	92.6 ± 105.9
XANT				
5	3.66	3.66	85.5 ± 110.1	90.2 ± 107.2
20	1.34	3.20	95.5 ± 103.2	94.3 ± 105.6
200	0.36	3.31	97.6 ± 101.5	95.8 ± 102.9

UA				
5	1.79	1.46	98.3 ± 100.8	92.6 ± 106.1
20	2.55	4.46	95.9 ± 103.9	96.9 ± 110.3
200	2.41	2.41	97.3 ± 102.0	98.9 ± 102.4

RSD%: percentage of relative standard deviation; RE%: percentage of recovery.

Table 4

Injection volume test.

Compound (50 ρmol)	1uL	10uL
ATP	29.98 ± 0.71	29.29 ± 0.84
ADP	22.38 ± 0.94	22.77 ± 1.65
AMP	28.54 ± 0.41	28.01 ± 0.25
ADO	31.80 ± 0.23	31.15 ± 0.19
GTP	26.27 ± 0.72	25.26 ± 0.51
GDP	25.00 ± 1.61	24.77 ± 0.95
GMP	34.73 ± 0.42	34.15 ± 0.33
GUO	28.24 ± 0.18	27.62 ± 0.31
IMP	28.78 ± 0.62	28.21 ± 0.59
INO	25.67 ± 0.34	24.98 ± 0.31
HIPOX	25.14 ± 0.16	24.99 ± 0.11
XAN	6.44 ± 0.12	6.43 ± 0.21
UA	9.21 ± 0.22	8.85 ± 0.10

Peak area (10^3) are expressed as mean ± SD, n = 4.

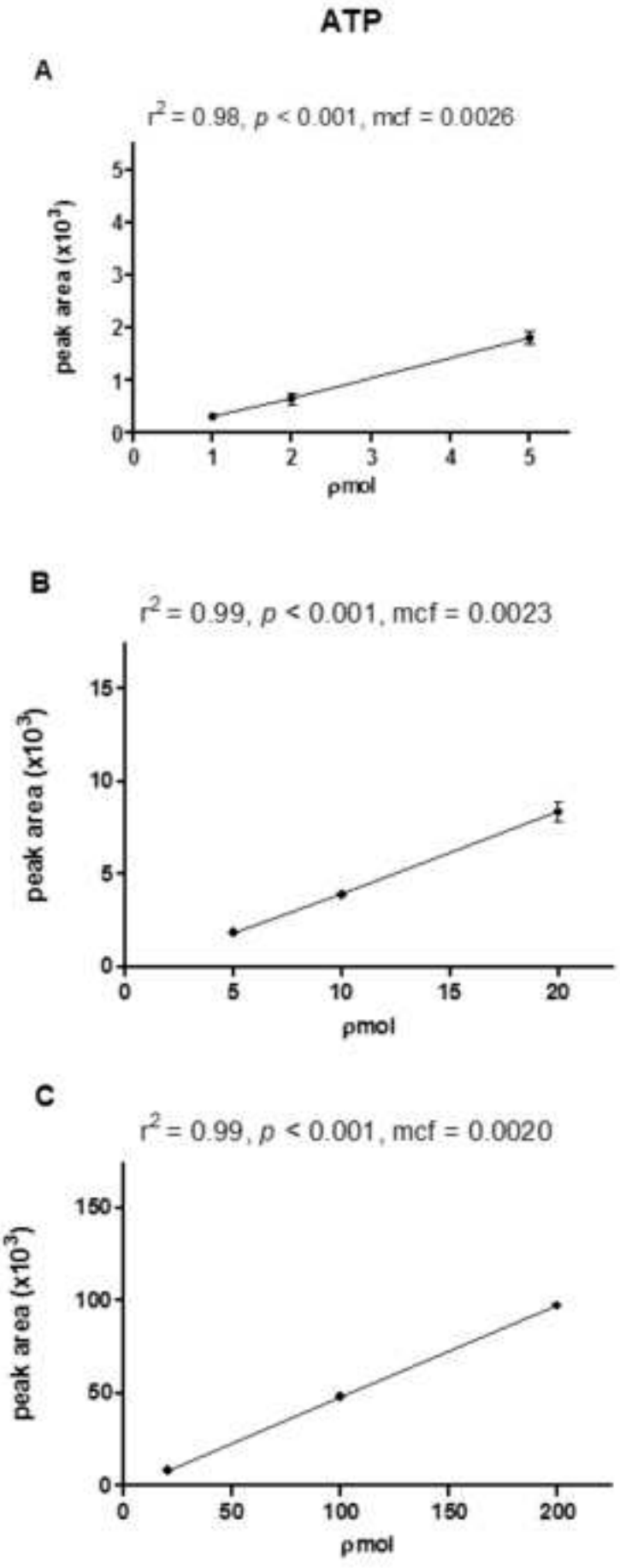
Tables

Table 5
Influence of different standard diluents on area and retention time profile.

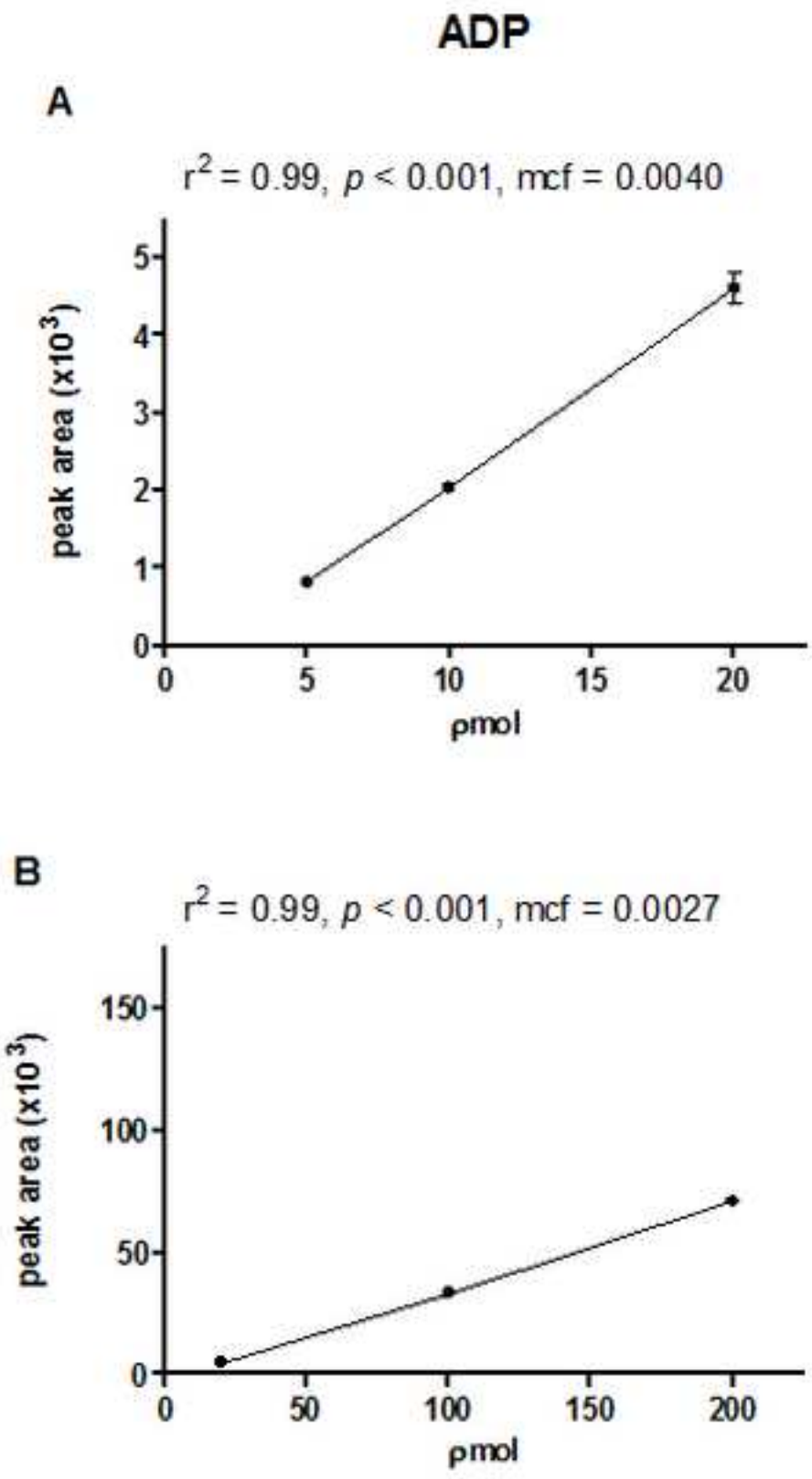
Standards (250 ρmol)	Water		ACSF		Buffer A	
	RSD %		RSD %		RSD %	
	Area	Retention time	Area	Retention time	Area	Retention time
ATP	1.30	0.50	1.34	0.50	1.28	0.18
ADP	1.45	0.34	1.50	0.47	1.03	0.34
AMP	1.58	0.32	0.63	0.33	1.54	0.32
ADO	1.19	0.12	1.58	0.23	1.19	0.48
GTP	1.77	0.35	1.93	0.33	1.55	0.38
GDP	1.30	0.37	1.35	0.47	1.50	0.42
GMP	1.28	0.34	1.28	0.57	1.37	0.47
GUO	0.79	0.08	1.55	0.28	1.83	0.31
IMP	1.39	0.32	1.03	0.43	1.54	0.13
INO	1.50	0.17	1.15	0.22	0.85	0.29
HIPOX	1.57	0.30	0.98	0.49	1.72	0.36
XAN	1.78	0.31	1.58	0.47	1.90	0.33
UA	1.85	0.37	1.85	0.25	1.95	0.43

Area and retention time are expressed as RSD % (percentage of relative standard deviation). ACSF: artificial cerebrospinal fluid. N = 5.

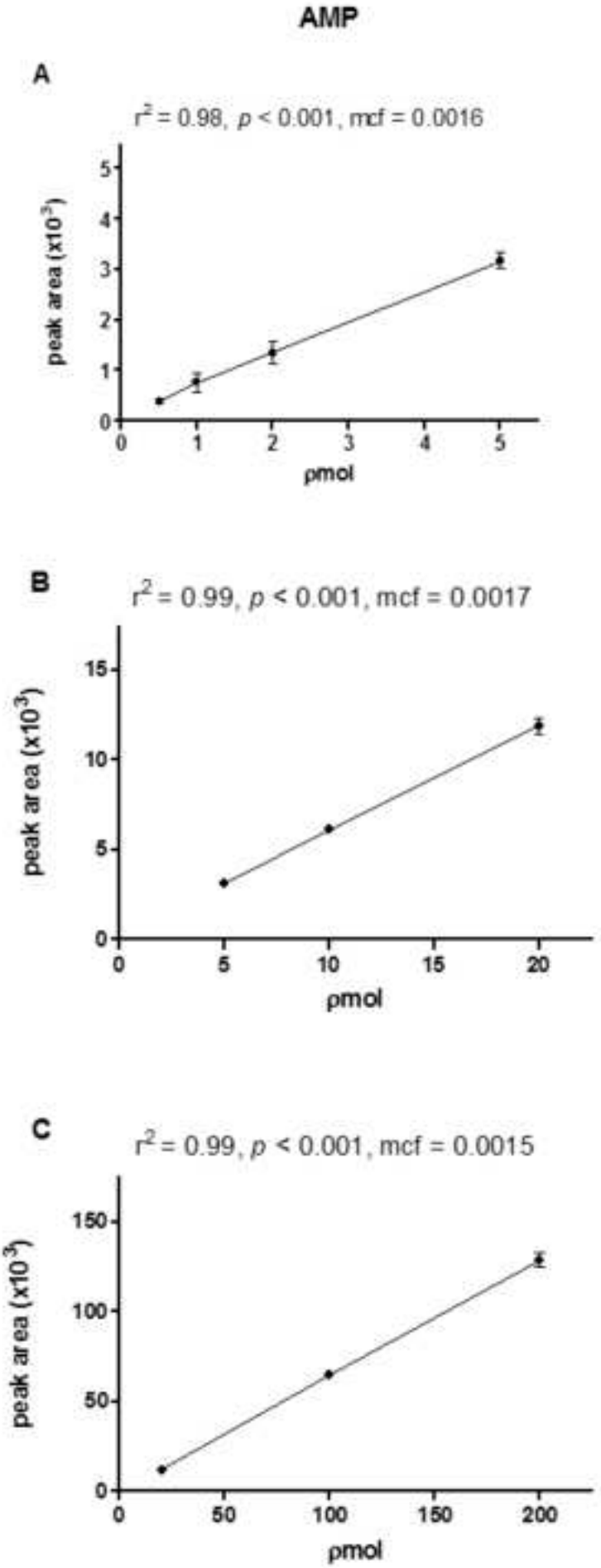
Electronic Supplementary Material (online publication only)
Click here to download high resolution image



Electronic Supplementary Material (online publication only)
Click here to download high resolution image

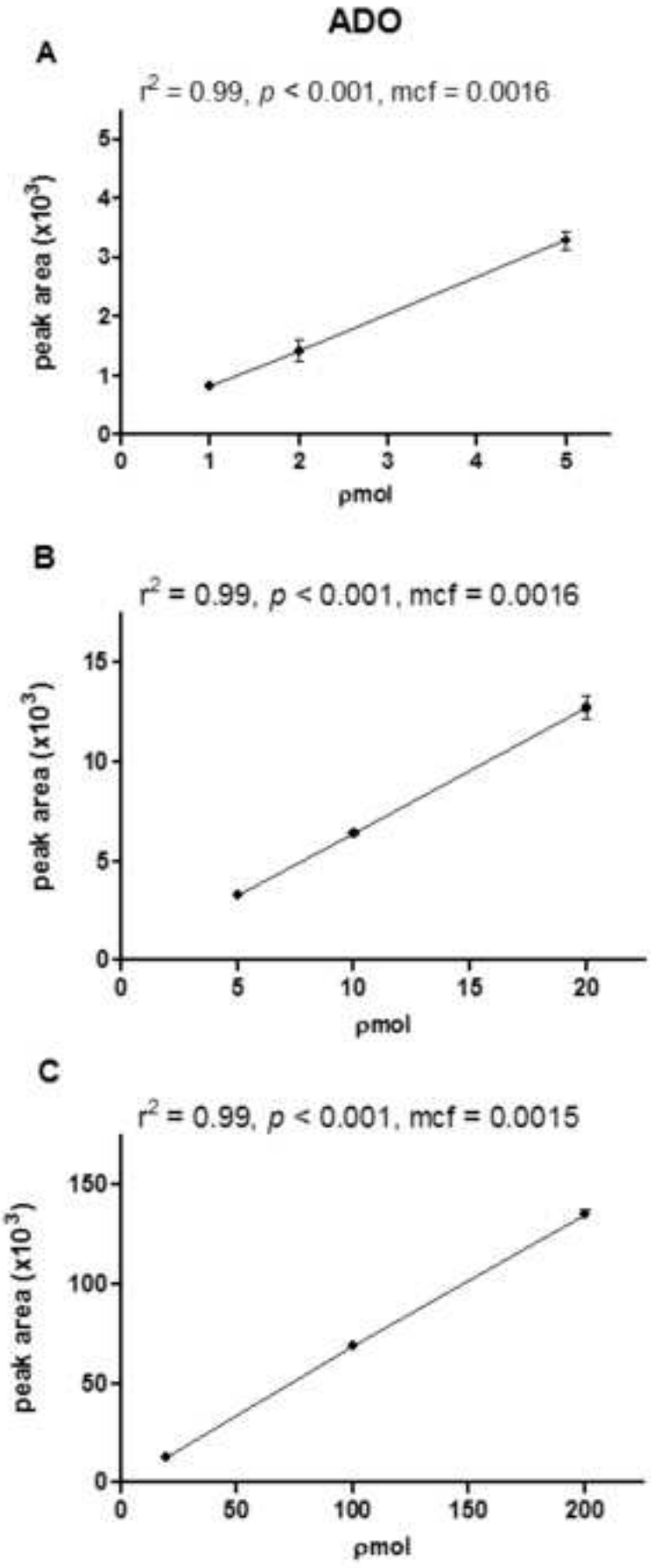


Electronic Supplementary Material (online publication only)
Click here to download high resolution image



