Universidade Federal do Rio Grande do Sul

Instituto de Ciência Básicas da Saúde

Departamento de Bioquímica

Curso de Pós-Graduação em Ciências Biológicas: Bioquímica

Estudo das alterações comportamentais e neuroquímicas induzidas pela sepse em modelo animal: possível papel terapêutico de antioxidantes

TATIANA BARICHELLO

Orientador:

Prof. Dr. Felipe Dal-Pizzol

Universidade Federal do Rio Grande do Sul

Instituto de Ciência Básicas da Saúde

Departamento de Bioquímica

Curso de Pós-Graduação em Ciências Biológicas: Bioquímica

Estudo das alterações comportamentais e neuroquímicas induzidas pela sepse em modelo animal: possível papel terapêutico de antioxidantes

TATIANA BARICHELLO

Orientador:

Prof. Dr. Felipe Dal-Pizzol

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica como requisito parcial para a obtenção do título de Doutor em Ciências Biológicas: Bioquímica

PORTO ALEGRE 2007

“O sistema límbico é a zona limite onde a psiquiatria encontra a neurologia”

“The limbic system is the border zone where psychiatry meets neurology”

Michael S. Mega et al. (1997)

Agradecimentos

Ao Prof. Dr. Felipe Dal-Pizzol pelo modelo de pesquisador, cuja busca incansável da verdade científica é a força propulsora que motiva um grande número de pesquisadores nos estudos da sepse em modelos animais e em estudos clínicos. Além disso, pela confiança em mim depositada quando aceitou a minha orientação e, mais recentemente, quando me convidou para integrar sua equipe de trabalho na Universidade.

Ao Prof. João Quevedo pela colaboração no desenvolvimento dos desenhos experimentais, na discussão dos resultados e no auxílio na preparação dos manuscritos.

Aos colaboradores Márcio Rodrigo Martins Adalisa Reinke, Gustavo Feier, Cristiane Ritter, Samira Valvassori, Gislaine Ziili Réus, Larissa Constantino, Roberta Machado, Ângeles Vitali, José Cláudio Moreira e Fabrícia Petronilho pelo companherismo e dedicação na realização dos diversos experimentos.

Aos orgãos de fomento (CNPq, FAPESC, UNESC e Instituto Cérebro e Mente) pelo fundamental auxílio financeiro necessário a execução dos experimentos que compõem essa Tese.

SUMÁRIO

Parte I

RESUMO

(Tatiana Barichello - **Estudo das alterações comportamentais e neuroquímicas induzidas pela sepse em modelo animal: possível papel terapêutico de antioxidantes**) – Este trabalho apresenta a compilação de 5 experimentos carreados ao longo de 2004-2007. Na **parte 1** apresenta sucintamente o marco teórico dos quatro trabalhos. Inicialmente são discutidos aspectos gerais da sepse. Após, são descritos alguns mecanismos conhecidos do envolvimento do sistema nervoso central com a sepse, como a permeabilidade diminuída da barreira sanguínea cerebral, alteração de alguns neurotransmissores, apoptose neuronal, ativação de algumas cascatas imunológicas, enfim, o processo inflamatório no sistema nervoso central. Os danos cognitivos ocasionados pela encefalopatia séptica também foram relatados, e por fim o estresse oxidativo e as defesas antioxidantes como um mecanismo que possa estar mediando os danos cognitivos observados em pacientes sobreviventes as sepse. Os experimentos (**Parte II**) comportamentais realizados 10 dias após a indução da sepse em ratos ocupam os **Capítulos 1 e 2**. No **capítulo 1** é apresentado um corpo de resultados que permite observar os déficits cognitivos nos testes de Habituação ao Campo Aberto, Esquiva Inibitória e Esquiva Inibitória de Múltiplos Treinos em ratos sobreviventes a sepse após 10 dias da indução pelo modelo animal de CLP. Estes dados demonstram uma incapacidade cognitiva no aprendizado e na memória dos animais sépticos. O **Capítulo 2** apresenta os resultados dos testes comportamentais de Reconhecimento de Objeto, Labirinto de Cruz Elevada e Teste de Natação Forçada, onde foi possível demonstrar que os ratos sépticos apresentaram uma incapacidade na memória e comportamento compatível com "depressão", mas não com ansiedade. **O Capítulo 3** estende os achados comportamentais observados no **Capítulo 1**, realizando os testes comportamentais de Habituação ao Campo Aberto, Esquiva Inibitória e Esquiva Inibitória de Múltiplos Treinos após 30 dias da indução de sepse em ratos pelo procedimento CLP. Os dados obtidos confirmam que a incapacidade de aprendizado e memória demonstrados no **Capítulo 1** persiste após 30 dias da cirurgia de CLP. A mensuração do estresse oxidativo no hipocampo, cerebelo, estriado e córtex de ratos submetidos à sepse foram descrito no **Capítulo 4**. Neste experimento foi observado que nas horas iniciais após a indução de sepse em ratos, houve danos oxidativos, avaliados pelo TBARS e carbonil em diversas regiões do cérebro. Entretanto, a exceção do estriado, foi demonstrado um aumento na atividade de SOD sem um proporcional aumento na atividade de CAT, com aumento conseqüente na relação de SOD e CAT, também nos períodos iniciais após a indução de sepse nos ratos (6 horas). O **Capítulo 6** aborda os resultados do experimento realizado com administração de antioxidantes NAC e DFX antes do Teste de Esquiva Inibitória, Esquiva Inibitória de Múltiplos Treinos após 10 e 30 dias da indução da sepse e Habituação ao Campo Aberto após 10 dias da cirurgia de CLP, onde foi demonstrando que, DFX e NAC associado, porém não isoladamente, revertem os danos cognitivos observados em ratos submetidos aos testes acima. A discussão geral (**parte III**) busca integrar esses achados descritos nos capítulos anteriores dentro de uma nova perspectiva para esclarecer os mecanismos neurobiológicos do dano cognitivo em sobreviventes a sepse. Além disso, abre discussões acerca de possíveis novas possibilidades de pesquisas.

ABSTRACT

(Tatiana Barichello – Investigation of behavioural and neurochemical sequelae in rats submitted to sepsis: possible therapeutic role for antioxidant agents) - This work presents the compilation of 5 experiments enters throughout 2004-2007. The **part I** presents lightly the theoretical landmark of the four works. Initially general aspects of sepsis are argued. After, some known mechanisms of the involvement of the central nervous system with sepsis are described, as the diminished permeability of the brain blood barrier, alteration of some neurotransmitters, neuronal apoptosis, activation of some inflammatory cascades, at last, the inflammatory process in the central nervous system. The cognitive damage caused by the septic encephalopathy had been also discussed, and finally it stress oxidative and the antioxidants defenses as a mechanism that can be causing the cognitive deficit in sepsis survivors patients. The main experiments (**part II**) carried through 10 days after the induction of sepsis in rats occupy **chapters 1 and 2**. In **chapter 1** a body of results is presented that allows observing the cognitive incapacities to the tests of Habituation to the Open Field, Inhibitory Avoidance and Continuous Multiple Trials Step-down Inhibitory Avoidance in rats surviving sepsis after 10 days of the induction for the animal model of CLP. These data demonstrate a cognitive incapacity in the memory and the learning of the septic animals. **chapter 2** presents the results of the complementary behavioural tests: Object Recognition, Elevated Plus-maze and Forced Swimming Test, where it was possible to demonstrate that the septic rats had presented incapacity in the memory and symptoms of depression, but not of anxiety. **chapter 3** extends the observed findings in **chapter 1**, carrying through the tests of Habituation to the Open Field, Continuous Multiple Trials Step-down Inhibitory Avoidance after 30 days of the induction of sepsis in rats for procedure CLP. The gotten data confirm that the incapacity of demonstrated learning and memory in **chapter 1** after persists 30 days of the surgery of CLP. The measure of it stress oxidative in hippocampus, cerebellum, striatum and cortex of rats submitted to sepsis was described in **chapter 4**. In this experiment it was observed that in the initial hours after the induction of sepsis in rats, had oxidative damages, evaluated for the TBARS and carbonyl in diverse regions of the brain. However, the exception of the striatum, was demonstrated an increase in the activity of SOD without a proportional increase in the activity of CAT, with consequent increase in the relation of SOD and CAT, also in the initials periods after induction of sepsis in the rats (6 hours). **chapter 5** approaches the results of the experiment carried through with antioxidants substance administration NAC and DFX before the Test of Inhibitory Avoidance and Continuous Multiple Trials Step-down Inhibitory Avoidance after 10 and 30 days of the induction of sepsis and Habituation to the Open Field after 10 days of the surgery of CLP, where it was demonstrating that, DFX and NAC associated, but not separately, above revert the observed cognitive damages in rats submitted to the tests. The general quarrel (**part III**) searches to integrate these described findings in the previous chapters inside of a new perspective to clarify the neurobiological mechanisms underlying brain damage in sepsis survivors. Moreover, it opens possible quarrels concerning new possibilities of research.