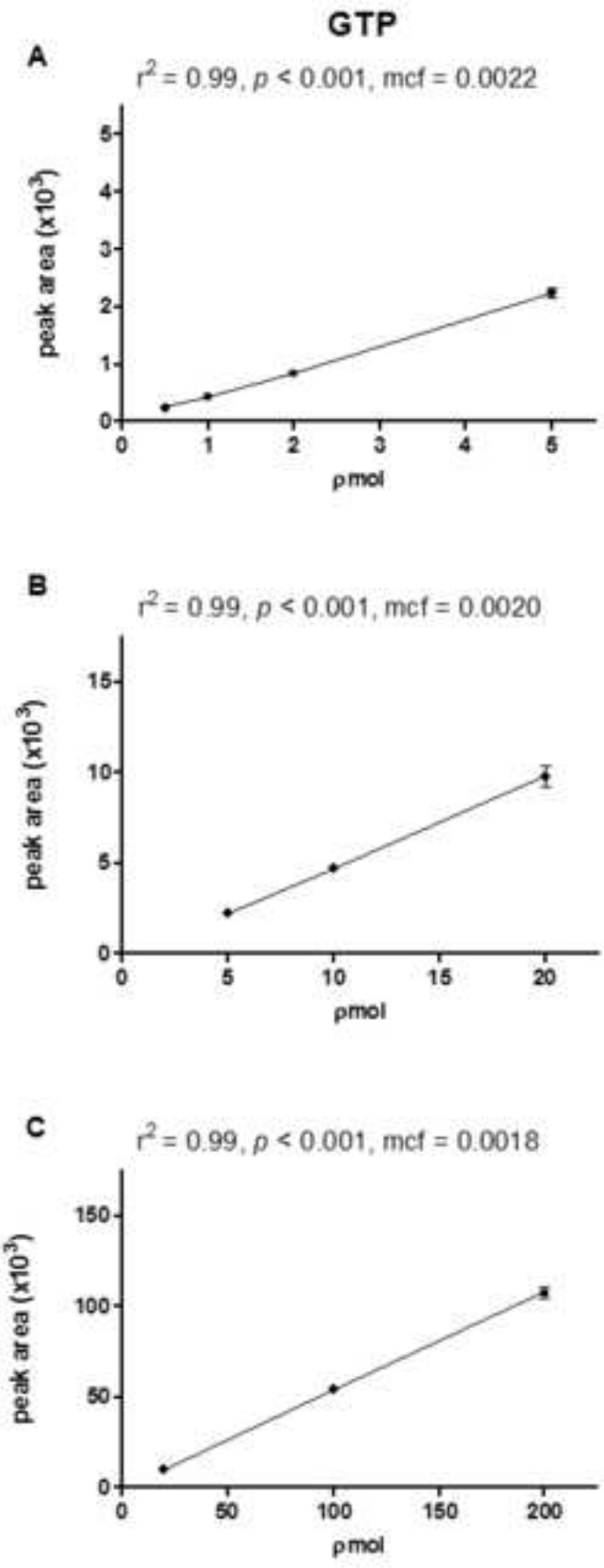
Electronic Supplementary Material (online publication only)

Click here to download high resolution image



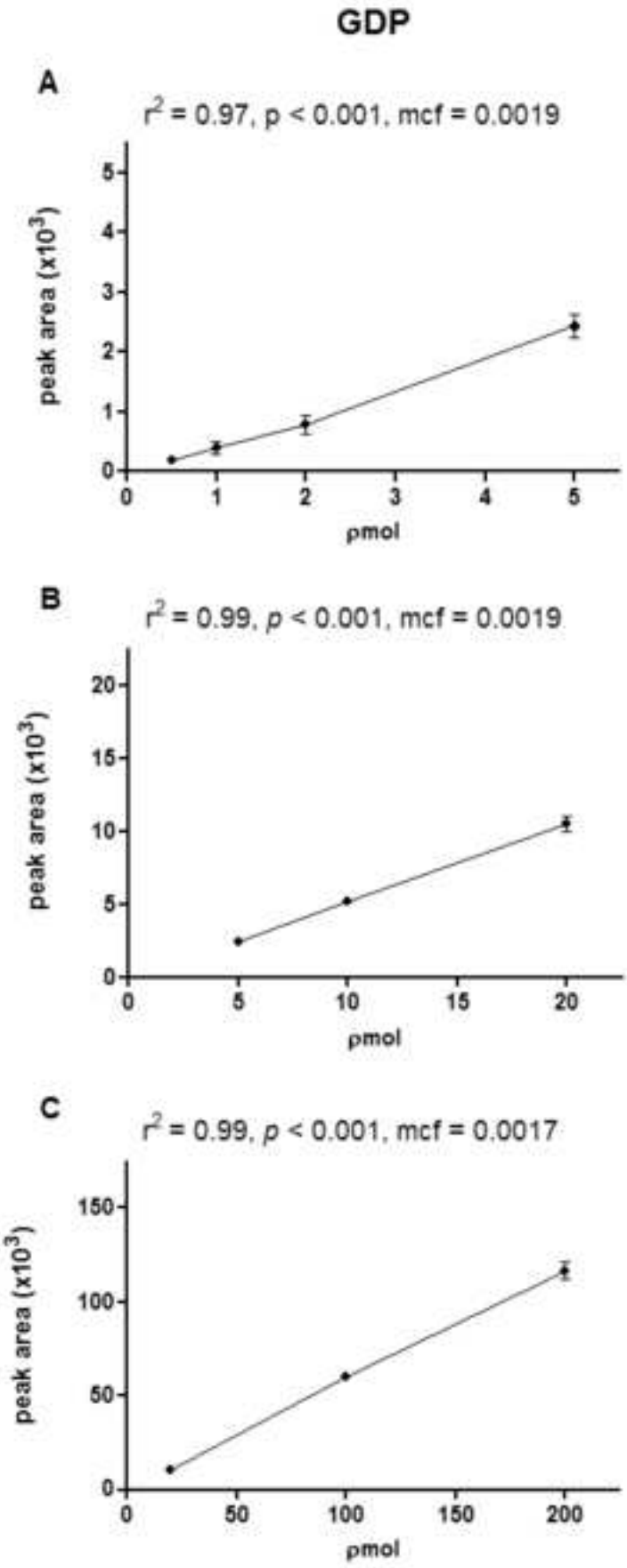
Electronic Supplementary Material (online publication only)

Click here to download high resolution image

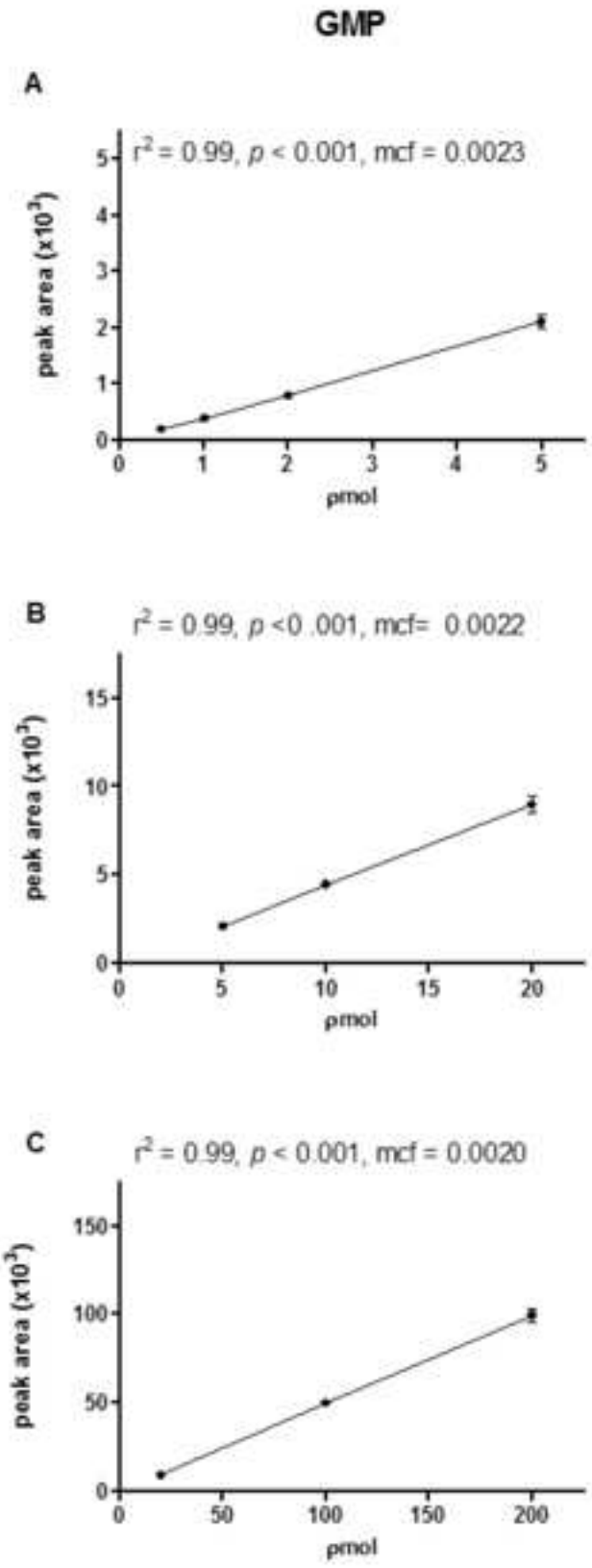


Electronic Supplementary Material (online publication only)

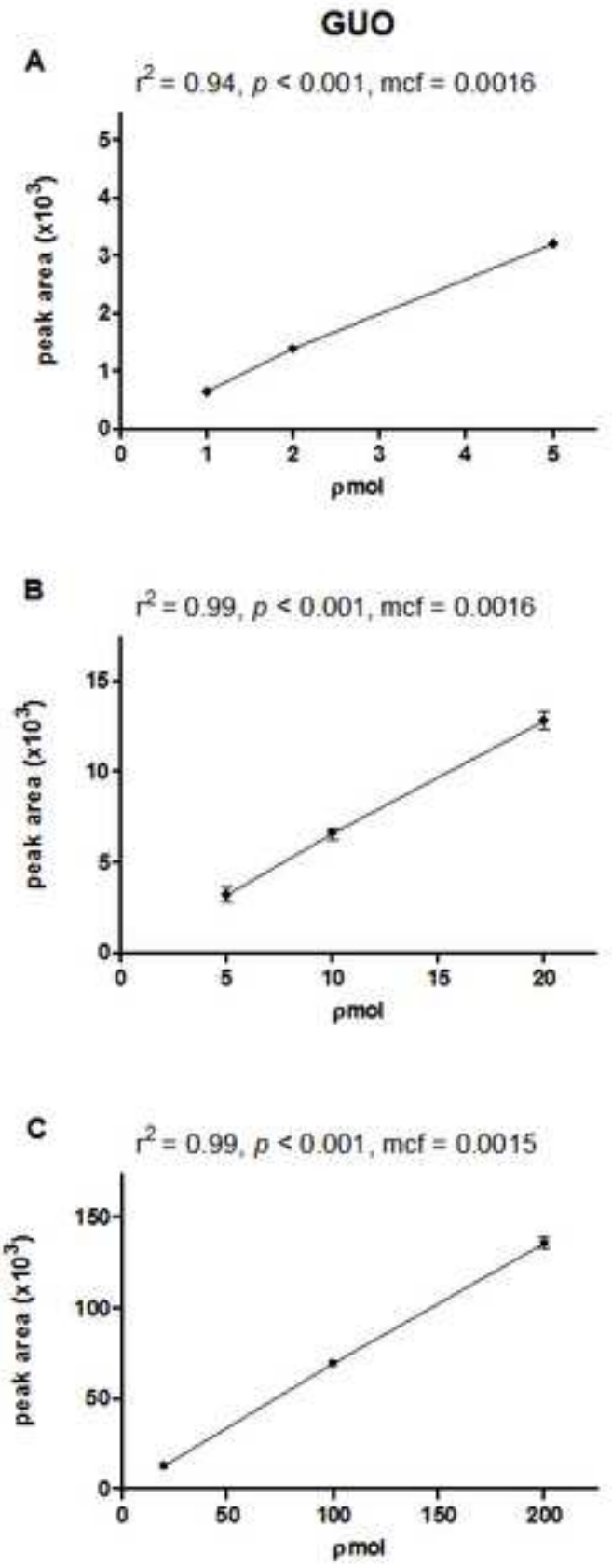
Click here to download high resolution image



Electronic Supplementary Material (online publication only)
Click here to download high resolution image

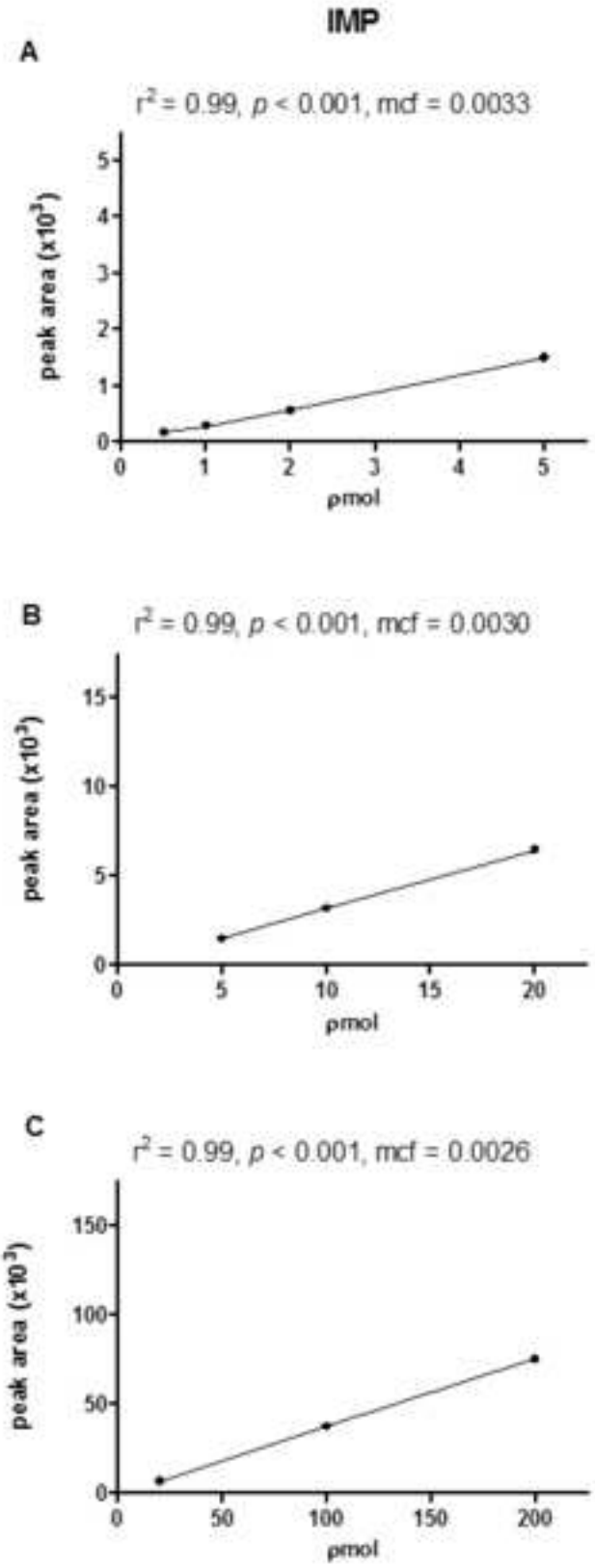


Electronic Supplementary Material (online publication only)
Click here to download high resolution image

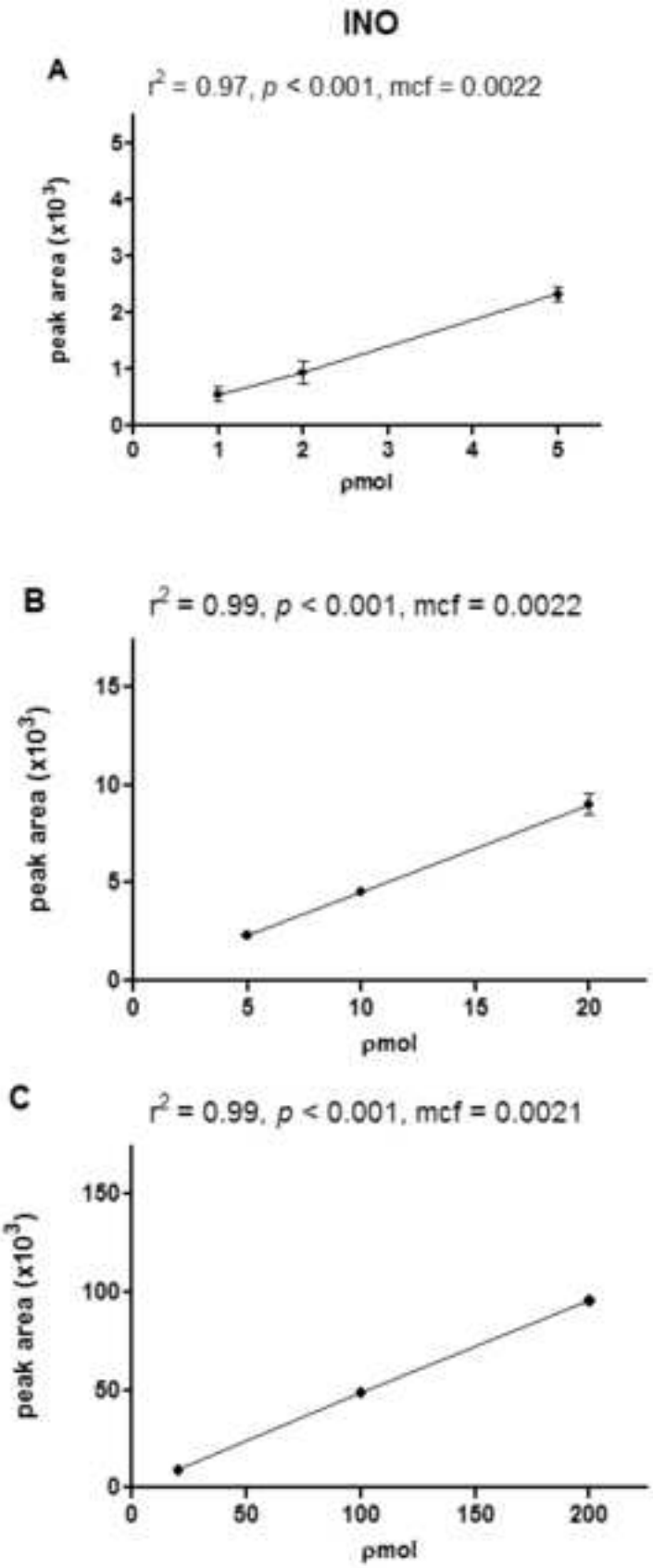


Electronic Supplementary Material (online publication only)