LISTA DE ABREVIATURAS

CAT - Catalase

CLP – Cecal Ligation and Perforation (Ligação e Perfuração Cecal)

CREB – (cAMP response element-binding)

DFX – Deferoxamina

DNA – Ácido Desoxirribonucléico

EAO – Espécies Reativas de Oxigênio

FAD - Flavina adenina dinucleotídeo

GABA – Ácido gama amino Butino

GPx – Glutationa Peroxidase

GSH – Glutationa

$HO_2^{\bullet}$ - Hidroperoxila

IL-1 – Interleucina 1

IL-6 – Interleucina 6

$O_2^{-\bullet}$ – Superóxido

OH - Hidroxila

NAC – N-acetilcisteína

NO – Óxido Nítrico

SOD – Peróxido Dismutase

TBARS – Ácido Tiobarbitúrico

TNF – Fator de Necrose Tumoral

TNFα – Fator α de necrose tumoral

VAChT – Vesículas Transportadoras de Acetilcolina

INTRODUÇÃO

Sepse

A Sepse é caracterizada por uma resposta inflamatória decorrente da reação do sistema imunológico à infecção, sendo assim uma doença com implicações clínicas relevantes (Vandijck *et al*, 2006). A incidência da sepse está aumentando continuamente e esta tendência epidemiológica reflete o envelhecimento da população e o aumentando do número de pacientes cronicamente doentes. Uma resistência crescente dos agentes infecciosos que causam a sepse também possui um papel importante no processo. O progresso no diagnóstico e no tratamento da sepse foi crescente nas últimas décadas, porém influenciando o prognóstico da doença somente ligeiramente. Um papel importante na patogenia da sepse é realizado pela resposta inflamatória, que pode causar danos aos tecidos que conduzem à falência orgânica. Esta reação é controlada pela resposta antiinflamatória, que pode ser conduzida exageradamente e assim, aumentando às infecções secundárias e levando a síndrome da respostas antiinflamatória compensatória (Holub, 2007).

A síndrome da resposta inflamatória sistêmica é um estado fisiopatológico multifatorial agressivo com uma taxa elevada de mortalidade de até 51% e a 10ª causa principal de morte nas unidades de terapia intensiva (Martin *et al*, 2005). O diagnóstico inclui uma exigência para dois ou mais dos seguintes sintomas: febre ou hipotermia, taquicardia, e leucocitose ou leucopenia (Bone,1992). A diferença principal entre a síndrome da resposta inflamatória sistêmica e a sepse é que a sepse é produzida por microorganismos infecciosos, visto que a síndrome da

resposta inflamatória sistêmica pode ocorrer na ausência de uma fonte documentada de infecção (Takala *et al*, 2002). Embora a causa seja difícil de verificar em doenças médicas complexas, há ampla evidência clínica e experimental para suportar o conceito da severidade da resposta inflamatória clínica e o resultado na sobrevivência (Sasse *et al*, 1995). No fato, a resposta inflamatória produzida durante a sepse pode estar dentro do espectro da resposta inflamatória sistêmica e envolve a amplificação rápida dos sinais e respostas além do tecido invadido (Takala et al, 2002).

A Sepse e suas complicações conduzem à mortalidade em cerca de 10-50% das ocorridas em unidades de terapia intensiva. Entretanto, os pacientes que sobrevivem a sepse podem ter a função comprometida de alguns órgãos, que podem resultar em sintomas tais como dispnéia, fadiga, depressão e alterações funcionais (Brun-Buisson, 2000; Friedman *et al*, 1998; Hotchkiss e Karl, 2003; Tran *et al*, 1990).

Um dos componentes principais que envolvem a fisiopatologia da sepse é a exacerbada ativação da resposta imune inata. O papel central do sistema imune inato durante a síndrome da resposta inflamatória sistêmica e a sepse é documentado pelo aumento dos fatores pró-inflamatórios após a infecção (Williams *et al*, 2000; Mastronardi *et al*, 2005; Mastronardi *et al*, 2001; Mastronardi *et al*, 2000; Wong *et al*, 1997; Wong *et al*, 1996; Mastronardi *et al*, 2001; v *et al*, 2003), com conseqüente aumento de citocinas pró-inflamatórias tais como o IL-1 e o TNF- para o sistema nervoso central (Takala *et al*, 2002). Como a maioria dos estudos envolvendo sepse focalizou órgãos periféricos como citados previamente, a participação do cérebro durante este processo ainda não está muito clara (Young *et al*, 1990).

Sepse e Sistema Nervoso Central

Os efeitos biológicos causados pela liberação de citocinas refletem no sistema nervoso central como febre (Dinarello, 2004; Maier *et al*, 1998), anorexia (Maier et al, 1998), e ativação do eixo hipotâlamo-pituitária-adrenal, tendo por resultado o aumento da produção dos corticóides adrenais (Licinio e Wong, 1997). As interações recíprocas entre o sistema nervoso central e sistema imunológico são considerados como os componentes principais da resposta inflamatória à sepse, o que acaba causando alterações nos sistemas neuroendócrino, autonômico (Chrousos, 1995), comportamental (Gordon *et al*, 2004) e distúrbios em quaisquer funções adaptáveis como as respostas imuno-inflamatórias e hemodinâmica (Sharshar *et al*, 2003; Sharshar *et al*, 2004; Spyer, 1989; Saper e Breder, 1994).

No caso da sepse e da síndrome da resposta inflamatória sistêmica há alteração na permeabilidade da barreira cerebral sanguínea e o processo inflamatório acaba afetando os sistemas de controle do sistema nervoso central, causando alteração as funções fisiológicas cruciais à homeostase levando a encefalopatia (Chrousos e Gold,1992). A encefalopatia séptica pode ocorrer em 8-70% dos pacientes sépticos, dependendo dos critérios de inclusão empregados (Sprung *et al*, 1990; Young *et al*, 1990), sendo a encefalopatia mais comum nas unidades de terapia intensiva (Bleck et al, 1993). O conceito de encefalopatia séptica como uma entidade que não possa ser explicada pela disfunção, hipotensão, ou pela hipóxia hepática ou renal, é relativamente novo, porém já está claro que a sepse e suas reações podem ser associadas a um largo espectro de danos e disfunções cerebrais (Papadopoulos *et al*, 2000). A disfunção da barreira hematoencefálica parece ter efeito central na fisiopatologia da encefalopatia séptica,

uma vez que permite a passagem para o SNC de endotoxinas que parecem influenciar diversos aspectos do metabolismo cerebral (Orlikowsk *et al*, 2003), além de alterar a função de células endoteliais, astrócitos e neurônios (Papadopoulos *et al*, 2000).

Além disto, o dano na estrutura da barreira hematoencefálica provoca uma alteração severa dos fluxos diferenciais de numerosas substâncias entre o plasma e o liquido cefalorraquidiano. Este fenômeno contribui para aumentar a relação entre os aminoácidos aromáticos e os aminoácidos ramificados diminuindo as concentrações intracerebrais de noradrenalina, dopamina e serotonina, enquanto que as concentrações de GABA são inalteradas (Descamps *et al*, 2003; Freund *et al*, 1985). Nos doentes sépticos, acredita-se que a alteração do estado mental seja por um excesso de ácidos aminos aromáticos, procedentes da parte proteolítica muscular e pela diminuição dos ácidos aminos ramificados (Basler *et al*, 2002; Komatsubara *et al*, 1995). A isto se associa a um aumento dos receptores benzodiazepínicos em ratos sépticos (Soejima *et al*, 1990).

Além das alterações de permeabilidade da barreira hemato-encefálica as modificações do débito sanguíneo cerebral e a sua auto-regulação (Bowie *et al*, 2003) foram implicadas na patogênese e a hipotensão foi associada significativamente ao desenvolvimento de dano isquêmico cerebral (Bowton *et al*, 1989, Booke *et al*, 2003).

A análise histológica demonstra, em casos fatais, várias alterações como proliferação de astrócitos e microglia no córtex, infartos cerebrais, púrpura cerebral, múltiplas hemorragias pequenas em substância branca e disseminação de microabsessos (Jackson *et al*, 1985). Nos sobreviventes, ao contrário, observam-se alterações reversíveis tais como a redução do fluxo sanguíneo cerebral, constrição

capilar, e disfunção da barreira hematoencefálica como já mencionado anteriormente (Bowton *et al*, 1989; Voigt *et al*, 2002).

Marcadores de danos cerebrais durante a sepse poderiam ter um valor considerável, ambos para esclarecer os mecanismos envolvidos e quantificar o grau de dado do cérebro. No estudo por Larsson e colaboradores (2005) a concentração plasmática da proteína S-100β, como possível marcador de danos gliais, encontra-se aumentada.

De qualquer maneira, a fisiopatologia da encefalopatia séptica está longe ser conhecida perfeitamente e vários aspectos merecem ser esclarecidos, em especial o início do desenvolvimento da encefalopatia. Tardiamente, as disfunções ou as insuficiências dos órgãos contribuem para o seu agravamento. Além disso, numerosos fatores de origem iatrogênicos também podem agravar o quadro.

Neste contexto, além dos estudos que envolvem humanos, os modelos de ligação e perfuração cecal (CLP) são clinicamente relevantes, pois produzem uma inflamação generalizada similar àquela observada durante a síndrome da resposta inflamatória sistêmica e a sepse (Ritter *et al*, 2004).

Sepse e Cognição

Recentemente, diversos estudos foram realizados mostrando que os sobreviventes de unidades de terapia Intensiva apresentam incapacidade cognitiva em longo prazo, incluindo alterações na memória, atenção, na concentração e ou na perda global da função cognitiva (Gordon *et al*, 2004; Granja *et al*, 2004; Hopkins *et al*, 2005; Hough e Curtis, 2005; Jackson *et al*, 2004).

O termo incapacidade cognitiva resulta de anormalidades clínicas significativas em uma ou mais funções do cérebro incluindo memória, atenção, função executiva, anormalidades espaciais e visuais, e a função intelectual. A incapacidade cognitiva pode ser suave, moderada, ou severa e pode limitar a habilidade de um indivíduo de pensar, raciocinar, e executar tarefas diárias. O termo declínio cognitivo relaciona-se a deterioração das habilidades cognitivas e não é necessariamente sinônimo de incapacidade cognitiva, pois não implica em um nível absoluto do funcionamento. Entretanto, este tipo de declínio pode causar incapacidades significativas na vida diária de uma pessoa que deseja executar níveis elevados da cognição em áreas ocupacionais e vocacionais. Alternativamente, o rápido declínio cognitivo em uma pessoa com coeficiente de inteligência abaixo da média poderia resultar no diagnóstico de incapacidade cognitiva, porém com impacto menor na função diária (Gordon *et al*, 2004).

Estudos precedentes que envolveram pacientes sobreviventes das unidades de terapia intensiva demonstraram algum grau de incapacidade cognitiva (Gordon *et al*, 2004; Granja *et al*, 2004; Hopkins *et al*, 2005; Hough e Curtis, 2005; Jackson *et al*, 2004). Em estudos em longo prazo a maioria dos pacientes mostrou uma melhora na função cognitiva total, porém algumas habilidades cognitivas, tais como a memória, não melhoraram completamente (Angus *et al*, 2001; Granja *et al*, 2005; Hopkins *et al*, 1994). É bem caracterizada a participação de vias inflamatórias apoptóticas e danos neuronais secundários a encefalopatia séptica (Messaris *et al*., 2004).

Muitos pacientes criticamente doentes possuem incapacidades neurocognitivas crônicas significativas em 2 meses (Jones *et al*, 2006), 6 meses (Weinert *et al*, 1997; Jackson *et al*, 2003b), 9 meses (Hopkins *et al,* 2006), 1 ano

(Hopkins *et al*, 1999), 2 anos (Hopkins *et al*, 2005), e em até 6 anos (Suchyta *et al*, 2004) após a alta hospitalar. As incapacidades neurocognitivas melhoram durante os primeiros 6 a 12 meses da saída dos pacientes do ambiente hospitalar podendo ser permanente ou não, e associados com as incapacidades na função diária, na baixa qualidade de vida, e em uma inabilidade de retornar ao trabalho (Sukantarat *et al*, 2005; Jackson *et al*, 2004).

Estudos concluíram que sobreviventes da sepse que tiveram incapacidades neurocognitivas na alta hospitalar, somente 45% mantiveram as incapacidades neurocognitivas após 1 ano (Hopkins *et al*, 1999). Não havia nenhuma melhoria adicional nas seqüelas neurocognitivas após 2 anos da alta hospitalar (Hopkins *et al*, 2003).

Outros significativos sintomas que sobreviventes de unidades de terapia intensiva apresentam são a depressão e a ansiedade (Scragg *et al*, 2001). A prevalência e a severidade dos transtornos afetivos incluindo sintomas de depressão e ansiedade variam de 10% a 58% (Jackson *et al*, 2003; Hopkins *et al*, 1999; Skozol e Vender, 2001, Al-Saidi *et al*, 2003; Schelling *et al*, 1998). A depressão foi relatada em até 30% dos sobreviventes (Jackson *et al*, 2003), e estima-se que 47% têm ansiedade clinicamente significativa (Scragg *et al*, 2001). Certamente, taxas elevadas de depressão entre sobreviventes de unidades de terapia intensiva sejam relacionadas à incapacidade cognitiva. Em alguns casos a depressão severa pode imitar sintomas de incapacidade cognitiva, embora as diferenças existam entre estas circunstâncias. No geral, os indivíduos com depressão retêm a habilidade de aprender, não se esquecem rapidamente e não indicam decréscimos significativos na linguagem (Hart *et al*, 1997; McGlynn e Schacter, 1989; Jones *et al*, 1992).

A neurobiologia da depressão pode ser explicada na maior parte na desregulação do eixo hipotalâmico-pituitário-adrenal e da sua ação no hipocampo que implicam no fator liberador de corticotrofinas, glucocorticoides, fator neurotrofico derivado do cérebro, e o CREB. Outras áreas do cérebro além do hipocampo provavelmente também estão envolvidas. Por exemplo, os núcleos acumbens, a amígdala, e o núcleo hipotalâmico são críticos na regulação da motivação como comer, dormir, nível de energia, ritmo circadiano, e em respostas aos estímulos de recompensa e aversivos, que estão anormais em pacientes deprimidos (Nestler *et al*, 2002)

Os sintomas de dano cognitivo como a memória, a depressão e a ansiedade devem ser melhor entendidos para posteriormente justificar os mecanismos que envolvem o dano cognitivo em sobreviventes de sepse. Neste contexto, devido a grande incidência, associado à gravidade da sepse seria por si só o suficiente para justificar o aprofundamento no conhecimento de sua fisiopatologia e a tentativa de novas possibilidades terapêuticas, pois como exposto nos parágrafos acima, a maioria dos sobreviventes de unidades de terapia intensiva apresentam algum dano cognitivo em longo prazo, portanto faz-se necessário a realização de estudos que possam investigar as possíveis incapacidades cognitivas apresentadas em curto e médio prazo em um modelo animal de ligação e perfuração cecal (CLP).

Sepse e Estresse Oxidativo

Diversos mecanismos de inflamação e dano celular são implicados na fisiopatologia da sepse, choque séptico e disfunção orgânica relacionada à sepse,

entre eles a geração de espécies ativas do oxigênio (EAO). O elemento oxigênio existe na atmosfera na forma diatômica O_2, com exceção de certos microorganismos unicelulares anaeróbios e aeróbios tolerantes, todos os animais, plantas e bactérias necessitam de O_2 para eficiente produção de energia através do uso do oxigênio dependente da cadeia transportadora de elétrons nas mitocôndrias de eucariontes (Halliwell & Gutteridge,1999).

A necessidade do O_2 obscurece o fato de que é um gás tóxico e mutagênico oferecendo sérios riscos, as espécies aeróbicas sobrevivem devido às defesas antioxidantes que as protegem. Os efeitos dos danos em organismos aeróbicos variam consideravelmente com o tipo de organismo, a idade, estado fisiológico e a dieta. A toxicidade do oxigênio é influenciada pela presença na dieta de variadas quantidades de vitaminas A, E, e C, metais tais como Zinco, Cobre e Ferro, antioxidantes sintéticos e ácidos graxos poliinsaturados (Halliwell & Gutteridge, 1999).

Para formar o oxigênio molecular O_2, os dois elétrons do subnível p de um elemento oxigênio fazem intercâmbio com os dois elétrons de outro elemento oxigênio, formando um composto estável com 12 elétrons na última camada (L). Quando no metabolismo normal ocorrer uma redução do oxigênio molecular O_2, este ganhará um elétron, formando o radical superóxido ($O_2^{-\bullet}$) (Mello *et al.*,1983). Em condições fisiológicas do metabolismo celular aeróbico, o O_2 sofre redução tetravalente, com aceitação de quatro elétrons, resultando na formação de água, durante este processo são formados intermediários reativos como os radicais superóxido ($O_2^{-\bullet}$), hidroperoxila ($HO_2^{\bullet}$), hidroxila (OH), e o peróxido de hidrogênio (H_2O_2) (Ferreira *et al.*, 1997, Cuzzocrea *et al*,2001). Normalmente a redução completa do O_2 ocorre na mitocôndria, e a reatividade das EAO é neutralizada pela

entrada de quatro elétrons (Cohen, 1989). Entre as espécies reativas de oxigênio formadas o radical superóxido ocorre em quase todas as células aeróbicas e é produzido durante a ativação máxima de neutrófilos, macrófagos, monócitos e eosinófilos (Halliwell & Gutteridge, 1990). O radical superóxido reage com alvos biológicos, sendo que o efeitos nos tecidos é resultado da formação secundaria de novos radicais livres em adição a reação do superóxido com lipídios, catecolaminas (Macarthur *et al*.,2000), e Dna (Dix *et al.,*1996).