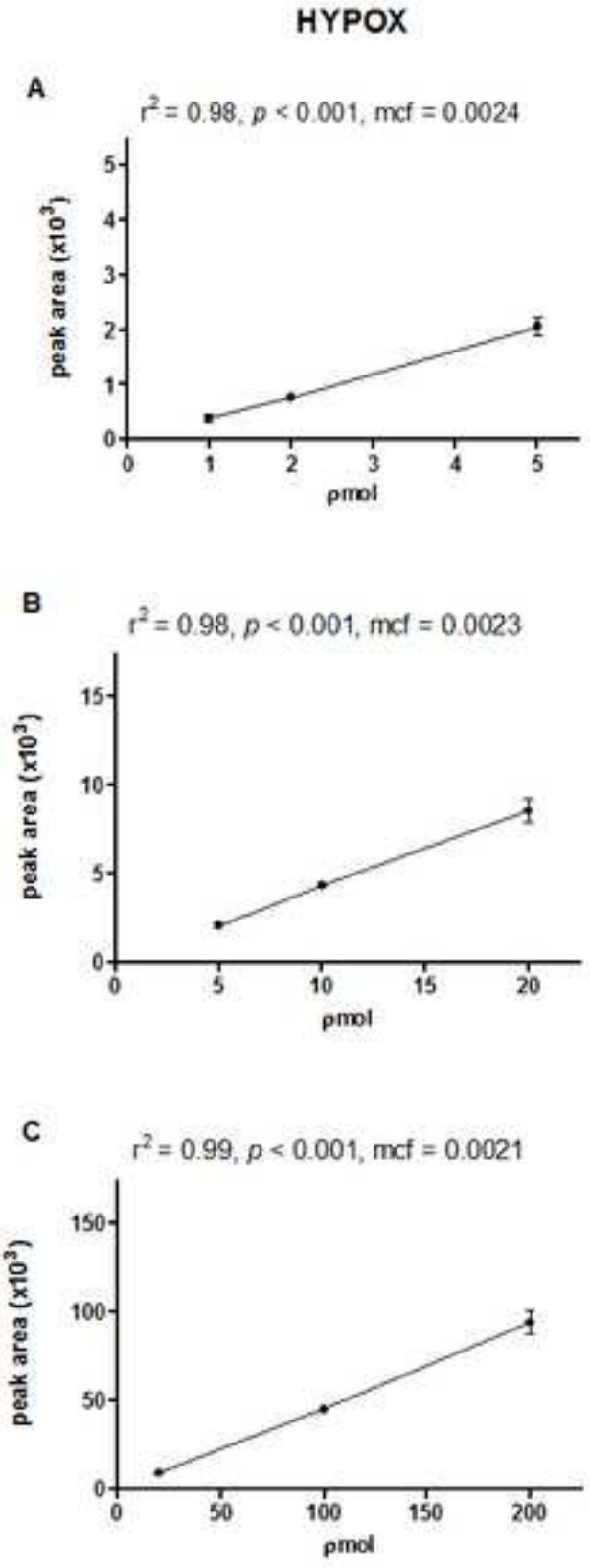
Click here to download high resolution image



Electronic Supplementary Material (online publication only)
Click here to download high resolution image

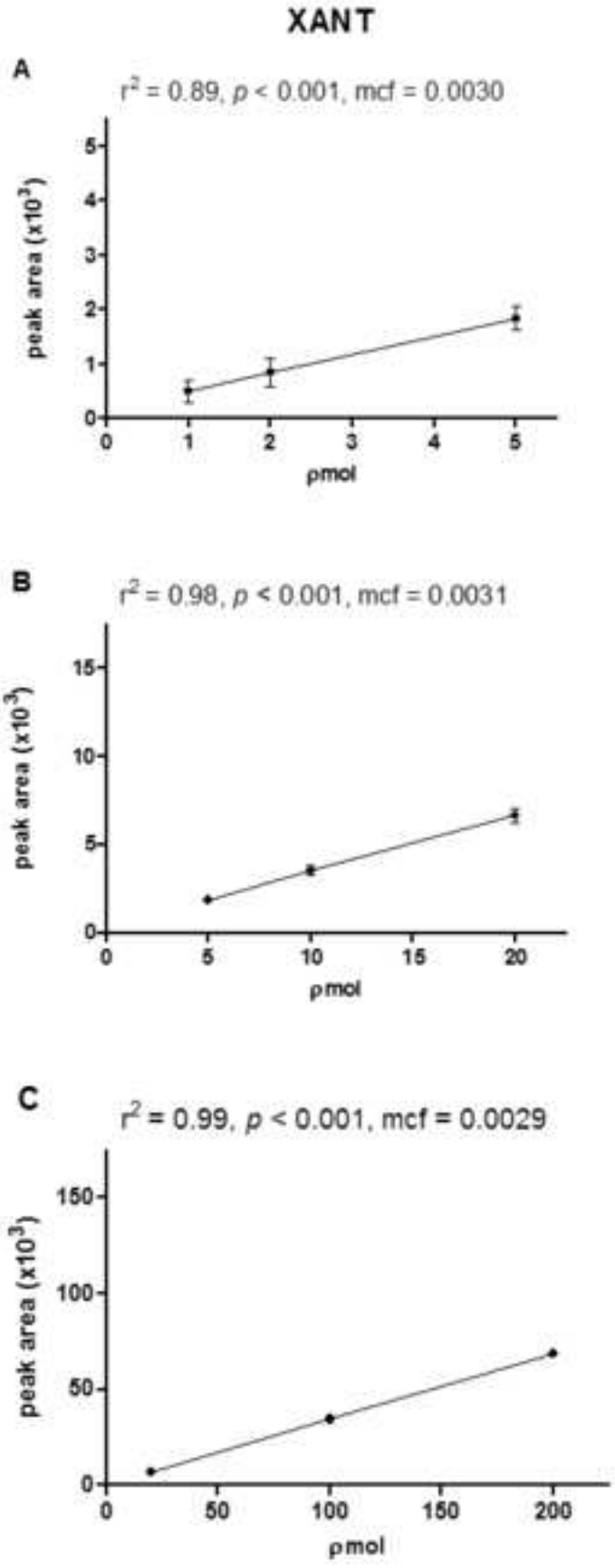


Electronic Supplementary Material (online publication only)
Click here to download high resolution image



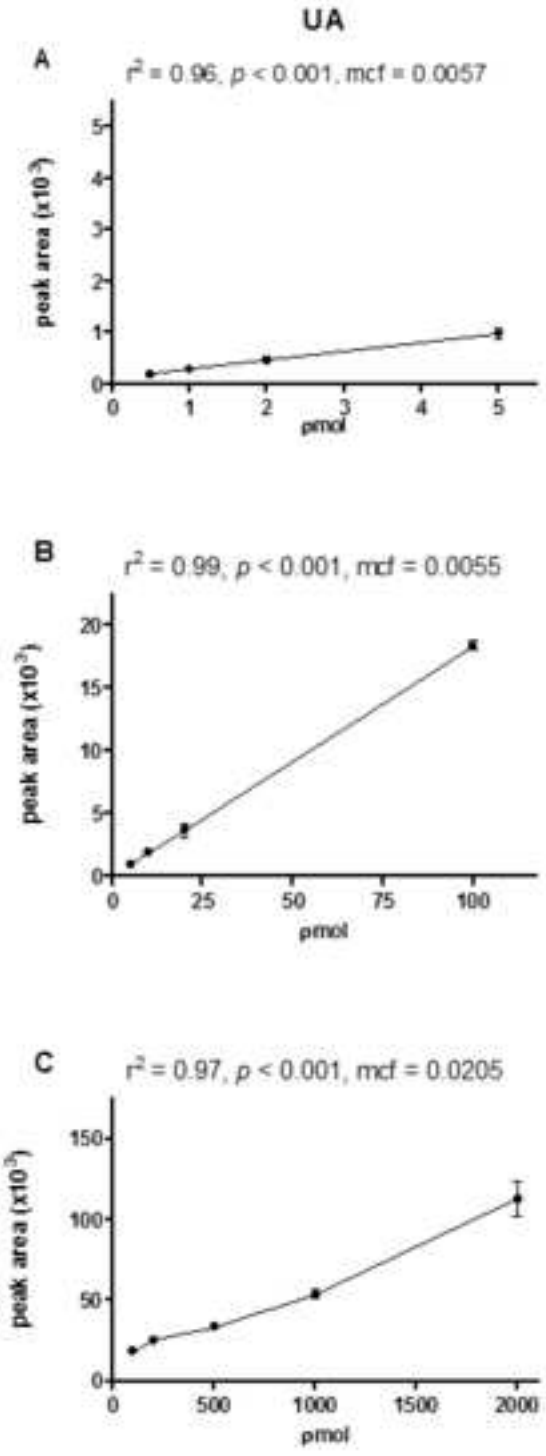
Electronic Supplementary Material (online publication only)

Click here to download high resolution image



Electronic Supplementary Material (online publication only)

Click here to download high resolution image



PARTE III

3. Discussão e conclusão

Apesar da ação esperada sobre o sistema imunológico, o tratamento em longo prazo com CsA está associado a efeitos adversos que prejudicam a qualidade de vida e até mesmo a sobrevivência de pacientes (Groetzner et al., 2005; Magnasco et al., 2008; Nath et al., 2005; Serkova et al., 2004; Shimmura et al., 2004; Subramanian and Trence, 2007). Os efeitos adversos podem ser mascarados pelo uso concomitante de outras drogas e até mesmo pelo órgão transplantado (Arnadottir et al., 1998; Herrero et al., 2000; Naesens et al., 2009; Roussel et al., 2008; Subramanian and Trence, 2007).

Com a finalidade de contribuir no esclarecimento deste tema, nosso grupo de pesquisa caracterizou um modelo de imunossupressão crônica em ratos não transplantados e avaliou os efeitos adversos do tratamento imunossupressor com CsA (capítulo I). Primeiramente, observamos que as doses de CsA 5 e 15 mg/kg administradas diariamente (via gavagem gástrica) por um período de 8 semanas são capazes de imunossuprimir ratos Wistar tratados, através da diminuição da produção de interleucinas (IL-1α, IL-1β e IL-2). Além disso, apesar de alterações histológicas leves nos rins e fígado de animais tratados com CsA, parâmetros de funcionalidade renal e hepática (creatinina, TGP e TGO, respectivamente) não foram alterados pelo tratamento. Desta forma, caracterizamos um protocolo de imunossupressão com CsA em ratos Wistar não transplantados sem prejudicar as funções renais e hepáticas.

Estudos posteriores (capítulo II) demonstraram que este mesmo tratamento crônico com CsA em ratos não transplantados induz dislipidemia e intolerância à glicose, com diminuição nos níveis de insulina e aumento nos níveis de tHcy circulante, um fator de risco independente para doença vascular (Antoniades et al., 2009; Potter et al., 2008). As alterações bioquímicas relatadas nesse trabalho sugerem o

desenvolvimento de um quadro diabético iminente em ratos tratados com CsA 15 mg/kg quando comparados aos controles. O tratamento com CsA gerou um perfil bioquímico coerente com o de intolerância à glicose, com diminuição dos níveis séricos de insulina em jejum e alterações no teste de tolerância à glicose. Além disso, também observamos que o tratamento com CsA 15 mg/kg aumenta o número de plaquetas no sangue, os níveis de fibrinogênio e tHcy circulante, aumentando o risco de eventos trombóticos.

Alguns trabalhos demonstram que além do aumento do número de plaquetas a CsA também aumenta a agregação plaquetária (Malyszko et al., 1996) . O aumentou dos níveis de fibrinogênio, uma proteína de fase aguda que está relacionada com a formação de placas ateroscleróticas e trombos (Sabeti et al., 2005), altera a viscosidade sangüínea, a sedimentação de eritrócitos e a agregação plaquetária (Lefkovits et al., 1995; Van Lente, 2000). Desta forma, o aumento do número de plaquetas e dos níveis de fibrinogênio ressalta um estado pró-inflamatório e pró-trombótico em ratos tratados com CsA.

A dislipidemia pós transplante é um evento comum e sua associação com o tratamento imunossupressor com CsA já foi descrita em estudos anteriores (Ballantyne et al., 1989; Raine et al., 1988; Vaziri et al., 2000; Verzola et al., 1999). Nesse trabalho, verificamos que a CsA aumenta os níveis de colesterol total, triglicerídeos e VLDL circulantes e este aumento, bem como o aumento dos níveis de fibrinogênio e a diminuição de insulina, se correlacionam com o aumento dos níveis de tHcy.

Embora não tenhamos estabelecido uma relação causal, as correlações entre tHcy e os parâmetros estudados sugerem um papel central da tHcy nas disfunções metabólicas observadas. Considerando a correlação bem estabelecida entre a resistência à insulina, dislipidemia, tHcy, hipercoagulabilidade e aterosclerose, podemos concluir que o tratamento com CsA aumenta os riscos dos indivíduos desenvolverem doenças

cardiovasculares e cerebrovasculares (Bensinger and Tontonoz, 2008; Demirag et al., 1998), independente da presença do órgão transplantado.

É importante compreender os efeitos da terapia imunossupressora sobre fatores de risco cardiovascular e cerebrovascular uma vez que estas complicações são a principal causa de morbidade e mortalidade entre os pacientes transplantados, superando até mesmo a infecção (Demirag et al., 1998; Serkova et al., 2004; Tellides and Pober, 2007). Portanto, procuramos elucidar outros possíveis mecanismos de ação pelos quais a CsA exerce efeitos vasculares deletérios.

Neste estudo (capítulo III) propusemos avaliar a atividade de ecto-nucleotidases sobre a hidrólise de nucleotídeos de adenina em soro, os níveis de nucleotídeos/nucleosídeos em plasma, correlações com os níveis séricos de tHcy em soro e a morfologia da artéria aorta de ratos Wistar não transplantados e tratados cronicamente com CsA.

Ecto-nucleotidases são ecto-enzimas capazes de hidrolisar nucleotídeos extracelulares aos seus respectivos nucleosídeos, assim, controlam a concentração de purinas no sangue. Uma cadeia de ecto-nucleotidases solúveis, composta por NTPDases (que hidrolisam nucleosídeos tri e difosfatados) e ecto-5'-nucleotidase (que hidrolisa nucleosídeos monofosfatados) modula as respostas mediadas pelos nucleotídeos e nucleosídeos no sistema vascular e podem ser alteradas em estados patológicos (Mishra et al., 2006; Robson et al., 2005). Nossos resultados demonstram que o tratamento com CsA 5 e 15 mg/kg inibe a atividade das enzimas estudadas, acarretando em uma diminuição de hidrólise dos nucleotídeos ATP, ADP e AMP. Este resultado correlaciona-se com o aumento dos níveis de tHcy sugerindo que a diminuição da hidrólise de ATP, ADP e AMP em soro de ratos tratados com CsA seja dependente do aumento das concentrações de tHcy. Os resultados encontrados em ratos tratados com

CsA estão de acordo com aqueles relatados para a hidrólise desses nucleotídeos *in vitro*, em que a Hcy inibiu a hidrólise de ATP, ADP e AMP de forma acompetitiva e dependente da concentração de Hcy (Bohmer et al., 2006).

No entanto, a E-NTPDase e ecto-5'-nucleotidase solúveis contribuem com uma pequena parcela da hidrólise de nucleotídeos circulantes (Yegutkin, 2008), e poderiam não representar o conjunto de enzimas nucleotidásicas solúveis e ancoradas na membrana de células vasculares. Com o objetivo de esclarecer esta questão, montamos uma metodologia de quantificação de purinas em HPLC (capítulo IV), que foi de fundamental importância para este estudo e também para estudos posteriores desta tese e do nosso grupo de pesquisa (Schmidt et al., 2008; Schmidt et al., 2009a; Schmidt et al., 2009b; Schmidt et al. 2010a; Schmidt et al. 2010b; Schmidt et al 2010c.).

Os resultados obtidos com a quantificação de purinas em plasma mostram que o tratamento com CsA diminui os níveis circulantes de ADO e aumenta os níveis de ADP, corroborando com os resultados obtidos com a hidrólise dos nucleotídeos em soro. Ao mesmo tempo, esses resultados reforçam a hipótese de as atividades da NTPDase e ecto-5'-nucleotidase solúveis podem estar representando o conjunto de enzimas que hidrolisam nucleotídeos no sistema vascular. Ainda, as concentrações de ADO e ADP encontradas também se correlacionam com os níveis de tHcy, de forma negativa e positiva, respectivamente, ou seja, quanto maiores as concentrações de tHcy, menores as concentrações de ADO e maiores as concentrações de ADP circulantes. A principal fonte de ADO circulante é a hidrólise intracelular e extracelular de AMP por endo e ecto-5'-nucleotidases (Deussen, 2000), mas uma fonte alternativa é a hidrólise intracelular de S-adenosilhomocisteina (SAH). Em condições fisiológicas, a enzima S-adenosilhomocisteína-hidrolase hidrolisa SAH até Hcy e ADO, mas na presença de altas concentrações de Hcy intracelular, a atividade reversa da enzima utiliza ADO para

produzir SAH (Riksen et al., 2005a; Riksen et al., 2003). Nestas circunstâncias os níveis intracelulares de ADO diminuem e ao mesmo tempo aumenta o gradiente de concentração transmembrana de ADO, diminuindo os níveis de ADO extracelular (Deussen, 2000). Desta forma, além da diminuição na atividade de hidrólise de ATP, ADP e AMP encontrada no soro de animais tratados com CsA, o aumento das concentrações de tHcy também pode ser responsável pela diminuição das concentrações de ADO encontradas em plasma.