Entre as EAO o oxigênio *singlet* que é a forma excitada do oxigênio molecular e não possui elétrons desemparelhados na ultima camada (Halliwell & Gutteridge, 1990) é reconhecido como um possível contribuinte para o estresse oxidativo nos sistemas vivos, altamente energético e mutagênico, sendo capaz de oxidar moléculas biológicas (Cuzzocrea *et al*.,2001; Ravanat *et al*.,1992).

O H_2O_2 é capaz de atravessar camadas lipídicas, podendo reagir com a membrana eritrocitária e com proteínas ligadas ao ferro, sendo assim é altamente tóxico para as células, podendo ser aumentada esta toxicidade em presença de ferro (Eaton,1991).

O radical mais reativo proposto muitos anos atrás é o $HO_2^{\bullet-}$**,** produzido pela interação do $O_2^{-\bullet}$ e H_2O_2 pela reação química conhecida como Haber-Weiss, traços do metal ferro na forma de íons, reage com H_2O_2, produzindo o radical hidroxil, o íon ferro não esta presente em vivo, mas os íons são produzidos pela ação do superóxido sobre os íons ferro armazenados em proteínas (Cuzzocrea *et al*.,2001). A liberação do ferro intracelular, a baixa capacidade liquórica de ligação ferro-proteína e a deficiência de enzimas antioxidantes no sistema nervoso central ampliam o risco de lesão induzida pelo trauma com a liberação de ferro (Ferreira *et al.,*1997). Estas espécies ativas de oxigênio são capazes de reagir

indiscriminadamente com qualquer tipo de molécula orgânica, extraindo elétrons e gerando novos radicais livres em reação em cadeias altamente citotóxicas (Ames *et al.*,1993).

Quando existe um aumento na produção ou diminuição das defesas antioxidantes existe uma condição chamada de estresse oxidativo, em que os radicais livres em excesso começam a produzir danos a lipídios, proteínas, DNA, carboidratos (Halliwell & Gutteridge, 1999), inibição das enzimas da cadeia respiratória mitocondrial, inativação do gliceraldeido-3-fosfato dehidrogenase, inibição da atividade da ATPase na membrana sódio/potássio, inativação da membrana no canal de sódio (Cuzzocrea *et al.*, 2001).

A capacidade das células em diminuir os efeitos do estresse oxidativo é determinada pelo balanço entre as quantidades de espécies oxidantes geradas, e, a capacidade dos processos metabólicos de produzir antioxidantes (Beutler *et al.*, 1989).

Antioxidantes e tratamento de sepse

Intervenções que reduzem a produção das EAO exercem efeitos benéficos em diversos modelos de endotoxemia e choque séptico. Estas intervenções incluem a N-acetilcisteina (NAC) (Atis *et al* 2006, Victor *et al* 2003, Ozdulger *et al* 2003), α-tocoferol (Durant et al 2004), alopurinol (Xiang *et al* 2003), deferoxamina (DFX) (Messaris et al 2004b), catalase (Supinski et al 1993), superoxide dismutase (Supinski e Callahan 2006), miméticos de superoxide dismutase (Salvemini e Cuzzocrea 2003), magnolol (Kong *et al* 2000) e tempol

(Matejovic *et al* 2005). Geralmente estas intervenções são administradas antes ou imediatamente após a indução da sepse o que pode limitar sua relevância clínica.

Entre os mais estudados antioxidantes no tratamento da sepse encontra-se a NAC é bem conhecida como precursora artificial de glutationa e utilizada clinicamente como droga mucolítica e no tratamento da intoxicação por paracetamol, com raros efeitos adversos. O NAC é um scavenger de peróxido de hidrogênio, ácido hipoclórico e radical hidroxil e por estas ações inibe a liberação de TNFα, a ativação de citocinas pró-inflamatórias e apoptose celular.

As evidências sugerem que a expressão do gene TNF é controlada pela transcrição do NF-kB, cuja a atividade pode ser induzida pelo peróxido de hidrogênio. NAC mostrou inibir a atividade do NF-kB em várias linhagens celulares, inclusive em macrófagos peritoneais de ratos (Pahan *et al* 1998). O peróxido de hidrogênio diretamente ou indiretamente através de sua redução a radical hidroxil via reação de Fenton, age como um mensageiro na síntese e ativação de mediadores inflamatórios. O NAC como scavenger destes radicais mostrou inibir a liberação destes mediadores.

Por estas razões é reconhecido o papel antioxidante da NAC na sepse, mas quando utilizada antes da indução da sepse e não depois. Em contraste alguns estudos demonstram um aumento no estresse oxidativo e mortalidade por sepse após uso de altas doses da NAC, possivelmente relacionado à sua capacidade para reduzir o ferro para sua forma catalicamente ativa (Sprong *et al* 1998), favorecendo a reação de Fenton.

O DFX é um quelante de ferro empregado com segurança no tratamento de várias doenças hematológicas. Experimentalmente, já foi citada em alguns estudos, como uma droga que diminuiu a injúria oxidativa, quando usada antes e

não depois da indução da sepse, melhorando mortalidade em um modelo animal de sepse abdominal (Messaris *et al* 2004).

As propriedades pró-inflamatórias dos EAO incluem dano às células endoteliais, formação de fatores quimiotáticos, recrutamento de neutrófilos, oxidação e peroxidação de lipídeos, dano ao DNA, liberação de TNF-α e IL-1β e formação de peroxinitrito (Azevedo *et al* 2006, Protti e Singer 2006). A hiperprodução de EAO e a falha nos mecanismos de "scavengers" naturais são implicados no dano endotelial, alterações miocárdicas e falência orgânica múltipla.

Os monócitos e polimorfonucleares sofrem alterações, descritas como ativação de leucócitos, em resposta a estimulação por TNF e interleucinas (IL), com um conseqüente aumento na produção de superóxido (O_2^-) por estas células. Primariamente o superóxido (O_2^-) tem um efeito pró-inflamatório, que é perpetuado pela formação de peroxinitrito (reação de superóxido com óxido nítrico). O peroxinitrito possui vários efeitos citotóxicos e pró-inflamatórios independentes que levam ao dano celular irreversível, como evidenciado no choque séptico (Alvarez e Evelson 2007).

O choque séptico é caracterizado por severa hipotensão e diminuição da perfusão tecidual em decorrência da hiporreatividade vascular a catecolaminas endógenas e exógenas, que pelo menos em parte é explicado pelo grande aumento na produção de óxido nítrico que ocorre na sepse (Fernandes *et al* 2006, Cuzzocrea *et al* , 2006).

O peróxido de hidrogênio (H_2O_2), apesar de ser considerado um oxidante estável, conta com um papel importante na fisiopatologia da sepse. O H_2O_2 pode ser metabolizado por duas enzimas antioxidantes, a glutationa peroxidase e a catalase, mas em presença de metais de transição, ele é decomposto em radical hidroxil via

reação de Fenton, um radical altamente tóxico e reativo. Os danos às células musculares e acidose aumentam a quantidade de ferro liberado da mioglobina e hemoglobina, facilitando esta reação. Recentemente foi demonstrado que alterações do metabolismo de ferro podem estar relacionadas com mortalidade em modelos animais de sepse (Wizorek *et al* 2003).

Associada a grande mortalidade envolvida, os gastos direto e indireto com a sepse atinge altas cifras em nosso país. A possibilidade de diminuir a mortalidade por sepse e reduzir os gastos com internação em Unidades de Terapia Intensiva (alto custo com antibióticos de largo espectro, recursos técnicos, humanos e tecnológicos associados com o manejo do paciente) justificam a necessidade de maior investimento no estudo desta patologia. Outrossim, são pouco conhecidos os efeitos da sepse no Sistema Nervoso Central e, através da determinação do Estresse Oxidativo, poderemos identificar e melhor compreender estes danos.