A inibição das atividades nucleotidásicas acarreta o prolongamento dos efeitos vasculares causados pelos nucleotídeos ATP, ADP e AMP em seus respectivos receptores (Imai et al., 1999; Gendron et al., 2002). O ADP, importante fator de ativação plaquetária, em concentrações mais elevadas na circulação, estimula a agregação plaquetária (Gachet, 2006; Ralevic and Burnstock, 2003). A ADO, por desempenhar um papel importante no controle da homeostasia cardiovascular (Belardinelli et al., 1989; Dubey et al., 1997; Ralevic and Burnstock, 2003), quando diminuída, tem sido relacionada à vasoconstrição, aterosclerose, trombose e outras complicações cardiovasculares. Riksen et al. (2005) propõe que a diminuição de ADO causada por elevados níveis de tHcy seria uma das alterações que contribuem para o desenvolvimento das doenças cardiovasculares (Riksen et al., 2005a).

A fim de confirmar esta hipótese, estudos morfológicos e imunohistoquímicos foram realizados na artéria aorta dos animais tratados. Observamos lesões na íntima do endotélio e reatividade endotelial na artéria aorta dos ratos tratados com CsA 15 mg/kg, o que corrobora com outro estudo da literatura que observou este mesmo efeito adverso do tratamento com CsA em ratos porém, num período de tratamento curto (7 dias) (Deuse et al., 2008). Lesão endotelial associada à dislipidemia e distúrbios coagulatórios são importantes parâmetros envolvidos na doença vascular obstrutiva

progressiva, uma vasculopatia que representa a principal causa de morte em pacientes transplantados (Demirag et al., 1998; Serkova et al., 2004; Tellides and Pober, 2007). Até o presente momento não há nenhum dado na literatura com respeito a alterações na atividade de ecto-nucleotidases solúveis em modelos de imunossupressão com CsA. Esses resultados contribuem para a compreensão dos mecanismos envolvidos nas complicações vasculares relatadas em pacientes que fazem uso da terapia imunossupressora com CsA.

Portanto, as evidências obtidas nesta tese podem ser relevantes para futuros trabalhos que tenham por objetivo investigar os mecanismos de ação da CsA sobe efeitos adversos que acompanham pacientes sob terapia imunossupressora com CsA. Além disso, contribuímos para a elucidação de um método de quantificação de purinas em plasma e líquor por HLPC. Esta metodologia vem sendo muito utilizada em amostra de humanos, camundongos e ratos, contribuindo para o esclarecimento dos mecanismos de ação das purinas.

4. Perspectivas

Temos o objetivo de dar continuidade aos estudos aqui apresentados, buscando elucidar as novas perguntas que emergiram durante o desenvolvimento desta Tese. Desta forma, as perspectivas são:

- aprofundar nossos estudos sobre aos mecanismos de neurotoxicidade da CsA;

- avaliar parâmetros de neuroinflamação em ratos tratados com CsA;

- avaliar a interação astrócito-vaso nestes animais;

- elucidar os mecanismos de ansiogênese da CsA (vide Anexo 1);

- avaliar a possível ação neuroprotetora da guanosina em ratos tratados com a CsA.

5. Referências bibliográficas

Antoniades, C, Antonopoulos, AS, Tousoulis, D, Marinou, K, Stefanadis, C. Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. Eur Heart J 2009;30:6-15.

Arnadottir, M, Hultberg, B, Vladov, V, Nilsson-Ehle, P, Thysell, H. Hyperhomocysteinemia in cyclosporine-treated renal transplant recipients. Transplantation 1996;61:509-512.

Arnadottir, M, Hultberg, B, Wahlberg, J, Fellstrom, B, Dimeny, E. Serum total homocysteine concentration before and after renal transplantation. Kidney Int 1998;54:1380-1384.

Avramut, M, Achim, CL. Immunophilins in nervous system degeneration and regeneration. Curr Top Med Chem 2003;3:1376-1382.

Ballantyne, CM, Podet, EJ, Patsch, WP, Harati, Y, Appel, V, Gotto, AM, Jr., Young, JB. Effects of cyclosporine therapy on plasma lipoprotein levels. Jama 1989;262:53-56.

Barnstable, CJ, Wei, JY, Han, MH. Modulation of synaptic function by cGMP and cGMP-gated cation channels. Neurochem Int 2004;45:875-884.

Baron, BM, Dudley, MW, McCarty, DR, Miller, FP, Reynolds, IJ, Schmidt, CJ. Guanine nucleotides are competitive inhibitors of N-methyl-D-aspartate at its receptor site both in vitro and in vivo. J Pharmacol Exp Ther 1989;250:162-169.

Bartynski, WS, Grabb, BC, Zeigler, Z, Lin, L, Andrews, DF. Watershed imaging features and clinical vascular injury in cyclosporin A neurotoxicity. J Comput Assist Tomogr 1997;21:872-880.

Bechstein, WO. Neurotoxicity of calcineurin inhibitors: impact and clinical management. Transpl Int 2000;13:313-326.

Belardinelli, L, Linden, J, Berne, RM. The cardiac effects of adenosine. Prog Cardiovasc Dis 1989;32:73-97.

Bensinger, SJ, Tontonoz, P. Integration of metabolism and inflammation by lipid-activated nuclear receptors. Nature 2008;454:470-477.

Bohmer, AE, Pochmann, D, Sarkis, JJ. In vitro effect of homocysteine on nucleotide hydrolysis by blood serum from adult rats. Chem Biol Interact 2006;160:159-164.

Bourne, HR, Sanders, DA, McCormick, F. The GTPase superfamily: a conserved switch for diverse cell functions. Nature 1990;348:125-132.

Boushey, CJ, Beresford, SA, Omenn, GS, Motulsky, AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. Jama 1995;274:1049-1057.

Burnstock, G. Potential therapeutic targets in the rapidly expanding field of purinergic signalling. Clin Med 2002a;2:45-53.

Burnstock, G. Purine and pyrimidine receptors. Cell Mol Life Sci 2007;64:1471-1483.

Burnstock, G. Purinergic signaling and vascular cell proliferation and death. Arterioscler Thromb Vasc Biol 2002b;22:364-373.

Burnstock, G, Fredholm, BB, North, RA, Verkhratsky, A. The birth and postnatal development of purinergic signalling. Acta Physiol (Oxf). 2010 *Epub ahead of print.*

Chang, R, Algird, A, Bau, C, Rathbone, MP, Jiang, S. Neuroprotective effects of guanosine on stroke models in vitro and in vivo. Neurosci Lett 2008;431:101-105.

Christensen, B, Refsum, H, Vintermyr, O, Ueland, PM. Homocysteine export from cells cultured in the presence of physiological or superfluous levels of methionine: methionine loading of non-transformed, transformed, proliferating, and quiescent cells in culture. J Cell Physiol 1991;146:52-62.

Cole, DE, Ross, HJ, Evrovski, J, Langman, LJ, Miner, SE, Daly, PA, Wong, PY. Correlation between total homocysteine and cyclosporine concentrations in cardiac transplant recipients. Clin Chem 1998;44:2307-2312.

Cristalli, G, Eleuteri, A, Volpini, R, Vittori, S, Camaioni, E, Lupidi, G. Adenosine deaminase inhibitors: synthesis and structure-activity relationships of 2-hydroxy-3-nonyl derivatives of azoles. J Med Chem 1994;37:201-205.

Cronstein, BN. Molecular therapeutics. Methotrexate and its mechanism of action. Arthritis Rheum 1996;39:1951-1960.

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

Demirag, A, Moray, G, Ozdemir, N, Hizel, N, Bilgin, N, Haberal, M. Comparison of serum lipids, lipoproteins, and fibrinogen in patients with kidney transplantation and hemodialysis. Transplant Proc 1998;30:2052-2053.

Deuse, T, Hoyt, G, Koyanagi, T, Robbins, RC, Schrepfer, S. Prevention and inhibition but not reversion of chronic allograft vasculopathy by FK778. Transplantation 2008;85:870-877.

Deussen, A. Metabolic flux rates of adenosine in the heart. Naunyn Schmiedebergs Arch Pharmacol 2000;362:351-363.

Dohgu, S, Kataoka, Y, Ikesue, H, Naito, M, Tsuruo, T, Oishi, R, Sawada, Y. Involvement of glial cells in cyclosporine-increased permeability of brain endothelial cells. Cell Mol Neurobiol 2000;20:781-786.

Dohgu, S, Yamauchi, A, Nakagawa, S, Takata, F, Kai, M, Egawa, T, et al. Nitric oxide mediates cyclosporine-induced impairment of the blood-brain barrier in cocultures of mouse brain endothelial cells and rat astrocytes. Eur J Pharmacol 2004;505:51-59.

Drachman, BM, DeNofrio, D, Acker, MA, Galetta, S, Loh, E. Cortical blindness secondary to cyclosporine after orthotopic heart transplantation: a case report and review of the literature. J Heart Lung Transplant 1996;15:1158-1164.

Dubey, RK, Jackson, EK, Rupprecht, HD, Sterzel, RB. Factors controlling growth and matrix production in vascular smooth muscle and glomerular mesangial cells. Curr Opin Nephrol Hypertens 1997;6:88-105.

Dunwiddie, TV, Masino, SA. The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci 2001;24:31-55.

Eikelboom, JW, Lonn, E, Genest, J, Jr., Hankey, G, Yusuf, S. Homocyst(e)ine and cardiovascular disease: a critical review of the epidemiologic evidence. Ann Intern Med 1999;131:363-375.

Faraci, FM. Hyperhomocysteinemia: a million ways to lose control. Arterioscler Thromb Vasc Biol 2003;23:371-373.

Fruman, DA, Klee, CB, Bierer, BE, Burakoff, SJ. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. Proc Natl Acad Sci U S A 1992;89:3686-3690.

Gachet, C. Regulation of platelet functions by P2 receptors. Annu Rev Pharmacol Toxicol 2006;46:277-300.

Groetzner, J, Reichart, B, Roemer, U, Reichel, S, Kozlik-Feldmann, R, Tiete, A, et al. Cardiac transplantation in pediatric patients: fifteen-year experience of a single center. Ann Thorac Surg 2005;79:53-60; discussion 61.

Grub, S, Persohn, E, Trommer, WE, Wolf, A. Induction of apoptosis by the O-hydroxyethyl-D(Ser)(8)-cyclosporine A derivative SDZ IMM 125 in rat hepatocytes. J Pharmacol Exp Ther 2000a;293:24-32.

Grub, S, Persohn, E, Trommer, WE, Wolf, A. Mechanisms of cyclosporine A-induced apoptosis in rat hepatocyte primary cultures. Toxicol Appl Pharmacol 2000b;163:209-220.

Herrero, JI, Quiroga, J, Sangro, B, Beloqui, O, Pardo, F, Cienfuegos, JA, Prieto, J. Hyperhomocysteinemia in liver transplant recipients: prevalence and multivariate analysis of predisposing factors. Liver Transpl 2000;6:614-618.

Huang, RF, Hsu, YC, Lin, HL, Yang, FL. Folate depletion and elevated plasma homocysteine promote oxidative stress in rat livers. J Nutr 2001;131:33-38.

Jansen, O, Krieger, D, Krieger, S, Sartor, K. Cortical hyperintensity on proton density-weighted images: An MR sign of cyclosporine-related encephalopathy. AJNR Am J Neuroradiol 1996;17:337-344.

Kawashima, Y, Nagasawa, T, Ninomiya, H. Contribution of ecto-5'-nucleotidase to the inhibition of platelet aggregation by human endothelial cells. Blood 2000;96:2157-2162.

Klawitter, J, Gottschalk, S, Hainz, C, Leibfritz, D, Christians, U, Serkova, NJ. Immunosuppressant neurotoxicity in rat brain models: oxidative stress and cellular metabolism. Chem Res Toxicol 2010;23:608-619.

Klawitter, J, Gottschalk, S, Hainz, C, Leibfritz, D, Christians, U, Serkova, NJ. Immunosuppressant neurotoxicity in rat brain models: oxidative stress and cellular metabolism. Chem Res Toxicol;23:608-619.

Kochi, S, Takanaga, H, Matsuo, H, Naito, M, Tsuruo, T, Sawada, Y. Effect of cyclosporin A or tacrolimus on the function of blood-brain barrier cells. Eur J Pharmacol 1999;372:287-295.

Kunapuli, SP, Daniel, JL. P2 receptor subtypes in the cardiovascular system. Biochem J 1998;336 (Pt 3):513-523.

Lara, DR, Schmidt, AP, Frizzo, ME, Burgos, JS, Ramirez, G, Souza, DO. Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. Brain Res 2001;912:176-180.

Latini, S, Pedata, F. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. J Neurochem 2001;79:463-484.

Lefkovits, J, Plow, EF, Topol, EJ. Platelet glycoprotein IIb/IIIa receptors in cardiovascular medicine. N Engl J Med 1995;332:1553-1559.

Loscalzo, J. The oxidant stress of hyperhomocyst(e)inemia. J Clin Invest 1996;98:5-7.

Magnasco, A, Rossi, A, Catarsi, P, Gusmano, R, Ginevri, F, Perfumo, F, Ghiggeri, GM. Cyclosporin and organ specific toxicity: clinical aspects, pharmacogenetics and perspectives. Curr Clin Pharmacol 2008;3:166-173.

Malinow, MR. Plasma homocyst(e)ine: a risk factor for arterial occlusive diseases. J Nutr 1996;126:1238S-1243S.

Malyszko, J, Malyszko, JS, Pawlak, K, Mysliwiec, M. The coagulo-lytic system and endothelial function in cyclosporine-treated kidney allograft recipients. Transplantation 1996;62:828-830.

Marchetti, P. New-onset diabetes after transplantation. J Heart Lung Transplant 2004;23:S194-201.

Markell, MS, Armenti, V, Danovitch, G, Sumrani, N. Hyperlipidemia and glucose intolerance in the post-renal transplant patient. J Am Soc Nephrol 1994;4:S37-47.

Mayer, EL, Jacobsen, DW, Robinson, K. Homocysteine and coronary atherosclerosis. J Am Coll Cardiol 1996;27:517-527.

McCully, KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol 1969;56:111-128.

Mishra, SK, Braun, N, Shukla, V, Fullgrabe, M, Schomerus, C, Korf, HW, et al. Extracellular nucleotide signaling in adult neural stem cells: synergism with growth factor-mediated cellular proliferation. Development 2006;133:675-684.

Moretto, MB, Arteni, NS, Lavinsky, D, Netto, CA, Rocha, JB, Souza, DO, Wofchuk, S. Hypoxic-ischemic insult decreases glutamate uptake by hippocampal slices from neonatal rats: prevention by guanosine. Exp Neurol 2005;195:400-406.

Moretto, MB, Boff, B, Lavinsky, D, Netto, CA, Rocha, JB, Souza, DO, Wofchuk, ST. Importance of schedule of administration in the therapeutic efficacy of guanosine: early intervention after injury enhances glutamate uptake in model of hypoxia-ischemia. J Mol Neurosci 2009;38:216-219.

Mubagwa, K, Mullane, K, Flameng, W. Role of adenosine in the heart and circulation. Cardiovasc Res 1996;32:797-813.

Naesens, M, Kuypers, DR, Sarwal, M. Calcineurin inhibitor nephrotoxicity. Clin J Am Soc Nephrol 2009;4:481-508.

Nath, DS, Gruessner, AC, Kandaswamy, R, Gruessner, RW, Sutherland, DE, Humar, A. Outcomes of pancreas transplants for patients with type 2 diabetes mellitus. Clin Transplant 2005;19:792-797.

Nouri-Majalan, N, Masoumi, R, Nafisi, R, Nogh, H, Ghafari, A, Moghaddasi, S. Relationship between serum homocysteine and other parameters in renal transplant patients. Transplant Proc 2009;41:2826-2828.

Potter, K, Hankey, GJ, Green, DJ, Eikelboom, J, Jamrozik, K, Arnolda, LF. The effect of long-term homocysteine-lowering on carotid intima-media thickness and flow-mediated vasodilation in stroke patients: a randomized controlled trial and meta-analysis. BMC Cardiovasc Disord 2008;8:24.

Raine, AE, Carter, R, Mann, JI, Morris, PJ. Adverse effect of cyclosporin on plasma cholesterol in renal transplant recipients. Nephrol Dial Transplant 1988;3:458-463.

Ralevic, V, Burnstock, G. Involvement of purinergic signaling in cardiovascular diseases. Drug News Perspect 2003;16:133-140.

Ralevic, V, Burnstock, G. Roles of P2-purinoceptors in the cardiovascular system. Circulation 1991;84:1-14.

Rathbone, M, Pilutti, L, Caciagli, F, Jiang, S. Neurotrophic effects of extracellular guanosine. Nucleosides Nucleotides Nucleic Acids 2008;27:666-672.

Riksen, NP, Rongen, GA, Blom, HJ, Boers, GH, Smits, P. Reduced adenosine receptor stimulation as a pathogenic factor in hyperhomocysteinemia. Clin Chem Lab Med 2005a;43:1001-1006.

Riksen, NP, Rongen, GA, Blom, HJ, Russel, FG, Boers, GH, Smits, P. Potential role for adenosine in the pathogenesis of the vascular complications of hyperhomocysteinemia. Cardiovasc Res 2003;59:271-276.

Riksen, NP, Rongen, GA, Boers, GH, Blom, HJ, van den Broek, PH, Smits, P. Enhanced cellular adenosine uptake limits adenosine receptor stimulation in patients with hyperhomocysteinemia. Arterioscler Thromb Vasc Biol 2005b;25:109-114.

Robson, SC, Wu, Y, Sun, X, Knosalla, C, Dwyer, K, Enjyoji, K. Ectonucleotidases of CD39 family modulate vascular inflammation and thrombosis in transplantation. Semin Thromb Hemost 2005;31:217-233.

Roussel, JC, Baron, O, Perigaud, C, Bizouarn, P, Pattier, S, Habash, O, et al. Outcome of heart transplants 15 to 20 years ago: graft survival, post-transplant morbidity, and risk factors for mortality. J Heart Lung Transplant 2008;27:486-493.

Sabeti, S, Exner, M, Mlekusch, W, Amighi, J, Quehenberger, P, Rumpold, H, Maurer, G, Minar, E, Wagner, O, Schillinger, M. Prognostic impact of fibrinogen in carotid atherosclerosis: nonspecific indicator of inflammation or independent predictor of disease progression? Stroke 2005;36:1400-1404.

Schmidt, AP, Avila, TT, Souza, DO. Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. Neurochem Res 2005;30:69-73.

Schmidt, AP, Bohmer, AE, Antunes, C, Schallenberger, C, Porciuncula, LO, Elisabetsky, E, Lara, DR, Souza, DO. Anti-nociceptive properties of the xanthine oxidase inhibitor allopurinol in mice: role of A1 adenosine receptors. Br J Pharmacol 2009a;156:163-172.

Schmidt, AP, Bohmer, AE, Leke, R, Schallenberger, C, Antunes, C, Pereira, MS, Wofchuk, ST, Elisabetsky, E, Souza, DO. Antinociceptive effects of intracerebroventricular administration of guanine-based purines in mice: evidences for the mechanism of action. Brain Res 2008;1234:50-58.

Schmidt, AP, Bohmer, AE, Schallenberger, C, Antunes, C, Pereira, MS, Leke, R, Wofchuk, ST, Elisabetsky, E, Souza, DO. Spinal mechanisms of antinociceptive action caused by guanosine in mice. Eur J Pharmacol 2009b;613:46-53.

Schmidt, AP, Bohmer, AE, Schallenberger, C, Antunes, C, Tavares, RG, Wofchuk, ST, Elisabetsky, E, Souza, DO. Mechanisms involved in the antinociception induced by systemic administration of guanosine in mice. Br J Pharmacol 2010a;159:1247-1263.

Schmidt, AP, Bohmer, AE, Soares, FA, Posso, IP, Machado, SB, Mendes, FF, Portela, LV, Souza, DO. Changes in purines concentration in the cerebrospinal fluid of patients experiencing pain: a case-control study. Neurosci Lett 2010b;474:69-73.

Schmidt, AP, Lara, DR, de Faria Maraschin, J, da Silveira Perla, A, Onofre Souza, D. Guanosine and GMP prevent seizures induced by quinolinic acid in mice. Brain Res 2000;864:40-43.

Schmidt, AP, Lara, DR, Souza, DO. Proposal of a guanine-based purinergic system in the mammalian central nervous system. Pharmacol Ther 2007;116:401-416.

Schmidt, AP, Paniz, L, Schallenberger, C, Bohmer, AE, Wofchuk, ST, Elisabetsky, E, Portela, LV, Souza, DO. Guanosine prevents thermal hyperalgesia in a rat model of peripheral mononeuropathy. J Pain 2010c;11:131-141.

Schreiber, SL, Crabtree, GR. The mechanism of action of cyclosporin A and FK506. Immunol Today 1992;13:136-142.

Serkova, NJ, Christians, U, Benet, LZ. Biochemical mechanisms of cyclosporine neurotoxicity. Mol Interv 2004;4:97-107.

Sheth, TN, Ichise, M, Kucharczyk, W. Brain perfusion imaging in asymptomatic patients receiving cyclosporin. AJNR Am J Neuroradiol 1999;20:853-856.

Shimmura, H, Tanabe, K, Tokumoto, T, Ishida, H, Ishikawa, N, Miyamoto, N, et al. Analysis of cause of death with a functioning graft: a single-center experience. Transplant Proc 2004;36:2026-2029.

Sklar, EM. Post-transplant neurotoxicity: what role do calcineurin inhibitors actually play? AJNR Am J Neuroradiol 2006;27:1602-1603.

Soares, FA, Schmidt, AP, Farina, M, Frizzo, ME, Tavares, RG, Portela, LV, Lara, DR, Souza, DO. Anticonvulsant effect of GMP depends on its conversion to guanosine. Brain Res 2004;1005:182-186.

Soslau, G, Youngprapakorn, D. A possible dual physiological role of extracellular ATP in the modulation of platelet aggregation. Biochim Biophys Acta 1997;1355:131-140.

Stanger, O, Herrmann, W, Pietrzik, K, Fowler, B, Geisel, J, Dierkes, J, Weger, M. Clinical use and rational management of homocysteine, folic acid, and B vitamins in cardiovascular and thrombotic diseases. Z Kardiol 2004;93:439-453.

Subramanian, S, Trence, DL. Immunosuppressive agents: effects on glucose and lipid metabolism. Endocrinol Metab Clin North Am 2007;36:891-905; vii.

Takata, F, Dohgu, S, Yamauchi, A, Sumi, N, Nakagawa, S, Naito, M, Tsuruo, T, Shuto, H, Kataoka, Y. Inhibition of transforming growth factor-beta production in brain pericytes contributes to cyclosporin A-induced dysfunction of the blood-brain barrier. Cell Mol Neurobiol 2007;27:317-328.

Tellides, G, Pober, JS. Interferon-gamma axis in graft arteriosclerosis. Circ Res 2007;100:622-632.

Ueland, PM. Homocysteine species as components of plasma redox thiol status. Clin Chem 1995;41:340-342.

Upchurch, GR, Jr., Welch, GN, Fabian, AJ, Freedman, JE, Johnson, JL, Keaney, JF, Jr., Loscalzo, J. Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 1997;272:17012-17017.

Van Lente, F. Markers of inflammation as predictors in cardiovascular disease. Clin Chim Acta 2000;293:31-52.

Vaziri, ND, Liang, K, Azad, H. Effect of cyclosporine on HMG-CoA reductase, cholesterol 7alpha-hydroxylase, LDL receptor, HDL receptor, VLDL receptor, and lipoprotein lipase expressions. J Pharmacol Exp Ther 2000;294:778-783.

Vercauteren, SB, Bosmans, JL, Elseviers, MM, Verpooten, GA, De Broe, ME. A meta-analysis and morphological review of cyclosporine-induced nephrotoxicity in auto-immune diseases. Kidney Int 1998;54:536-545.

Verzola, A, Bedani, PL, Gilli, P. Effect of ciclosporin on triglyceridemia in long-term renal transplant recipients. Nephron 1999;81:240-241.

Vinade, ER, Schmidt, AP, Frizzo, ME, Izquierdo, I, Elisabetsky, E, Souza, DO. Chronically administered guanosine is anticonvulsant, amnesic and anxiolytic in mice. Brain Res 2003;977:97-102.

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 2005;79:248-253.

Welch, GN, Loscalzo, J. Homocysteine and atherothrombosis. N Engl J Med 1998;338:1042-1050.

Yegutkin, GG. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochim Biophys Acta 2008;1783:673-694.

Zhang, X, Chen, BC, Xiang, FL, Chang, S, Zhou, HM, Du, DF, Yuan, J, Chen, ZK. Inhibition of MD-1 expression by immunosuppressants or antisense oligodeoxynucleotides on skin allograft survival in mice. Transplant Proc 2005;37:1965-1967.

Zimmermann, H. 5'-Nucleotidase: molecular structure and functional aspects. Biochem J 1992;285 (Pt 2):345-365.

PARTE IV

Anexo 1

Anxiety-like behavior and astrocytic morphology in chronic cyclosporine treated rats: lack of correlation with the astrocytic glutamatergic system.

Débora Guerini Souza, Marcelo Ganzella, Gisele Hansel, Júlia Dubois Moreira, Letícia Rodrigues, Vanessa Kazlauckas, Matilde Achaval, Luis Valmor Portela, Diogo Onofre Souza, Ana Elisa Böhmer.

Este trabalho foi desenvolvido durante a o período desta tese. No entanto, não está no corpo da tese e nem foi submetido a nenhum periódico com a intenção de aprimorar a discussão dos resultados obtidos. Nosso objetivo é discutir com a banca os resultados a fim de enriquecer e ampliar nossa visão e conhecimento em relação às alterações astrocitárias inauditas deste trabalho.

Anxiety-like behavior and astrocytic morphology in chronic cyclosporine treated rats: lack of correlation with the astrocytic glutamatergic system.

Débora Guerini Souza [1], Marcelo Ganzella [1], Gisele Hansel [1], Júlia Dubois Moreira [1], Letícia Rodrigues [1], Vanessa Kazlauckas [1], Matilde Achaval [2], Luis Valmor Portela [1], Diogo Onofre Souza [1], Ana Elisa Böhmer (✉)[1]

[1] Department of Biochemistry, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

[2] Department of Morphological Sciences, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

(✉) Ana Elisa Böhmer

Avenida Ramiro Barcelos, 2600-Anexo

Zip Code: 90.035-003

Porto Alegre - RS - Brazil

Phone: (55-51) 33085559 / 33085558

Fax: (55-51) 33085540 / 33085535

E-mail: anaelisab@gmail.com

1. Introduction

Maintenance of viability and function of a transplanted organ requires life-long immunosuppressive therapy. Today, most immunosuppressive protocols are based upon cyclosporine (CsA) administration (Vercauteren, Bosmans et al. 1998; Subramanian and Trence 2007; Tellides and Pober 2007). Despite the expected action on the immune system, CsA long-term treatment is associated with adverse effects that have deleterious outcome on the quality of life and survival of patients (Serkova, Christians et al. 2004; Groetzner, Reichart et al. 2005; Subramanian and Trence 2007; Magnasco, Rossi et al. 2008).

Neurotoxicity is one of the most prominent adverse effects of CsA administration and responsible for neuropsychological problems including anxiety, depression, headaches, tremor, hallucinations and seizures (Bechstein 2000; Serkova, Christians et al. 2004; Magnasco, Rossi et al. 2008), however, the clinical predisposition and mechanisms of CsA-induced neurotoxicity remain controversial and poorly understood. Neurotoxicity is more frequently associated with measured steady-state minimum blood concentrations higher than recommended levels, but also occurs during long-term treatment when CsA concentrations blood are within the therapeutic range (Serkova, Christians et al. 2004). Anxiogenic effect of CsA is widely reported by patients under chronic treatment (Calne, Rolles et al. 1979; Hauben 1996; Gijtenbeek, van den Bent et al. 1999; Wijdicks 2001). Anxiety disorders are common psychiatric diseases and is characterized by the presence of physical symptoms, especially autonomic ones (as tachycardia, sweating, dyspnea) associated with feelings of fear, discomfort, or psychological stress, causing great suffering to patients (Hoffman and Mathew 2008).

Growing evidence indicates that glia pathology and amino-acid neurotransmitter system abnormalities contribute to the pathophysiology and possibly to the pathogenesis of

major mood disorders (Sanacora, Rothman et al. 2003; Krystal, Tolin et al. 2009). Astrocytes serve numerous and complex functions, such as maintaining synaptic transmission, providing nutrients to neurons, generating response to injury and participating in immune defense (Pekny and Nilsson 2005; Pellerin, Bouzier-Sore et al. 2007; Belanger and Magistretti 2009).

The glutamatergic system has received considerable attention over the last years as potential target for anxiolytic drugs (Chojnacka-Wojcik, Klodzinska et al. 2001; Cryan, Kelly et al. 2003; Sanacora, Rothman et al. 2003; Bergink, van Megen et al. 2004; Palucha and Pilc 2007; Kapus, Gacsalyi et al. 2008). Glutamate is the main excitatory neurotransmitter in mammalian central nervous system (CNS) and is involved in several plastic brain processes (Ozawa 1998; Segovia, Porras et al. 2001; Izquierdo, Bevilaqua et al. 2006; Schmidt, Bohmer et al. 2008). However, glutamate is involved in various acute and chronic brain diseases when its concentration increase over the physiological range in the synaptic cleft (excitotoxicity), including neurodegenerative diseases, traumatic brain injury, cerebral ischemia, and seizures (Lipton and Rosenberg 1994; Meldrum 1994; Maragakis and Rothstein 2006; Sheldon and Robinson 2007). Glutamate exerts its excitatory action via ionotropic (AMPA, NMDA and kainate) and metabotropic (coupled to G proteins) glutamate receptors (Kew and Kemp 2005). The high affinity uptake through excitatory amino acid transporters is responsible for the clearance of glutamate from the synaptic cleft, and the main mechanism to modulate its physiological activity and to avoid the glutamatergic excitotoxicity (Danbolt 2001). Astrocytes play a major role in glutamate recycling. Glutamate uptake activity is predominantly astrocytic (Belanger and Magistretti 2009), and glutamine synthetase (GS) is an exclusively astrocytic enzyme (Gjessing, Gjesdahl et al. 1972; Danbolt 2001), able to convert glutamate to glutamine.

There are only a few experimental studies in the literature relating CsA treatment and anxiety. Moreover, only acute and high-dose CsA administration effects were previously studied with rodents (von Horsten, Exton et al. 1998; Borlongan, Kwanbara et al. 1999; Sato, Takayanagi et al. 2007). Regarding clinical studies, it is difficult to determine in transplanted patients the adverse effects specifically related to CsA treatment. Other confounding variables, such as allograft rejection, impaired renal function and effects due to other drug therapies mask the adverse effects that could be related solely to CsA treatment. Here, we aimed to investigate the effects of a chronic CsA treatment in non transplanted rats upon anxiety related behavior and the putative role of astrocytes and glutamatergic system on the behavioral effects.

2. Materials and methods

Animals and treatments

Twenty nine adult male Wistar rats (250-300 g), were kept on a 12-hour light/dark cycle (light on at 7:00 am) at temperatures of 22 º ± 1 ºC, housed in plastic cages (5 per cage) with tap water and commercial food *ad libitum.* Animals were obtained from our animal house. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

Rats were divided in 2 groups according to the experimental design. The control group (N = 10) received vehicle (corn oil). CsA-treated group (N = 19) received CsA 15 mg/kg/day diluted in corn oil. The drug or vehicle administration was performed by daily gastric gavage for 8 weeks.

Drug and Reagents

CsA was from Novartis (France). The anesthetic sodium thiopental was obtained from Cristália (SP, Brazil). Antibody against GLAST and GLT-1 were kindly provided by Dr. Niels C. Danbolt from the Center for Molecular Biology and Neuroscience, University of Oslo. Antibody against GS was purchased from Sigma Aldrich (USA) and polyclonal rabbit anti-GFAP from Dako (Denmark). The horseradish peroxidase-conjugated secondary antibody against rabbit and mouse, as the ECL, was purchased from Amersham Pharmacia Biotech. X-ray films were purchased from Kodak X-Omat, Rochester, NY, USA. L- [^{3}H] glutamate (specific activity 30 Ci/mmol) was purchased from Amersham International, UK. All other chemicals were of high grade of quality.

Behavioral procedures

The behavioral tasks aimed to evaluate the possible anxiolytic effects of CsA, as well as its actions in rat's locomotion and spacial exploration. Behavioral sessions were carried out in an experimental room with constant temperature (21 ± 2°C) and light conditions (60-W light), except for the plus maze exploration task (see details below). Before the sessions, animals were allowed to adapt to the experimental room for at least 1 h. Inter-task intervals were of 2 days and all behavioral tasks were performed between 9:00-16:00 h. All behavioral parameters in open field and plus maze tasks were recorded and analyzed by the video tracking system Any-maze (Stoelting, USA).

Open field session

An open field apparatus was used to analyze free locomotor and exploratory activities of animals, as previously described (Ghisleni, Kazlauckas et al. 2008). All the

animals were gently placed in the same initial position of a 60 cm diameter square black arena (walls 50 cm high) facing the wall and left free to explore for 15 min. The floor was divided into 12 equal squares by black lines. The area was divided in center and periphery and the room was illuminated by two halogen lamps pointed towards the room walls. Animal movements were recorded by an aerial camera and the videos were analyzed by Any-maze software, which provided data about distance traveled (first 5 min – considered exploratory activity and the last 10 min – consideres locomotor activity) mobile and immobile time, and center and periphery entries. A trained and blinded-to-treatment observer counted manually number of rearings, groomings and fecal boli. After each trial, apparatus was cleaned with an ethanol solution (70%) and wiped with a dry tissue.

Elevated Plus Maze session

The elevated plus-maze apparatus consisted of a central platform (10 × 10 cm) with two open and two closed arms (45 × 10-cm), arranged in such a way that the 2 arms of each type were opposite to each other (Pellow and File 1986). The maze was kept 88 cm above the floor and the sessions were carried out in a room lighted only with a dim red light (Walf and Frye 2007). Animals were individually placed in the central platform facing an open arm and their activities were recorded by an aerial camera for 5 min. Videos were analyzed by Any-maze software and data about time spent in open and closed arms, entries in open and closed arms and distance traveled in open and closed arms were obtained. A trained and blinded-to-treatment observer recorded manually the number of rearings. After each trial, apparatus was cleaned with an ethanol solution (70%) and wiped with a dry tissue.