Modelo animal de sepse

Estudos de sepse em humanos são difíceis devido a severidade da doença, a necessidade de intervenções terapêuticas imediatas, a heterogeneidade dos pacientes. Assim, modelos animais têm sido usados extensivamente para explorar a patogênese e gerarem dados pré-clínicos de intervenções terapêuticas. Para estas propostas, deve-se utilizar um modelo animal que reproduza a vasodilatação, hipotensão, aumento do débito cardíaco, resposta ao tratamento e mortalidade vistos em pacientes sépticos. Tem-se utilizado para isto modelo de sepse abdominal, sepse cutânea, sepse induzida pela administração de lipopolissacarídeo (LPS) ou fator de necrose tumoral. Porém os modelos que

induzem peritonite são mais amplamente usados. A peritonite pode ser induzida por inoculação direta de bactérias ou de conteúdo fecal na cavidade peritoneal. Entretanto o modelo mais aceito na literatura, e que parece simular mais adequadamente o quadro clínico de sepse, é o chamado CLP. A CLP se baseia na ligação do ceco logo abaixo da válvula ileo-cecal (mantendo desta maneira o trânsito intestinal), perfuração do ceco com tamanho padronizado e liberação de conteúdo fecal para a cavidade peritoneal, conforme classicamente descrito por Wichterman e cols (1980). Desta maneira além da peritonite se induz isquemia mesentérica simulando as grandes síndromes clínicas de sepse abdominal (p.ex. apendicite, isquemia mesentérica). Recentemente este modelo foi modificado para melhor simular as características clínicas dos pacientes com sepse abdominal, introduzindo desta maneira a ressucitação volêmica e emprego de antibióticos de amplo espectro (Hollenberg *et al* 2001).

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar as alterações comportamentais e neuroquímicas induzidas pela sepse em modelo animal com um possível papel terapêutico de antioxidantes.

2.2 Objetivos Específicos:

- Avaliar os efeitos da sepse após 10 e 30 dias sobre o aprendizado e memória;
- Avaliar os efeitos da sepse leve e grave após 10 dias sobre a ansiedade;

- Avaliar os efeitos da sepse leve e grave após 10 dias sobre sintomas depressivos em ratos de diferentes idades;
- Determinar uma relação temporal entre a indução da sepse e parâmetros de estresse oxidativo nas estruturas cerebrais dissecadas: hipocampo, estriado, córtex e cerebelo em um modelo animal de sepse.
- Avaliar os efeitos do tratamento com antioxidantes na incapacidade cognitiva precoce e tardia e no estresse oxidativo em hipocampo.

Parte II

Capítulo 1.

Cognitive impairment in sepsis survivors from cecal ligation and perforation

Tatiana Barichello, Márcio R. Martins, Adalisa Reinke, Gustavo Feier, Cristiane Ritter, João Quevedo, Felipe Dal-Pizzol

Critical Care Medicine (2005) 33:221-223

Cognitive impairment in sepsis survivors from cecal ligation and perforation*

Tatiana Barichello, PharmD, MSc; Márcio R. Martins, BSc; Adalisa Reinke, BSc; Gustavo Feier, Bsc; Cristiane Ritter, MD, MSc; João Quevedo, MD, PhD; Felipe Dal-Pizzol, MD, PhD

Objective: **Critical illness survivors present long-term cognitive impairment, including problems with memory and learning. We evaluated cognitive performance in rats that survived from sepsis induced by cecal ligation and puncture (CLP).**

Design: **Prospective, controlled experiment.**

Setting: **Animal basic science laboratory.**

Subjects: **Male Wistar rats, weighing 300–350 g.**

Interventions: **The rats underwent CLP (sepsis group) with "basic support" (saline at 50 mL/kg immediately and 12 hrs after CLP plus ceftriaxone at 30 mg/kg and clindamycin at 25 mg/kg 6, 12, and 18 hrs after CLP) or sham-operated (control group).**

Measurements and Main Results: **Ten days after surgery, the animals underwent three behavioral tasks: a) inhibitory avoidance task; b) habituation to an open field; and c) continuous multiple-trials step-down inhibitory avoidance task (CMSIA). In the habituation to an open-field task, there were no differences in the number of crossings and rearings. The sepsis group showed significantly decreased performance in latency retention compared with the sham group in inhibitory avoidance. Furthermore, when tested by the habituation to an open-field task, the sepsis group did not show any difference between training and test, indicating memory impairment. In the CMSIA, the sepsis group showed a significant increase in the number of training trials required to reach the acquisition criterion.**

Conclusion: **Our data provide the first experimental demonstration that survivors from CLP show learning and memory impairment after complete physical recovery from sepsis. (Crit Care Med 2005; 33:221–223)**

KEY WORDS: sepsis; survivors; cecal ligation and puncture; learning; memory; rat

Despite major improvements in intensive care and antibiotic therapy, the mortality and morbidity rates due to severe sepsis and septic shock remain high. Critical illness survivors present long-term cognitive impairment, including alterations in memory, attention, concentration, and/or global loss of cognitive function (1–4). The development of an animal model that mimics the cognitive alterations observed in patients is of great value. In this context, murine models of cecal ligation and perforation (CLP) are clinically relevant since they induce a polymicrobial sepsis that mimics human sepsis (5). CLP models have contributed to elucidate the pathogenesis and to determine new therapies in sepsis (5, 6). In this article, we evaluated learning and memory performance in rats after sepsis induced by CLP compared with sham-operated rats.

MATERIALS AND METHODS

Under anesthesia (ketamine, 80 mg/kg, and xylazine, 10 mg/kg), 105 male Wistar rats (300–350 g) underwent CLP (sepsis group) and 40 rats underwent sham operation (control group) as previously described (5). After surgery, the sepsis group received "basic support" (saline at 50 mL/kg immediately and 12 hrs after CLP plus ceftriaxone at 30 mg/kg and clindamycin at 25 mg/kg every 6 hrs over a total of 3 days). The sham-operated group received only saline, 50 mL/kg, immediately and 12 hrs after surgery, and the volume of saline corresponded to antibiotic administration. Survival in the sham group was 100%, and in the sepsis group, it was 40% (40 rats). The number of survivals is in accordance with our previous reports (5, 6). Ten days after surgery, the animals separately underwent three behavioral tasks: a) the step-down inhibitory avoidance task (single-training); b) continuous multiple-trials step-down inhibitory avoidance task; and c) the open-field task. The behavioral tests were performed by the same person, who was blinded as to group (sham or CLP). All experimental procedures involving animals were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and with the approval of the local ethics committee.

The step-down inhibitory avoidance apparatus and procedures have been described in previous reports (7). Briefly, the training apparatus was a 50 × 25 × 25-cm acrylic box (Albarsch, Porto Alegre, Brazil) whose floor consisted of parallel caliber stainless steel bars (1-mm diameter) spaced 1 cm apart. A 7-cm-wide, 2.5-cm-high platform was placed on the floor of the box against the left wall. In the training trial, animals were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, the animals received a 0.4-mA, 2.0-sed foot shock and returned to their home cage. A retention test trial was performed 24 hrs after training. The retention test trial was procedurally identical to training, except that no foot shock was presented. The retention test step-down latency (maxi-

*See also p. 262.

From the Laboratório de Fisiopatologia Experimental (TB, AR, CR, FD-P), the Laboratório de Neurotoxicologia (MRM, GF, JQ), Universidade do Extremo Sul Catarinense, Criciúma, Brazil.

Supported, in part, by UNESC (Brazil), CNPq (Brazil), FUNCITEC (Brazil). None of the authors have any financial interests to disclose.

Address requests for reprints to: Felipe Dal-Pizzol, MD, PhD, Laboratório de Fisiopatologia Experimental, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil, Avenida Universitária, 1105, 88006–000. E-mail: piz@unesc.rct-sc.br

DOI: 10.1097/01.CCM.0000150741.12906.BD

mum, 180 secs) was used as a measure of inhibitory avoidance retention.

Habituation to an open field was carried out in a 40 × 60-cm open field surrounded by 50-cm high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. Animals were gently placed on the left rear quadrant, and left to explore the arena for 5 mins (training session). Immediately following this, the animals were taken back to their home cage, and 24 hrs later submitted again to a similar open-field session (test session). Crossing of the black lines and rearing performed in both sessions were counted. The decrease in the number of crossings and rearings between the two sessions was taken as a measure of the retention of habituation (8).

Continuous multiple-trials step-down inhibitory avoidance task was performed in the same step-down inhibitory avoidance apparatus described above. However, in the training session, the animal was placed on the platform and immediately after stepping down on the grid, received a 0.3-mA, 2.0-sec foot shock. This procedure continued until the rat remained on the platform for 50 secs. The animal was then returned to the home cage. The number of training trials required to reach the acquisition criterion of 50 secs on the platform was recorded. The retention test was performed 24 hrs later.

Data from the inhibitory avoidance task and retention test latencies from continuous multiple-trials step-down inhibitory avoidance were expressed as median and interquartile ranges. Statistical significances were determined by the Mann-Whitney U test, $p < .05$. Data from the open-field task and the number of training trials from continuous multiple-trials step-down inhibitory avoidance were expressed as mean ± SEM. Statistical significances were determined by paired samples Student's t-test, $p < .05$.

RESULTS AND DISCUSSION

After 10 days, the animals seemed to be free from infection. We performed blood cultures that were all negative in this period. The animals recovered their weight and grooming habits, blood counts returned to control levels, and polymerase chain reaction values were negative. In addition, we had previously demonstrated that 5 days after CLP, organic oxidative stress, mitochondrial dysfunction, and neutrophil infiltration return to control levels in the present CLP model (6).

No differences between groups were demonstrated in the inhibitory avoidance training session. In the test session, the step-down latency was significantly decreased in the sepsis group compared with the sham group ($p < .01$) (Fig. 1*A*). In the habituation to the open-field training session, no difference in motor and exploratory activity was demonstrated between groups, e.g., in the number of crossings and rearings. This finding supports our hypothesis that no active infection remained in the CLP group.

In the open-field task, there were no differences in the number of crossings and rearings between groups in the habituation to the open-field training session, demonstrating no difference in motor and exploratory activity between groups, which reinforces the idea of no active infection in the CLP group (Fig. 1*B*). In the test session, there was a significant reduction in both crossings and rearings of the sham group compared with the sepsis group ($p < .001$) (Fig. 1*B*). In addition, the sepsis group did not show a difference in crossings and rearings between training test sessions, demonstrating memory impairment in this group (Fig. 1*B*). On the other hand, the sham group showed a decreased number of crossings ($p < .005$) and rearings ($p < .005$), indicating habituation to the task environment (Fig. 1*B*).

The continuous multiple-trials step-down inhibitory avoidance showed a significant increase in the number of training trials required to reach the acquisition criterion (50 secs on the platform) in the sepsis group compared with the sham group ($p < .05$) (Fig. 2*A*). In the retention test, there was no difference between groups (Fig. 2*B*). The results of this task showed that the sepsis group required approximately two times more stimulus to reach the acquisition criterion compared with the sham group, indicating a learning impairment (acquisition of new knowledge) in the sepsis group.

These results do not appear to be secondary to antibiotic administration since we performed the open-field and inhibitory avoidance in a sham-operated group with antibiotic administration and did

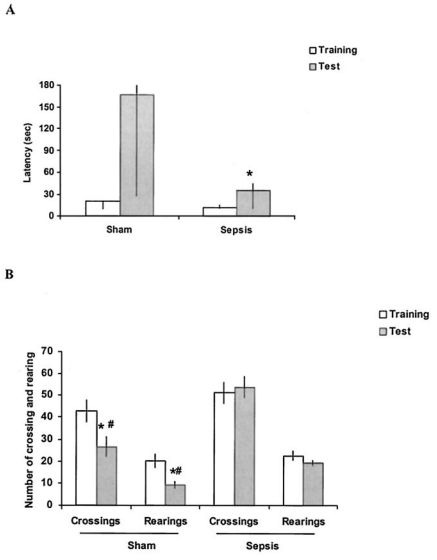


Figure 1. *A*, inhibitory avoidance task. Data are expressed as median (interquartile ranges) training and test session latencies, in seconds. *Significant difference compared with Sham group (Mann-Whitney U test, $p < .01$). Both groups (n = 14 in the sham group and n = 13 in the sepsis group) showed significant training test differences (Wilcoxon's test, $p < .01$). *B*, Open-field task. Data are expressed as mean ± SEM of crossings and rearings of training (*white columns*) and test (*gray columns*) session (n = 14 in the sham group and n = 17 in the sepsis group). *Significant difference between training test (paired samples Student's t-test, $p < .005$). #Significant difference between groups in training and test sessions (Student's t-test, $p < .0001$).

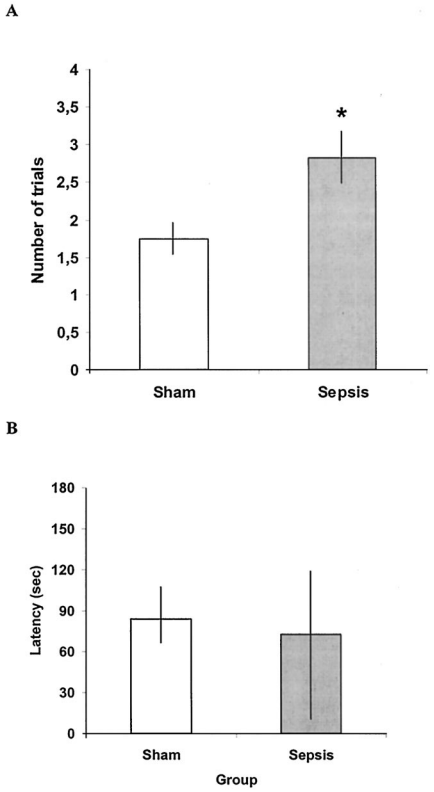


Figure 2. *A*, continuous multiple-trials step-down inhibitory avoidance. Data are expressed as mean ± SEM of the number of training trials required to reach acquisition criterion (50 secs on the platform). *Significant difference between groups (Student's t-test, $p < .0001$). *B*, retention test latencies, expressed as median (interquartile ranges). No difference was observed between groups (n = 12 per group).

Our data provide the first experimental demonstration that survivors from cecal ligation and puncture show learning and memory impairment after complete physical recovery from sepsis.

not detect significant differences in comparison with the sham-operated group (data not shown).

Previous reports describing human intensive care unit sepsis survivors demonstrated that the vast majority of such patients experienced cognitive impairment at hospital discharge (1, 4). At 1-yr follow-up, most patients showed improvement in overall cognitive function; however, some cognitive skills, such as memory, did not completely improve (1, 2, 4). Our data provide the first experimental demonstration in a clinically relevant model of animal sepsis. The rats showed impairment in learning and memory. In this context, Shimizu et al. demonstrated that 24 hrs after CLP, animals showed learning impairment in passive avoidance retention (9). Our results are of greater significance since 10 days after CLP, but not 24 hrs, the animals are fully recovered, with no signs of infection or motor alterations (5, 6). In addition, our survival study design using fluids and antibiotics replicates more closely the supportive therapy performed in the clinical setting, when compared with the study of Shimizu et al. (9). The deficits demonstrated here mimics, at least in part, the cognitive alterations observed in patients surviving sepsis, particularly memory impairment. In this way, we believe that the CLP model of sepsis will help us to investigate the biological mechanisms involved in the cognitive deficits associated with sepsis and to determine therapeutic approaches to this problem.

REFERENCES

1. Hopkins RO, Weaver LK, Pope D, et al: Neuropsychological sequelae and impaired health status in survivors of severe acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1999; 161:50–56
2. Angus DC, Musthafa AA, Clermont G, et al: Quality-adjusted survival in the first year after the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2001; 163:1389–1394
3. Granja C, Dias C, Costa-Pereira A, et al: Quality of life of survivors from severe sepsis and septic shock may be similar to that of others who survive critical illness. *Crit Care* 2004; 8:R91–R98
4. Heyland DK, Hopman W, Coo H, et al: Long-term health-related quality of life in survivors of sepsis. Short Form 36: a valid and reliable measure of health-related quality of life. *Crit Care Med* 2000; 28:3599–3605
5. Ritter C, Andrades ME, Reinke A, et al: Treatment with *N*-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis. *Crit Care Med* 2004; 32:342–349
6. Ritter C, Andrades M, Frota Junior ML, et al: Oxidative parameters and mortality in sepsis induced by cecal ligation and perforation. *Intensive Care Med* 2003; 29:1782–1789
7. Quevedo J, Vianna MR, Roesler R, et al: Two time windows of anisomycin-induced amnesia for inhibitory avoidance training in rats: Protection from amnesia by pretraining but not pre-exposure to the task apparatus. *Learn Mem* 1999; 6:600–607
8. Vianna MR, Alonso M, Viola H, et al: Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat. *Learn Mem* 2000; 7:333–340
9. Shimizu I, Adachi N, Liu K, et al: Sepsis facilitates brain serotonin activity and impairs learning ability in rats. *Brain Res* 1999; 830: 94–100

Capítulo 2.