Light-dark box session

Rats were subjected to the light/dark task as previously described (Crawley and Goodwin 1980) with some modifications. The dark–light box consisted of 2 compartments: a light compartment (30 cm - 20 cm - 20 cm), with a transparent acrylic cover in the light side to allow observation of the rat and illuminated under a 60-W light, (the other surrounding walls and floor were painted white), and a dark compartment (15 cm - 20 cm - 20 cm) with the surrounding walls and floor painted black that were not illuminated. The floor of the light compartment was divided into 12 equal squares by black lines and the dark compartment was divided into 9 equal squares by white lines. The two compartments were separated by a wall, which had a small opening (80 x 50 mm, height x length) at floor level. The rats were gently placed in the corner of the light compartment facing the wall opposite to the opening. The following parameters were recorded during 5 min: latency time for the first entry in the dark compartment, number of entries in the dark compartment, total time spent in the light compartment, number of crossings (horizontal activity) in light compartment, number of fecal boli in each compartment, and the risk assessment behavior index (the number of investigations of the light compartment by placing some but not all paws on it). After each trial, apparatus was cleaned with an ethanol solution (70%) and wiped with a dry tissue. Among other parameters, a low exploratory activity in the light area is taken as an index of anxiety.

Astrocytic immunohistochemical analyzes

Rats were perfused through the left cardiac ventricle with 200 mL of saline solution, followed by 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and left for post-fixation in the same fixative solution at 4^{o}C for 2 h at room temperature. Hereafter this, the material was cryoprotected by immersing the brain in

30% sucrose in phosphate buffer at 4 ^{o}C. The brains were sectioned (45 μm) in a cryostat (Leitz) and the free-floating sections were preincubated in 2% bovine serum albumin in phosphate-buffered saline containing 0.3% Triton X-100 for 30 min and then incubated with polyclonal anti-GFAP (Dako) from rabbit, diluted 1:200 in 2% BSA in PBS-Triton X-100, for 48 h at 4^{o} C. After washes, tissue sections were incubated in Alexa Fluor® 594 anti-rabbit IgG (Invitrogen, USA), diluted 1:200 in PBS, at room temperature and protected from light for 2 h. The sections were mounted on slides with Fluor Save® (Dako, Denmark) and covered with coverslips. Images were viewed with a Nikon Eclipse E-600 microscope and images transferred to a computer with a digital camera.

The number of GFAP-stained astrocytes/mm^2 in the hippocampus - CA1 was estimated according to previously reported (Martinez, Hermel et al. 2006) with Nikon NIS Elements AR 2.30 software. All lighting conditions and magnifications were kept constant. The images were captured and a square region of interest (ROI) was created considering the minor size of the organ/layer. The ROI square was overlaid in all sections of *stratum radiatum* of CA1, with blood vessels and artifacts being avoided. Eight images were analyzed per field from a total of five animals per group. GFAP-reactive astrocytes located inside this square or intersected by the lower and/or right edges of the square were counted. Astrocytes intersected by the upper and/or left edges of the square were not counted (Viola, Rodrigues et al. 2009). This analysis was performed within an area of the hippocampus extending between the Bregma 2.30 and 4.16 (Plate 29–36, according to Paxinos & Wattson Atlas). To determine general and astrocytic optic density fluorescence intensity, Nikon NIS Elements AR 2.30 software was used, as already described (Viola, Rodrigues et al. 2009). Morphological analysis was performed by three separate observers who were blind to the experimental groups, and results were averaged in the final results.

Na^+- dependent [^{3}H] glutamate Uptake

The animals were decapitated, their brains immediately removed and humidified with Hank's balanced salt solution (HBSS). Hippocampus, striatum and cortex were dissected and slices (0.4 mm) were obtained using a McIIwain tissue chopper. Hippocampus, striatum and cortex slices were pre-incubated with HBSS at 35° C for 15 min, followed by the addition of 0.66 and 0.33 $CimL^{-1}$ l-[^{3}H]glutamate for hippocampus/striatum and cortex, respectively, and 100µM (final concentration) glutamate. Incubation was stopped after 3, 5 or 7 min for striatum, hippocampus and cortex, respectively, with 2 ice-cold washes of l mL HBSS. After washing, 0.5N NaOH was immediately added to the slices and they were stored overnight. Na^+-independent uptake was measured using the above-described protocol with alterations in the temperature (4° C) and the composition of the medium (N-methyl-D-glucamine instead of sodium chloride). Results (Na^+-dependent uptake) were measured as the difference between the total uptake and the Na^+-independent uptake. Each incubation was performed in triplicate (Thomazi, Godinho et al. 2004). Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409).

Glutamine Synthetase activity

The enzymatic assay was performed as previously described (Petito, Chung et al. 1992), with some modifications. Briefly, hippocampus, striatum and cortex slices (0.4 mm) were homogenized in phosphate buffer. A 0.1 ml aliquot was added to 0.1 ml of reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole–HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine–HCl; 10 ATP and incubated for 15 min at

37 °C. The reaction was stopped by the addition of 0.4 ml of a solution containing (in mM): 370 ferric chloride; 670 HCl; 200 trichloroacetic acid. After centrifugation, the supernatant's absorbance was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate, treated with ferric chloride reagent.

Western blotting of neural proteins

In order to analyze brain immunocontent of GLAST, GLT-1 and Glutamine Synthetase, striatum, hippocampus and cortex were homogenized in a 25 mM Hepes (pH 7,4), 0,1% sodium dodecyl sulphate and a protease and phosphatase inhibitor cocktail (Sigma). Samples were centrifuged at 5,000 x g for 10 min, supernatant free from cell debris was separated and stored at -20 °C for further analyzes. In order to determine the adequate amount of protein to be assayed, different protein concentrations were carried out in the same gel for each protein tested. Samples' proteins (20 µg protein/well) were separated in an 8% SDS-PAGE mini-gel and transferred to nitrocellulose membrane using a Trans-Blot system (Bio-Rad, Hercules CA). Membranes were processed as follow: (1) blocking with 5% casein for 2 h; (2) incubation with primary antibody overnight: GLT-1 (1:1000), GLAST (1:1000), Glutamine Synthetase (1:1000) and β-Actin (1:2000); (3) incubation with horseradish peroxidase-conjugated secondary antibody for rabbit 1:2000 and mouse 1:2000 (β-actin only) for 2 h; (4) chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and band intensities were analyzed using Image J software (developed at the U.S. National Institutes of Health and available on the Internet at

http://rsb.info.nih.gov/nih-image/). Results are expressed as GLAST and GLT-1 Optic Density (OD) / β-actin OD and GS OD / load protein OD.

Protein determination

Protein was measured by the Coomassie Blue method (Bradford 1976), using bovine serum albumin as standard.

Cerebrospinal fluid (CSF) sampling

Animals were anesthetized with sodium thiopental (40 mg/kg, 1 mL/kg i.p.; supplemented as necessary) and placed in a stereotaxic apparatus; CSF samples were drawn (40–60 μL per rat) by direct puncture of the cisterna magna with an insulin syringe (27 gauge x 1/2 in length) (Schmidt, Tort et al. 2009). Samples were centrifuged at 10,000 x g in an Eppendorf centrifuge for 10 min (4 °C) to obtain cell free supernatants and stored at -70°C until analysis.

HPLC analyzes

High-performance liquid chromatography (HPLC) was performed with CSF cell-free supernatants aliquots to quantify aspartate (Asp) and glutamate (Glu) levels (Schmidt, Tort et al. 2009). Chromatographic separation was carried out with a reverse phase column (Supelcosil LC-18, 250 mm x 4.6 mm, Supelco) in a Shimadzu Instruments liquid chromatography (50 μL loop valve injection). The mobile phase flowed at a rate of 1.4 mL/min and column temperature was 24º C. Buffer composition: Buffer A: 0.04 mol/L NaH_2PO_4 buffer, pH 5.5, containing 20% of metanol; Buffer B: 0.01 mol/L NaH_2PO_4 buffer, pH 5.5, containing 80% of methanol). The gradient profile was modified according

to the content of buffer B in the mobile phase: 0% at 0.00 min, 25% at 13.75 min, 100% at 15.00 - 20.00 min, 0% at 20.01 - 25.00 min. Absorbance was read at 360 nm and 455 nm, excitation and emission respectively. Concentration is expressed in μM (mean ± SEM).

Statistical analysis

Data are expressed as mean ± SEM. Data were submitted to Kolmogorov-Smirnov test for normal distribution evaluation. Data from open field, elevated plus maze and light-dark box test were analyzed by Student t test. The number of animals used at each experiment is described in the respective figure legend. All results with $P \leq 0.05$ were considered statistically significant.

3. Results

Open field task

Table 1. Both Control and CsA groups presented the same pattern of locomotor activity the 15 min task, decreasing in both groups after 5 min of the session. Additionally, there were no difference in the total number of crossed squares, total number of rearings, mobile time and number of fecal boli. However, rats from the CsA group presented lower locomotion in the first 5 min (exploratory activity) and number of entries into the central part of the arena (Table 1, $P \leq 0.01$) and showed an increased number of grooming during this time, compared to controls (Table 1, $P \leq 0.01$).

Elevated plus maze task

Table 2 and Fig. 1. CsA treated animals showed anxiogenic behavior in this task. CsA administration significantly decreased the time spent in open arms (Fig. 1A, $P \leq 0.01$),

the number of entries in open arms (Fig. 1B, $P \leq 0.001$) and the distance traveled in open arms (Fig. 1C, $P \leq 0.01$), and increased the time in closed arms (Fig. 1D, $P \leq 0.01$), compared with Control group. The number of rearings in open and closed arms, the entries and the distance in closed arms did not differ between CsA and control groups (Table 2). Moreover, there was a positive correlation between time spent in open arms of the elevated plus maze task and the time spent in the central area of the arena in the open field task ($R^2 = 0.70$, $P \leq 0.01$).

Light-dark box task

Compared with Control group, CsA rats spent less time in the light compartment (Fig. 2A, $P \leq 0.001$). The total number of entries into dark compartment and the number of risk assessment behavior (Fig. 2B and C), as well as the number of crossings in light compartment, the latency for the first entry into the dark compartment and the total fecal boli were not different between Control and CsA groups (Table 3).

Immunohistochemistry

Astrocytic cell bodies and processes were identified in the CA1 *stratum radiatum*. Quantitative results for number of astrocytes, regional GFAP OD and GFAP OD within individual astrocytes are shown in Table 4. There was no significant difference in any of these parameters between the CsA and control groups, demonstrating that the number of astrocytes and GFAP density were not affected by CsA treatment. Regarding qualitative analyzes (Figura 3), astrocytes observed in the control group showed two types of GFAP immunoreactive (GFAP-ir) cells, displaying fusiform (4.55 ± 0.26 cells/ROI) or stellate astrocytic shapes (2.00 ± 0.16 cells/ROI). The cells presented their processes perpendicular

to the *stratum pyramydale*. In the same region, CsA treatment changed the morphology of the GFAP-ir cells. The stellate cells do not change compared to control group (2.00 ± 0.24 cells/ROI) whereas the fusiform astrocytes decreased in comparison to control group (2.10 ± 0.19 cells/ROI). However, a different astrocytic shape appeared. These cells also showed the perpendicular processes to the *pyramidal stratum*, however, other processes originated from the lateral region of the cells, being parallel to the *pyramidal stratum*. Thus, these astrocytes took an intermediate shape, between the fusiform and stellate astrocytes. Controls cells also showed this kind of cells (0.61 ± 0.09 cells/ROI), nevertheless, the number of astrocytes with this intermediate shape predominate after CsA treatment (3.67 ± 0.24 cells/ROI, $P < 0.0001$) compared to control. Figure 4 and 5 show a representative image of the CA1 *stratum radiatum* from control and CsA treated group, respectively.

Glutamate uptake

CsA chronic treatment did not affect glutamate uptake in hippocampus, striatum and cortex slices compared to control group (Table 5).

Glutamine Synthetase (GS) activity

CsA treatment did not affect the GS activity in hippocampus, striatum and cortex (Table 6).

Immunocontent of glutamate transporters and GS

We assessed GLAST and GLT1 immunocontent in hippocampus, striatum and cortex homogenates and, in accordance to glutamate uptake data, there was no difference between CsA and control groups (Table 7). Also, GS immunocontent in hippocampus,

striatum and cortex was not affected by CsA chronic treatment compared to controls (Table 7).

CSF aspartate and glutamate levels

CsA chronic treatment did not affect the CSF excitatory amino acids concentration. Glutamate levels ranged 4.00 ± 0.62 μM in control animals and 3.32 ± 0.41 μM in CsA treated group. Aspartate levels ranged 1.40 ± 0.32 μM in control animals and 1.64 ± 0.38 μM in CsA treated group.

4. Discussion

In this study we investigated the effects of chronic CsA treatment in rats on three classic behavioral tasks, open field, elevated plus maze and light/dark box.

Regarding the open field task, it is well known that rodents prefer to walk close to the walls, a behavior called thigmotaxis. In fact, anxiety behavior in the open field is triggered by two factors: individual testing (the animal is separated from its social group) and agoraphobia (the arena is very large relative to the animal's breeding or natural environment). Thus, in experiments involving rodents, we are not measuring the effects of treatments only over exploration, but also the effects on the reaction of the subjects to a stressful event (Prut and Belzung 2003). In the present study, CsA treatment had no effect over locomotor activity, showing that CsA treatment did not cause motor alteration and that our results are reflection of behavioral alterations. Despite no alteration was observed in the total distance traveled during the total 15 minutes of the session, CsA decreased the distance traveled in the first 5 minutes of the session and the total number of entries into the central area of the open field arena. Reduction of the time spent in the central area, as well

as of the ratio central/total locomotion or increased latency to enter the central part are indications of low exploratory behavior and anxiety (Prut and Belzung 2003).

Elevated plus maze task results showed that CsA induced clear signs of anxiety-like behavior, as showed by the differences of time spent in closed arms and distance traveled in open arms of apparatus comparing to control groups. Elevated plus maze task is a widely used behavioral assay for anxiety behavior of rodents. This is demonstrated by anxiogenic drugs reducing time spent on the open arms and anxiolytic drugs increasing the time spent on the open arms (Pellow and File 1986). In addition, there was a positive correlation between time spent in open arms of the elevated plus maze apparatus and the time spent in the central area of the open field arena, reinforcing the anxiogenic results achieved.

The light/dark task is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stressors, that is, novel environment and light (Crawley and Goodwin 1980). Naturally, rodents prefer the darker areas since they are nocturnal animals, but they also tend to explore lighted areas at least for a short period of time, as observed here with the control rats. The low index of exploration of the light compartment in the light-dark box reinforces an anxiogenic effect of CsA, reflected in more time spent in dark compartment in comparison to control rats.

Although anxiety is a well known side effect of chronic CsA treatment in humans, to our knowledge no previous experimental animal study with a chronic use of CsA evidenced the anxiogenic-like behavior. Thus, the experimental animal model used in this study becomes an important tool for the search of mechanisms by which CsA induces an anxyogenic-like behavior.

A newly emerging line of evidence in clinical and preclinical studies has implicated glial abnormalities in the pathophysiology and novel treatment direction for mood disorders

like anxiety (Sanacora, Rothman et al. 2003; Lee, Gaskins et al. 2007). Here, regarding astrocytes histological parameters, none alterations in the general and astrocytic GFAP density were observed, neither the number of astrocytes were altered by CsA treatment compared to controls. Although these parameters have not been altered, morphological changes were detected concerning astrocytic shape. While astrocytic fusiform shape predominates in the CA1 *stratum radiatum* of control animals, CsA treatment decreased the number of fusiform astrocytes. At the same time, the astrocytes that predominate at CsA treated rats presented an intermediate shape, between the fusiform and stellate ones. It is interesting to think that astrocyte morphology/function may elicit behavioral disorders. But it is unclear whether glial changes are a consequence or a cause of brain illness (Giaume, Koulakoff et al.). Sanacora and colleagues proposed in recent reviews that glia pathology and amino-acid neurotransmitter system abnormalities (especially glutamate) contribute to the pathophysiology of major mood disorders (Sanacora, Rothman et al. 2003; Kugaya and Sanacora 2005).

In searching for a possible mechanism involved in the anxiogenic-like behavior and astrocytic morphological changes observed in CsA treated rats, we evaluated some astrocytic glutamatergic parameters on brain structures. The glutamatergic system is involved with the pathoetiology and some behavioral stereotypes related to psychiatric disorders such as anxiety and depression (Cryan, Kelly et al. 2003; Bergink, van Megen et al. 2004; Lee, Gaskins et al. 2007). Astrocytes play a crucial role in keeping glutamatergic system homeostasis, being the main responsible by the glutamate uptake and recycling (Danbolt 2001). Also, they control the number and function of neuronal synapses and blood flow in the brain, being capable to change their shapes due to cerebral insult in a hypertrophic way (Wilhelmsson, Bushong et al. 2006). In this way, morphological

(astrocyte swelling) as well as metabolic alterations of astrocytes were already observed after CsA *in vitro* treatment including reduction of the energy state and intracellular levels of glutamate and glutamine (Serkova, Christians et al. 1997).

Concerning the glutamatergic parameters investigated, none alterations were observed in CsA treated rats compared to controls. Glutamate uptake and glutamine synthetase activity as well as the immunocontent of astrocytic glutamate transporters (GLT-1 and GLAST) and glutamine synthetase were not affected by CsA chronic treatment. Neither glutamate nor aspartate CSF levels were altered. Thus, suggesting that profound or persistent astrocytic glutamatergic modulations are not involved with the anxiogenic-like behavior in this chronic CsA treated animals. However, it is also important to note that other parameters of the glutamatergic system, mainly the neuronal ones, were not investigated in this work. Therefore we cannot exclude the involvement of the glutamatergic system in behavioral outcomes and astrocytic morphological changes observed. Also, different neurotransmitter systems could be involved in the behavioral and morphological alterations observed, thus more studies are necessary to clarify the neurochemical alterations related chronic CsA treatment.