Behavioral deficits in sepsis-surviving rats induced by cecal ligation and perforation

Tatiana Barichello, Márcio Rodrigo Martins, Adalisa Reinke, Larissa S. Constantino, Roberta Albino Machado, Samira S. Valvassori, José Cláudio F. Moreira, João Quevedo, Felipe Dal-Pizzol

Brazilian Journal of Medical and Biological Research (2007) 40: 831-7

Brazilian Journal of Medical and Biological Research (2007) 40: 831-837
ISSN 0100-879X

Behavioral deficits in sepsis-surviving rats induced by cecal ligation and perforation

T. Barichello[1], M.R. Martins[2], A. Reinke[1], L.S. Constantino[1], R.A. Machado[1], S.S. Valvassori[2], J.C.F. Moreira[3], J. Quevedo[2] and F. Dal-Pizzol[1]

[1]Laboratório de Fisiopatologia Experimental, [2]Laboratório de Neurociências, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brasil
[3]Centro de Estresse Oxidativo, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil

Abstract

Sepsis and its complications are the leading causes of mortality in intensive care units, accounting for 10-50% of deaths. Intensive care unit survivors present long-term cognitive impairment, including alterations in memory, attention, concentration, and/or global loss of cognitive function. In the present study, we investigated behavioral alterations in sepsis-surviving rats. One hundred and ten male Wistar rats (3-4 months, 250-300 g) were submitted to cecal ligation and puncture (CLP), and 44 were submitted to sham operation. Forty-four rats (40%) survived after CLP, and all sham-operated animals survived and were used as control. Twenty animals of each group were used in the object recognition task (10 in short-term memory and 10 in long-term memory), 12 in the plus-maze test and 12 in the forced swimming test. Ten days after surgery, the animals were submitted individually to an object recognition task, plus-maze and forced swimming tests. A significant impairment of short- and long-term recognition memory was observed in the sepsis group (recognition index 0.75 *vs* 0.55 and 0.74 *vs* 0.51 for short- and long-term memory, respectively ($P < 0.05$). In the elevated plus-maze test no difference was observed between groups in any of the parameters assessed. In addition, sepsis survivors presented an increase in immobility time in the forced swimming test (180 *vs* 233 s, $P < 0.05$), suggesting the presence of depressive-like symptoms in these animals after recovery from sepsis. The present results demonstrated that rats surviving exposure to CLP, a classical sepsis model, presented recognition memory impairment and depressive-like symptoms but not anxiety-like behavior.

Correspondence
F. Dal-Pizzol
Laboratório de Fisiopatologia Experimental
Universidade do Extremo Sul Catarinense
88006-000 Criciúma, SC
Brasil
E-mail: pizzol.ez@terra.com.br

Research supported by FAPESC, CNPq and UNESC.

Received October 4, 2006
Accepted April 18, 2007

Key words
- Sepsis survivors
- Recognition memory
- Plus-maze test
- Forced swimming test
- Cecal ligation and puncture
- Cognitive impairment

Introduction

Sepsis and its complications are a leading cause of mortality, accounting for 10-50% of deaths on intensive care units (1-4). Several studies have been performed to investigate the role of peripheral organs such as lungs, liver, gut, and kidneys in sepsis development (5), but the participation of the central nervous system during sepsis has been studied less. Septic encephalopathy represents brain dysfunction due to sepsis or the systemic inflammatory response syndrome, and has been reported to occur in 8-70% of septic patients depending on the inclusion criteria employed (6-8).

Survivors of critical care, including septic patients, may have persistenty compromised organ function, which may result in symptoms such as dyspnea, fatigue, depression, and impaired functional status. Recently, several studies have demonstrated that critical care survivors present long-term cognitive impairment, including alterations in memory, attention, concentration, and/or global loss of cognitive function (9-17). However, the neurobiological mechanisms involved in this cognitive impairment remain unclear.

A recent study evaluating apoptosis and vulnerability of different brain regions induced by systemic inflammation concluded that the hippocampus is the most vulnerable region during experimental sepsis (18). In this context, murine models of cecal ligation and perforation (CLP) are clinically relevant since they induce a polymicrobial sepsis that mimics human sepsis (19-22). The CLP model has contributed to the elucidation of the pathogenesis and to the determination of new therapies in sepsis (19,20). Shimizu et al. (23) demonstrated that 24 h after CLP animals presented learning impairment in passive avoidance retention. Moreover, we recently reported that CLP-induced sepsis survivors presented learning, aversive and spatial memory impairment when submitted to behavioral tasks 10 days after CLP (21) and learning and aversive memory impairment lasting up to 30 days after CPL (22). Thus, CLP seems to be a good model for the study of cognitive and emotional alterations as late manifestation of sepsis.

Therefore, the objective of the present study was to determine changes in recognition memory and the presence of anxiety- or depressive-like symptoms in severe sepsis-surviving rats.

Material and Methods

Subjects

Male Wistar rats (3-4 months, 220-310 g) were obtained from our breeding colony (UNESC). The animals were housed 5 to a cage with food and water available *ad libitum* and were maintained on a 12-h light/dark cycle (lights on at 7:00 am). All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

Cecal ligation and perforation surgery

The animals were subjected to CLP as previously described (19-22). Briefly, rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), given intraperitoneally. Under aseptic conditions, a 3-cm midline laparotomy was performed to allow exposure of the cecum with the adjoining intestine. The cecum was tightly ligated with a 3.0-silk suture at its base, below the ileocecal valve, and was perforated once with a 14-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site returned to the peritoneal cavity, and the laparotomy was closed with 4.0-silk sutures. Animals were resuscitated with normal sa-

line (50 mL/kg subcutaneous) immediately and 12 h after CLP. All animals were returned to their cages with free access to food and water. In this model septic rats become bacteremic with Gram-negative enteric organisms. In the sham-operated group the rats were submitted to all surgical procedures but the cecum was neither ligated nor perforated.

Treatment protocols

After surgery, the sepsis group received "basic support" (50 mL/kg saline immediately and 12 h after CLP plus 30 mg/kg ceftriaxone and 25 mg/kg clindamycin every 6 h for a total of 3 days). The sham-operated group received only 50 mL/kg saline immediately and 12 h after surgery and the volume of saline corresponding to antibiotic administration. To perform behavior experiments 44 animals were sham-operated and the survival in this group was 100%; 10 animals were used as control to the short-term memory in the object recognition task, 10 animals were used as control to the long-term memory in the object recognition task, 12 animals were used as control to the plus-maze test, and 12 animals were used as control to the forced swimming test. One hundred and ten animals were submitted to CLP and 40% of these animals survived to perform behavioral tests (N = 44). These animals were divided as follows: 10 animals were used in the short-term memory object recognition task, 10 animals were used in the long-term memory object recognition task, 12 animals were used in the plus-maze test, and 12 animals were used in the forced swimming test. The number of survivors agreed with previous reports from our group (19-20).

Behavioral tests

Ten days after surgery the animals were submitted individually to the object recognition task, elevated plus-maze or forced swimming tests. All behavioral procedures were conducted between 13:00 and 16:00 h in a sound-isolated room. All behavioral tests were recorded by an observer who was blind to the animal group.

Object recognition

The apparatus and procedures for the object recognition task have been described elsewhere (24,25). Briefly, the task took place in a 40 x 50-cm open field surrounded by 50-cm high walls made of plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. All animals were submitted to a habituation session where they were allowed to freely explore the open field for 5 min. No objects were placed in the box during the habituation trial. Crossings of the black lines and rearings performed in this session were evaluated as locomotor and exploratory activity, respectively.

Twenty-four hours after habituation, training was conducted by placing individual rats for 5 min in the field, in which two identical objects (objects A1 and A2; both being cubes) were positioned in two adjacent corners, 10 cm from the walls. In a short-term recognition memory test given 1.5 h after training, the rats explored the open field for 5 min in the presence of one familiar (A) and one novel (B, a pyramid with a square-shaped base) object. All objects had similar textures (smooth), colors (blue), and sizes (weight 150-200 g), but distinctive shapes. A recognition index calculated for each animal is reported as the ratio TB/(TA + TB) (TA = time spent exploring the familiar object A; TB = time spent exploring the novel object B). Between trials the objects were washed with 10% ethanol solution. In a long-term recognition memory test given 24 h after training, the same rats were allowed to explore the field for 5 min in

the presence of the familiar object A and a novel object C (a sphere with a square-shaped base). Recognition memory was evaluated as done for the short-term memory test. Exploration was defined as sniffing (exploring the object 3-5 cm away from it) or touching the object with the nose and/or forepaws.

Elevated plus-maze

The elevated plus-maze task used in animal models of anxiety has been described in detail elsewhere (26,27). Briefly, the apparatus consisted of two open arms (50 x 10 cm) and two enclosed arms (50 x 10 x 40 cm) arranged in such a way that the two arms of each type were opposite to each other, and a central platform (5 x 5 cm). The maze's height was 50 cm and the tests were conducted under dim red light. Animals were exposed for 5 min to the red light in their own home cages before the testing procedure. Next, they were placed individually on the central platform of the plus-maze facing an open arm. During a 5-min test period the following measurements were recorded by two observers: the number of entries, the time spent in the open and closed arms, and the total number of arm entries.