It is important to comment that cerebrovascular complications were most frequent in transplanted patients treated with CsA (Martinez 1998) and that in a recent published article we showed that the chronic CsA treatment, as used in this work, altered biochemical parameters related to cardiovascular and cerebrovascular risk factors (Bohmer, Souza et al.). Thus, alterations in the cerebrovascular system promoted by the CsA treatment might be related to the observed behavior and morphological changes. Besides astrocytes, other brain morphological changes was already observed and correlated to clinical symptoms of CsA-dependent neurotoxicity, such as hypodensity of white matter, cerebral edema and

morphological alterations related to metabolic encephalopathy, and hypoxic damage (Berden, Hoitsma et al. 1985; Adams, Ponsford et al. 1987; de Groen, Aksamit et al. 1987; Scheinman, Reinitz et al. 1990; McManus, O'Hair et al. 1992; Belli, De Carlis et al. 1993; Wijdicks, Wiesner et al. 1995; Drachman, DeNofrio et al. 1996; Bartynski, Grabb et al. 1997; Gopal, Thorning et al. 1999; Helderman, Bennett et al. 2003). Astroglial networks are likely to be independent from each other and are probably not as elaborate as neuronal circuits in terms of size and the specificity of their connections (Giaume, Koulakoff et al.). However, it is conceivable that their shape and extend vary with time and the activity of their environment.

In conclusion, this study indicates that the CsA treatment under our experimental protocol is able to induce consistent anxiogenic-like behavior in rats without causing modulation on astrocytic glutamatergic system but promoting changes on astrocytic morphology. Thus, the experimental animal model used in this work becomes an interesting model to investigate new therapeutic perspectives and mechanism involving the psychiatric effects of the chronic CsA administration.

Acknowledgments

This work was supported by CNPq, FAPERGS and MCT-CNPq/MS-SCTIE-DECIT-DAF (Nº 54/2005), IBNet, INCT for Excitotoxicity and Neuroprotection/CNPq.

Legends

Figure 1

Behavioral pattern in the elevated plus maze task. Data are mean ± SEM of the: (A) time spent in open arms (%); (B) number of entries into open arms and (C) the total distance traveled in open arms (m). Data are mean ± SEM and were analyzed by Student t test. N= 10 (control group) and 19 (CsA group). * $P \leq 0.01$ and ** $P \leq 0.001$ as compared to control, Student t test.

Figure 2

Behavioral pattern in the light / dark test. Data are mean ± SEM of the: (A) time spent in light compartment (%); (B) the total number of entries into dark compartment and (C) the total number of risk assessment behavior. Data are mean ± SEM and were analyzed by Student t test. N= 10 (control group) and 19 (CsA group). ** $P \leq 0.001$ as compared to control, Student t test.

Figure 3

Predominant astrocytes shape according to treatment. Graphs show A) a reduced number of fusiform shaped astrocytes in CsA (black bar) compared to Control (white bar) treated group ($P < 0.0001$); B) no changes in stellate shaped astrocytes amount and C) increased amount of intermediate shaped astrocytes in CsA treated rats compared to controls ($P < 0.0001$). Data are given as mean ± SEM; n = 5.

Figure 4

Representative photomicrographs (from 5 animals) of the CA1 *stratum radiatum* of a control animal. Glial fibrilary acid protein (GFAP) immunohistochemistry (original magnification 400x).

Figure 5

Representative photomicrographs (from 5 animals) of the CA1 *stratum radiatum* of a CsA treated animal. Glial fibrilary acid protein (GFAP) immunohistochemistry (original magnification 400x).

References

Adams, D. H., S. Ponsford, et al. (1987). "Neurological complications following liver transplantation." Lancet **1**(8539): 949-51.

Bartynski, W. S., B. C. Grabb, et al. (1997). "Watershed imaging features and clinical vascular injury in cyclosporin A neurotoxicity." J Comput Assist Tomogr **21**(6): 872-80.

Bechstein, W. O. (2000). "Neurotoxicity of calcineurin inhibitors: impact and clinical management." Transpl Int **13**(5): 313-26.

Belanger, M. and P. J. Magistretti (2009). "The role of astroglia in neuroprotection." Dialogues Clin Neurosci **11**(3): 281-95.

Belli, L. S., L. De Carlis, et al. (1993). "Dysarthria and cerebellar ataxia: late occurrence of severe neurotoxicity in a liver transplant recipient." Transpl Int **6**(3): 176-8.

Berden, J. H., A. J. Hoitsma, et al. (1985). "Severe central-nervous-system toxicity associated with cyclosporin." Lancet **1**(8422): 219-20.

Bergink, V., H. J. van Megen, et al. (2004). "Glutamate and anxiety." Eur Neuropsychopharmacol **14**(3): 175-83.

Bohmer, A. E., D. G. Souza, et al. "Long-term cyclosporine treatment in non-transplanted rats and metabolic risk factors of vascular diseases." Chem Biol Interact **185**(1): 53-8.

Borlongan, C. V., Y. Kwanbara, et al. (1999). "Cyclosporine-A reduces spontaneous place preference in adult rats." Neurosci Lett **267**(3): 169-72.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-54.

Calne, R. Y., K. Rolles, et al. (1979). "Cyclosporin A initially as the only immunosuppressant in 34 recipients of cadaveric organs: 32 kidneys, 2 pancreases, and 2 livers." Lancet **2**(8151): 1033-6.

Chojnacka-Wojcik, E., A. Klodzinska, et al. (2001). "Glutamate receptor ligands as anxiolytics." Curr Opin Investig Drugs **2**(8): 1112-9.

Crawley, J. and F. K. Goodwin (1980). "Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines." Pharmacol Biochem Behav **13**(2): 167-70.

Cryan, J. F., P. H. Kelly, et al. (2003). "Antidepressant and anxiolytic-like effects in mice lacking the group III metabotropic glutamate receptor mGluR7." Eur J Neurosci **17**(11): 2409-17.

Danbolt, N. C. (2001). "Glutamate uptake." Prog Neurobiol **65**(1): 1-105.

de Groen, P. C., A. J. Aksamit, et al. (1987). "Central nervous system toxicity after liver transplantation. The role of cyclosporine and cholesterol." N Engl J Med **317**(14): 861-6.

Drachman, B. M., D. DeNofrio, et al. (1996). "Cortical blindness secondary to cyclosporine after orthotopic heart transplantation: a case report and review of the literature." J Heart Lung Transplant **15**(11): 1158-64.

Ghisleni, G., V. Kazlauckas, et al. (2008). "Diphenyl diselenide exerts anxiolytic-like effect in Wistar rats: putative roles of GABAA and 5HT receptors." Prog Neuropsychopharmacol Biol Psychiatry **32**(6): 1508-15.

Giaume, C., A. Koulakoff, et al. "Astroglial networks: a step further in neuroglial and gliovascular interactions." Nat Rev Neurosci **11**(2): 87-99.

Gijtenbeek, J. M., M. J. van den Bent, et al. (1999). "Cyclosporine neurotoxicity: a review." J Neurol **246**(5): 339-46.

Gjessing, L. R., P. Gjesdahl, et al. (1972). "The free amino acids in human cerebrospinal fluid." J Neurochem **19**(7): 1807-8.

Gopal, A. K., D. R. Thorning, et al. (1999). "Fatal outcome due to cyclosporine neurotoxicity with associated pathological findings." Bone Marrow Transplant **23**(2): 191-3.

Groetzner, J., B. Reichart, et al. (2005). "Cardiac transplantation in pediatric patients: fifteen-year experience of a single center." Ann Thorac Surg **79**(1): 53-60; discussion 61.

Hauben, M. (1996). "Cyclosporine neurotoxicity." Pharmacotherapy **16**(4): 576-83.

Helderman, J. H., W. M. Bennett, et al. (2003). "Immunosuppression: practice and trends." Am J Transplant **3 Suppl 4**: 41-52.

Hoffman, E. J. and S. J. Mathew (2008). "Anxiety disorders: a comprehensive review of pharmacotherapies." Mt Sinai J Med **75**(3): 248-62.

Izquierdo, I., L. R. Bevilaqua, et al. (2006). "Different molecular cascades in different sites of the brain control memory consolidation." Trends Neurosci **29**(9): 496-505.

Kapus, G. L., I. Gacsalyi, et al. (2008). "Antagonism of AMPA receptors produces anxiolytic-like behavior in rodents: effects of GYKI 52466 and its novel analogues." Psychopharmacology (Berl) **198**(2): 231-41.

Kew, J. N. and J. A. Kemp (2005). "Ionotropic and metabotropic glutamate receptor structure and pharmacology." Psychopharmacology (Berl) **179**(1): 4-29.

Krystal, J. H., D. F. Tolin, et al. (2009). "Neuroplasticity as a target for the pharmacotherapy of anxiety disorders, mood disorders, and schizophrenia." Drug Discov Today **14**(13-14): 690-7.

Kugaya, A. and G. Sanacora (2005). "Beyond monoamines: glutamatergic function in mood disorders." CNS Spectr **10**(10): 808-19.

Lee, Y., D. Gaskins, et al. (2007). "Glia mechanisms in mood regulation: a novel model of mood disorders." Psychopharmacology (Berl) **191**(1): 55-65.

Lipton, S. A. and P. A. Rosenberg (1994). "Excitatory amino acids as a final common pathway for neurologic disorders." N Engl J Med **330**(9): 613-22.

Magnasco, A., A. Rossi, et al. (2008). "Cyclosporin and organ specific toxicity: clinical aspects, pharmacogenetics and perspectives." Curr Clin Pharmacol **3**(3): 166-73.

Maragakis, N. J. and J. D. Rothstein (2006). "Mechanisms of Disease: astrocytes in neurodegenerative disease." Nat Clin Pract Neurol **2**(12): 679-89.

Martinez, A. J. (1998). "The neuropathology of organ transplantation: comparison and contrast in 500 patients." Pathol Res Pract **194**(7): 473-86.

Martinez, F. G., E. E. Hermel, et al. (2006). "Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity in the medial amygdala subnuclei across the estrous cycle and in castrated and treated female rats." Brain Res **1108**(1): 117-26.

McManus, R. P., D. P. O'Hair, et al. (1992). "Cyclosporine-associated central neurotoxicity after heart transplantation." Ann Thorac Surg **53**(2): 326-7.

Meldrum, B. S. (1994). "The role of glutamate in epilepsy and other CNS disorders." Neurology **44**(11 Suppl 8): S14-23.

Ozawa, S. (1998). "[Ca2+ permeation through the ionotropic glutamate receptor]." Tanpakushitsu Kakusan Koso **43**(12 Suppl): 1589-95.

Palucha, A. and A. Pilc (2007). "Metabotropic glutamate receptor ligands as possible anxiolytic and antidepressant drugs." Pharmacol Ther **115**(1): 116-47.

Pekny, M. and M. Nilsson (2005). "Astrocyte activation and reactive gliosis." Glia **50**(4): 427-34.

Pellerin, L., A. K. Bouzier-Sore, et al. (2007). "Activity-dependent regulation of energy metabolism by astrocytes: an update." Glia **55**(12): 1251-62.

Pellow, S. and S. E. File (1986). "Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat." Pharmacol Biochem Behav **24**(3): 525-9.

Petito, C. K., M. C. Chung, et al. (1992). "Brain glutamine synthetase increases following cerebral ischemia in the rat." Brain Res **569**(2): 275-80.

Prut, L. and C. Belzung (2003). "The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review." Eur J Pharmacol **463**(1-3): 3-33.

Sanacora, G., D. L. Rothman, et al. (2003). "Clinical studies implementing glutamate neurotransmission in mood disorders." Ann N Y Acad Sci **1003**: 292-308.

Sato, Y., Y. Takayanagi, et al. (2007). "Impact of cyclosporine upon emotional and social behavior in mice." Transplantation **83**(10): 1365-70.

Scheinman, S. J., E. R. Reinitz, et al. (1990). "Cyclosporine central neurotoxicity following renal transplantation. Report of a case using magnetic resonance images." Transplantation **49**(1): 215-6.

Schmidt, A. P., A. E. Bohmer, et al. (2008). "Antinociceptive effects of intracerebroventricular administration of guanine-based purines in mice: evidences for the mechanism of action." Brain Res **1234**: 50-8.

Schmidt, A. P., A. B. Tort, et al. (2009). "The NMDA antagonist MK-801 induces hyperalgesia and increases CSF excitatory amino acids in rats: reversal by guanosine." Pharmacol Biochem Behav **91**(4): 549-53.

Segovia, G., A. Porras, et al. (2001). "Glutamatergic neurotransmission in aging: a critical perspective." Mech Ageing Dev **122**(1): 1-29.

Serkova, N., U. Christians, et al. (1997). "Assessment of the mechanism of astrocyte swelling induced by the macrolide immunosuppressant sirolimus using multinuclear nuclear magnetic resonance spectroscopy." Chem Res Toxicol **10**(12): 1359-63.

Serkova, N. J., U. Christians, et al. (2004). "Biochemical mechanisms of cyclosporine neurotoxicity." Mol Interv **4**(2): 97-107.

Sheldon, A. L. and M. B. Robinson (2007). "The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention." Neurochem Int **51**(6-7): 333-55.

Subramanian, S. and D. L. Trence (2007). "Immunosuppressive agents: effects on glucose and lipid metabolism." Endocrinol Metab Clin North Am **36**(4): 891-905; vii.

Tellides, G. and J. S. Pober (2007). "Interferon-gamma axis in graft arteriosclerosis." Circ Res **100**(5): 622-32.

Thomazi, A. P., G. F. Godinho, et al. (2004). "Ontogenetic profile of glutamate uptake in brain structures slices from rats: sensitivity to guanosine." Mech Ageing Dev **125**(7): 475-81.

Vercauteren, S. B., J. L. Bosmans, et al. (1998). "A meta-analysis and morphological review of cyclosporine-induced nephrotoxicity in auto-immune diseases." Kidney Int **54**(2): 536-45.

Viola, G. G., L. Rodrigues, et al. (2009). "Morphological changes in hippocampal astrocytes induced by environmental enrichment in mice." Brain Res **1274**: 47-54.

von Horsten, S., M. S. Exton, et al. (1998). "Cyclosporine A affects open field behavior in DA rats." Pharmacol Biochem Behav **60**(1): 71-6.

Walf, A. A. and C. A. Frye (2007). "The use of the elevated plus maze as an assay of anxiety-related behavior in rodents." Nat Protoc **2**(2): 322-8.

Wijdicks, E. F. (2001). "Neurotoxicity of immunosuppressive drugs." Liver Transpl **7**(11): 937-42.

Wijdicks, E. F., R. H. Wiesner, et al. (1995). "Neurotoxicity in liver transplant recipients with cyclosporine immunosuppression." Neurology **45**(11): 1962-4.

Wilhelmsson, U., E. A. Bushong, et al. (2006). "Redefining the concept of reactive astrocytes as cells that remain within their unique domains upon reaction to injury." Proc Natl Acad Sci U S A **103**(46): 17513-8.

Figure 1

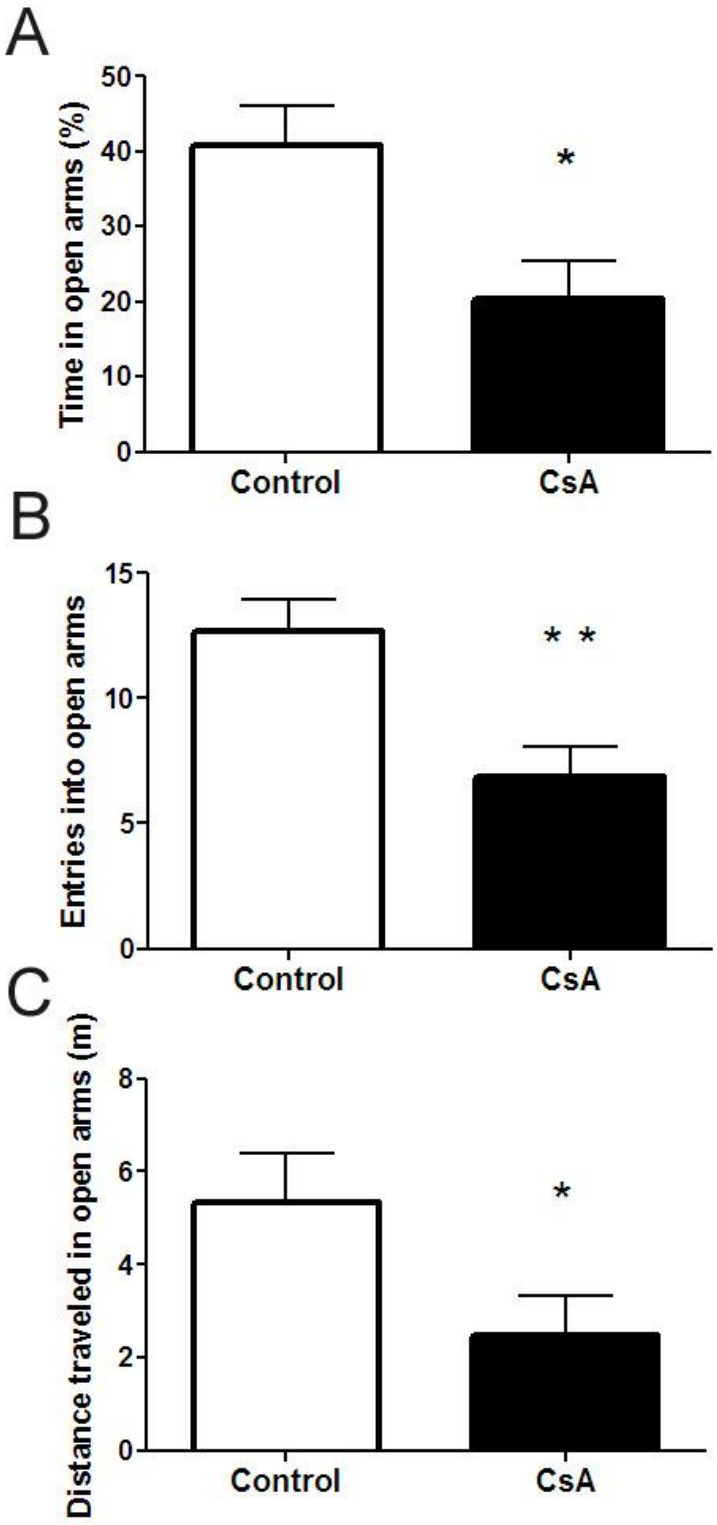

A
Time in open arms (%)
50
40
30
20
10
0
*
Control
CsA
B
Entries into open arms
15
10
5
0
* *
Control
CsA
C
Distance traveled in open arms (m)
8
6
4
2
0
*
Control
CsA

Figure 2

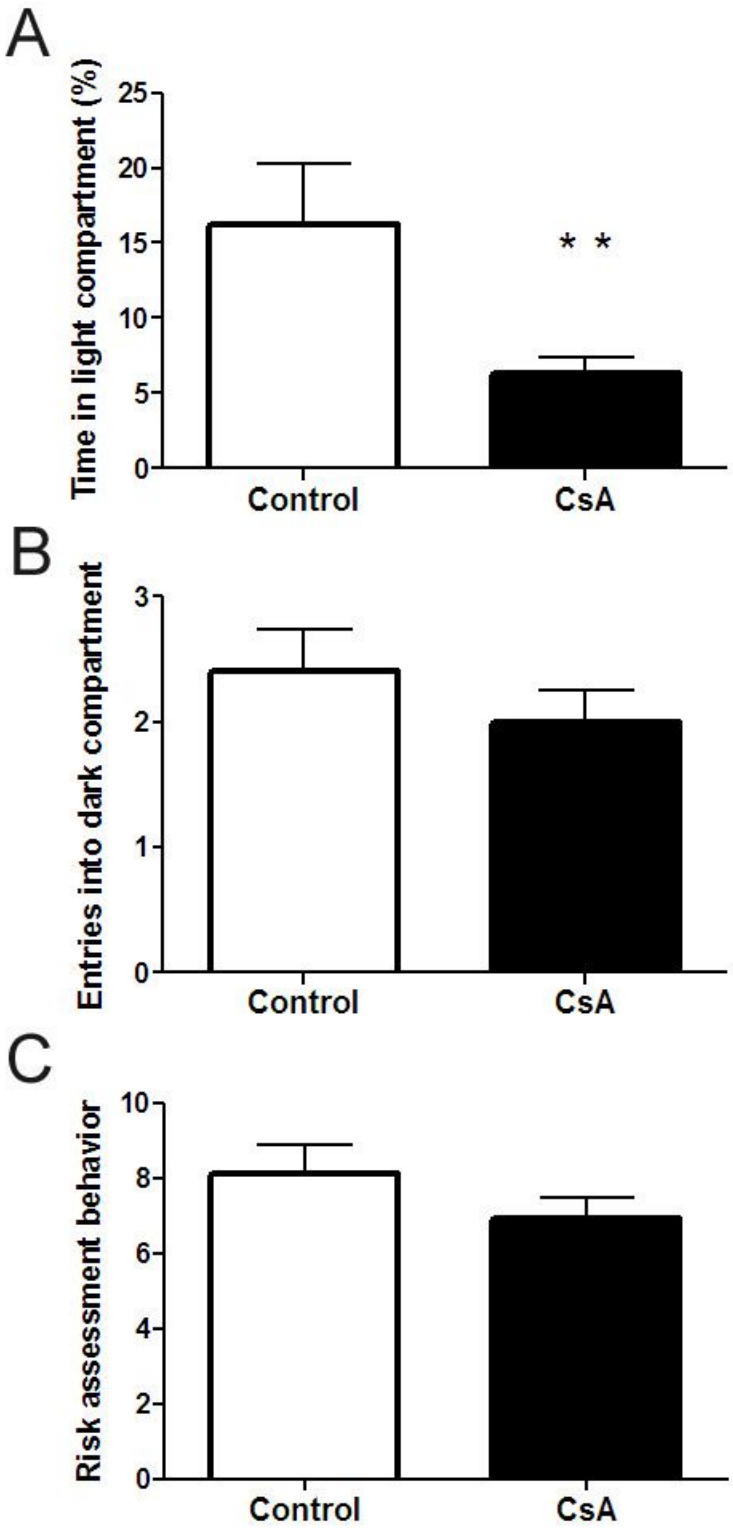

A
Time in light compartment (%)
25
20
15
10
5
0
* *
Control
CsA
B
Entries into dark compartment
3
2
1
0
Control
CsA
C
Risk assessment behavior
10
8
6
4
2
0
Control
CsA

Figure 3

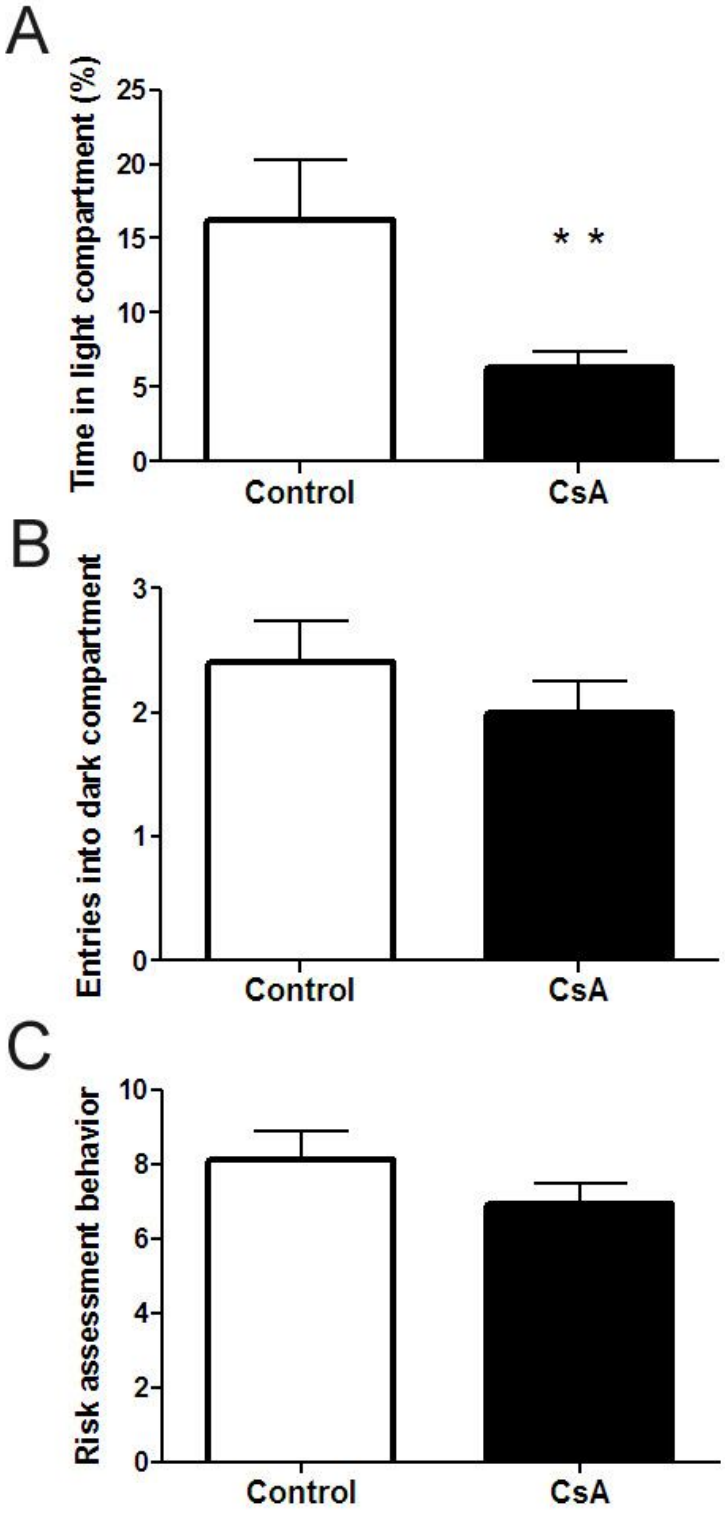

A
Time in light compartment (%)
25
20
15
10
5
0
* *
Control
CsA
B
Entries into dark compartment
3
2
1
0
Control
CsA
C
Risk assessment behavior
10
8
6
4
2
0
Control
CsA

Table 1

Open field test.

Parameters analyzed	Control	CsA
Distance traveled in the first 5 minutes (m)	13.9 ± 3.2	8.5 ± 0.8 *
Distance traveled in the last 10 minutes (m)	12.3 ± 2.9	11.0 ± 2.0
Total distance traveled (m)	26.2 ± 6.0	19.6 ± 3.2
Crossings	143.2 ± 18.3	117.1 ± 12.1
Entries into the central part of the arena	6.3 ± 1.3	3.2 ± 0.6 *
Mobile time (%)	35.6 ± 6.0	28.3 ± 3.0
Immobile time (%)	64.4 ± 6.0	71.6 ± 3.0
Rearing in the first 5 min	16.7 ± 2.9	11.8 ± 1.4
Rearing in the last 10 min	14.2 ± 2.9	10.0 ± 1.2
Total rearing	30.9 ± 5.3	21.9 ± 2.4
Groomings in the first 5 min	0.9 ± 0.3	1.8 ± 0.2 *
Groomings in the last 10 min	4.0 ± 0.4	3.5 ± 0.5
Total groomings	4.9 ± 0.6	5.4 ± 0.6
Total fecal boli	4.8 ± 0.9	4.7 ± 1.2

Table 2

Elevated plus Maze session.

Parameters analyzed	Control	CsA
Time in closed arms (%)	38.8 ± 4.5	62.0 ± 6.4 *
Entries into closed arms	8.7 ± 1.3	7.4 ± 1.0
Distance traveled in closed arms (m)	5.5 ± 0.8	5.5 ± 0.6
Rearing in open arms	3.1 ± 1.2	2.5 ± 0.9
Rearing in closed arms	17.6 ± 3.4	14.1 ± 1.4

Effect of CsA treatment on parameters related to the elevated plus maze test. Data are mean ± SEM and were analyzed by Student t test. N= 10 (control group) and 19 (CsA group). * $P \leq 0.01$ as compared to control Student t test.

Table 3

Light / dark session.

Parameters analyzed	Control	CsA
Crossings in light compartment	21.1 ± 4.1	15.0 ± 2.5
Latency for the first entry into the dark compartment	17.4 ± 3.6	17.4 ± 9.3
Total fecal boli	2.6 ± 0.9	2.0 ± 0.7

Effect of CsA treatment on parameters related to the Light / Dark session. Data are mean ± SEM and were analyzed by Student t test. N= 10 (control group) and 19 (CsA group).

Table 4

Effects of chronic CsA treatment on astrocitary parameters of CA1 *stratum radiatum*

	Control	**CsA 15mg/kg**
GFAP general optical density/ROI (A.U.)	0.9268 ± 0.01	0.9208 ± 0.01
GFAP astrocytic optical density/ROI (A.U.)	0.3129 ± 0.03	0.3262 ± 0.01
Astrocytes number/ROI	27.46 ± 2.2	27.22 ± 1.6

Data is given as mean ± SEM; n=5/group; ROI, Region of Interest; A.U., arbitrary units

Table 5

Glutamate uptake on brain structures of control and CsA-treated rats

	Control	**CsA 15mg/kg**
Hippocampus (nmol/mg/min)	0.7317 ± 0.05	0.7332 ± 0.06
Striatum (nmol/mg/min)	0.8124 ± 0.05	0.8444 ± 0.08
Cortex (nmol/mg/min)	1.023 ± 0.08	0.955 ± 0.12

Data is given as mean ± SEM; n=10-16/group

Table 6

Glutamine Synthetase acitivity on brain structures of control and CsA-treated rats

	Control	**CsA 15mg/kg**
Hippocampus (mmol/h/mg)	1.499 ± 0.18	1.352 ± 0.05
Striatum (mmol/h/mg)	1.205 ± 0.07	1.442 ± 0.12
Cortex (mmol/h/mg)	1.966 ± 0.08	2.105 ± 0.10

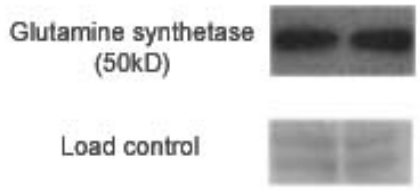
Data is given as mean ± SEM; n=10-11/group

Table 7

GLAST, GLT1, EAAC1 and Glutamine Synthetase (GS) immunocontent in control and CsA-treated rats

Variable	Control	CsA 15mg/kg
GLAST hippocampus (GLAST OD/β-actin OD)	0.9527 ± 0.03	0.9976 ± 0.10
GLAST striatum (GLAST OD/β-actin OD)	1.341 ± 0.05	1.313 ± 0.13
GLAST cortex (GLAST OD/β-actin OD)	1.039 ± 0.12	0.9761 ± 0.05
GLT1 hippocampus (GLT1 OD/β-actin OD)	0.8660 ± 0.10	1.093 ± 0.13
GLT1 striatum (GLT1 OD/β-actin OD)	0.8277 ± 0.11	1.113 ± 0.08
GLT1 cortex (GLT1 OD/β-actin OD)	0.9926 ± 0.26	0.9892 ± 0.12
GS hippocampus (GS OD/load OD)	0.5767 ± 0.07	0.5831 ± 0.08
GS striatum (GS OD/load OD)	0.9952 ± 0.02	1.031 ± 0.06
GS cortex (GS OD/load OD)	0.9836 ± 0.06	1.048 ± 0.05

Data is given as mean ± SEM; n=5-9/group



Anexo 2

Participação em outros trabalhos desenvolvidos durante o período da tese de doutorado:

Schmidt AP, Böhmer AE, Soares FA, Posso IP, Machado SB, Mendes FF, Portela LV, Souza DO. Changes in purines concentration in the cerebrospinal fluid of patients experiencing pain: a case-control study. Neurosci Lett. 2010 Apr 26;474(2):69-73.

Schmidt AP, Böhmer AE, Schallenberger C, Antunes C, Tavares RG, Wofchuk ST, Elisabetsky E, Souza DO. Mechanisms involved in the antinociception induced by systemic administration of guanosine in mice. Br J Pharmacol. 2010 Mar;159(6):1247-63.

Haas L, Portela LV, Böhmer AE, Oses JP, Lara DR. Increased plasma levels of brain derived neurotrophic factor (BDNF) in patients with fibromyalgia. Neurochem Res. 2010 May;35(5):830-4.

Schmidt AP, Paniz L, Schallenberger C, Böhmer AE, Wofchuk ST, Elisabetsky E, Portela LV, Souza DO. Guanosine prevents thermal hyperalgesia in a rat model of peripheral mononeuropathy. J Pain. 2010 Feb;11(2):131-41.

Schmidt AP, Böhmer AE, Schallenberger C, Antunes C, Pereira MS, Leke R, Wofchuk ST, Elisabetsky E, Souza DO. Spinal mechanisms of antinociceptive action caused by guanosine in mice. Eur J Pharmacol. 2009 Jun 24;613(1-3):46-53.

Schmidt AP, Böhmer AE, Antunes C, Schallenberger C, Porciúncula LO, Elisabetsky E, Lara DR, Souza DO. Anti-nociceptive properties of the xanthine oxidase inhibitor allopurinol in mice: role of A1 adenosine receptors. Br J Pharmacol. 2009 Jan;156(1):163-72.

Schmidt AP, Tort AB, Silveira PP, Böhmer AE, Hansel G, Knorr L, Schallenberger C, Dalmaz C, Elisabetsky E, Crestana RH, Lara DR, Souza DO. The NMDA

antagonist MK-801 induces hyperalgesia and increases CSF excitatory amino acids in rats: reversal by guanosine. Pharmacol Biochem Behav. 2009 Feb;91(4):549-53.

Busnello JV, Oses JP, da Silva RS, Feier G, Barichello T, Quevedo J, Böhmer AE, Kapczinski F, Souza DO, Sarkis JJ, Portela LV. Peripheral nucleotide hydrolysis in rats submitted to a model of electroconvulsive therapy. Prog Neuropsychopharmacol Biol Psychiatry. 2008 Dec 12;32(8):1829-33.

Schmidt AP, Böhmer AE, Leke R, Schallenberger C, Antunes C, Pereira MS, Wofchuk ST, Elisabetsky E, Souza DO. Antinociceptive effects of intracerebroventricular administration of guanine-based purines in mice: evidences for the mechanism of action. Brain Res. 2008 Oct 9;1234:50-8.

Oses JP, Viola GG, de Paula Cognato G, Júnior VH, Hansel G, Böhmer AE, Leke R, Bruno AN, Bonan CD, Bogo MR, Portela LV, Souza DO, Sarkis JJ. Pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid. Epilepsy Res. 2007 Jul;75(2-3):104-11.