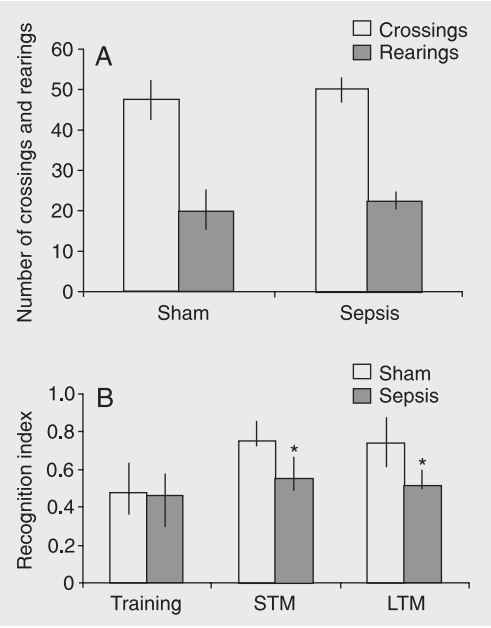


Figure 1. Object recognition task. No significant difference was observed in the numbers of crossings and rearings in the habituation session (Student *t*-test). Data are reported as means ± SEM (A). The sepsis group presented a significant impairment of novel object recognition memory compared to the sham group (B). Results are reported as median (interquartile ranges) recognition indexes in training, short-term (STM) and long-term memory (LTM) retention test trials. N = 10 animals per group. *P < 0.05 compared to the sham group (Mann-Whitney U-test).

Forced swimming test

The forced swimming test was conducted according to previous reports (28-30). Briefly, the test involves two exposures to a cylindrical water tank in which rats cannot touch the bottom or from which they cannot escape. The tank is made of transparent Plexiglas and is 80 cm tall, 30 cm in diameter, and filled with water (22-23ºC) to a depth of 40 cm. Water in the tank was changed after each rat. For the first exposure, rats were placed in the water for 15 min (pre-test session). Twenty-four hours later the rats were placed in the water again for a 5-min session (test session). Behavior was videotaped for later analysis, and the periods of immobility, swimming, and struggling time were recorded. The rats were judged to be immobile whenever they stopped swimming and remained floating in the water, with their head just above water level.

Statistical analysis

Data for recognition indexes are reported as median and interquartile ranges. Comparisons among groups were performed using Mann-Whitney U-tests. Recognition indexes within individual groups were analyzed by Wilcoxon tests. The data for the elevated plus-maze and forced swimming tests are reported as means ± SEM and were analyzed by the Student *t*-test. In all comparisons, $P < 0.05$ indicated statistical significance.

Results

Object recognition

In the habituation session, no significant differences were observed in the number of crossings or rearings (P = 0.56; Figure 1A).

In Figure 1B, Wilcoxon tests showed that the sham group, but not the sepsis group, spent a significantly higher percentage of time exploring the novel object during either short- or long-term retention test sessions in comparison with the training trial. In addition, the sepsis group presented a significant reduction in the recognition index in short- and long-term recognition retention tests compared to the sham group (Mann-Whitney U-test, $P < 0.05$). The results indicate that sepsis survivors presented an impairment of novel object recognition memory.

Elevated plus-maze

No statistically significant difference was observed in the number of entries ($P = 0.65$) or in the time spent in the arms ($P = 0.51$) between groups.

Forced swimming test

In the test session (5 min), 24 h after the pretest session (15 min), we observed a significant increase in the immobility time in the sepsis group compared to the sham group ($P < 0.05$), as shown in Figure 2.

Discussion

Previous reports involving intensive care unit survivors demonstrated cognitive impairment at discharge from the hospital (9-17). In long-term follow-up studies most patients showed improvement in overall cognitive function; however, some cognitive skills, such as memory, were not completely recovered (9,13,14). The mechanisms involved in these cognitive impairments remain unclear. In recent reports we demonstrated that CLP, a clinically relevant model of sepsis in rats, presented impairment in learning and memory. Our results were clinically relevant since 10 and 30 days after CLP the animals had fully recovered with no signs of infection or motor alterations (21,22).

We have recently reported aversive and spatial memory impairments in severe sepsis-surviving rats (21,22). The present study investigated this issue in rats trained in an object recognition task. This task, originally developed by Ennaceur and Delacour (31), is based on the tendency of rodents to explore a novel object more than a familiar one. Because no rewarding or aversive stimulation is used during training, the learning occurs under conditions of relatively low stress or arousal (31). Here we found that sepsis-surviving rats presented significant impairment of novel object recognition memory. These findings are relevant since the novel object recognition task in rodents is a nonspatial, nonaversive memory test, in contrast to our previous reports (21,22). In addition, the object recognition task has also been increasingly used as a powerful experimental tool to assess drug effects on memory and to investigate the neural mechanisms underlying learning and memory (24,25,32-35).

In the elevated plus-maze, a validated test to evaluate anxiety-like behavior (26), no differences were demonstrated between groups, indicating that sepsis survivors did not presented anxiety-like symptoms after recovery from disease.

Sepsis survivors presented depressive-like symptoms assessed in the forced swimming test. The time of immobility was significantly longer in the sepsis group. The original view of the forced swimming test offered by Porsolt (28) was that of a model of depression with features similar to those

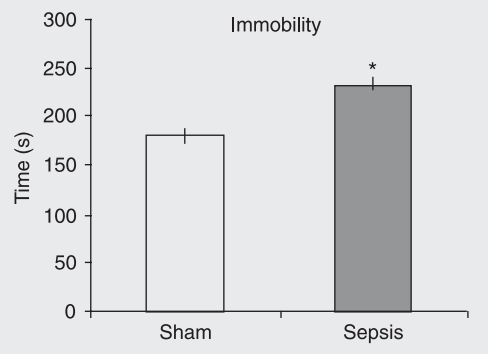


Figure 2. Forced swimming test. The sepsis group showed a significant increase in the time of immobility compared to the sham group. Data are reported as means ± SEM for N = 12 animals per group. *$P < 0.05$ compared to the sham group (Student *t*-test).

of the learned helplessness model but technically easier to produce. The internal affective state of rodents after exposure to the initial swim in the forced swimming test was labeled as 'behavioral despair'. The pretest swim induction procedure has been proposed to be similar to the initial session that induces learned helplessness by exposing rats to inescapable stress. Induction of learned helplessness produces broad-ranging behavioral deficits in affect, cognition, sleep, and motor performance that closely resemble many of the symptoms of depression (36). Additionally, as described above, the sepsis-surviving group did not present locomotor activity impairment, supporting the idea that the longer immobility time in the sepsis group was related to depressive-like symptoms. This finding agrees with clinical studies that show depressive symptoms in survivors of severe diseases such as sepsis and septic shock (9,11-13,15).

In summary, our results demonstrated that survivors of CLP, a classical sepsis model, presented recognition memory impairment and depressive-like symptoms but not anxiety-like behavior. These findings, together with our previous reports (21,22), indicate that the CLP model could be a good research tool for the study of the biological mechanisms involved in the behavioral alterations secondary to sepsis.

References

1. Brun-Buisson C. The epidemiology of the systemic inflammatory response. *Intensive Care Med* 2000; 26 (Suppl 1): S64-S74.
2. Friedman G, Silva E, Vincent JL. Has the mortality of septic shock changed with time. *Crit Care Med* 1998; 26: 2078-2086.
3. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. *N Engl J Med* 2003; 348: 138-150.
4. Tran DD, Groeneveld AB, van der Meulen J, Nauta JJ, Strack van Schijndel RJ, Thijs LG. Age, chronic disease, sepsis, organ system failure, and mortality in a medical intensive care unit. *Crit Care Med* 1990; 18: 474-479.
5. O'Brien JM Jr, Ali NA, Abraham E. Year in review in critical care, 2004: sepsis and multi-organ failure. *Crit Care* 2005; 9: 409-413.
6. Sprung CL, Peduzzi PN, Shatney CH, Schein RM, Wilson MF, Sheagren JN, et al. Impact of encephalopathy on mortality in the sepsis syndrome. The Veterans Administration Systemic Sepsis Cooperative Study Group. *Crit Care Med* 1990; 18: 801-806.
7. Young GB, Bolton CF, Austin TW, Archibald YM, Gonder J, Wells GA. The encephalopathy associated with septic illness. *Clin Invest Med* 1990; 13: 297-304.
8. Bleck TP, Smith MC, Pierre-Louis SJ, Jares JJ, Murray J, Hansen CA. Neurologic complications of critical medical illnesses. *Crit Care Med* 1993; 21: 98-103.
9. Angus DC, Musthafa AA, Clermont G, Griffin MF, Linde-Zwirble WT, Dremsizov TT, et al. Quality-adjusted survival in the first year after the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2001; 163: 1389-1394.
10. Gordon SM, Jackson JC, Ely EW, Burger C, Hopkins RO. Clinical identification of cognitive impairment in ICU survivors: insights for intensivists. *Intensive Care Med* 2004; 30: 1997-2008.
11. Granja C, Dias C, Costa-Pereira A, Sarmento A. Quality of life of survivors from severe sepsis and septic shock may be similar to that of others who survive critical illness. *Crit Care* 2004; 8: R91-R98.
12. Granja C, Lopes A, Moreira S, Dias C, Costa-Pereira A, Carneiro A. Patients' recollections of experiences in the intensive care unit may affect their quality of life. *Crit Care* 2005; 9: R96-R109.
13. Heyland DK, Hopman W, Coo H, Tranmer J, McColl MA. Long-term health-related quality of life in survivors of sepsis. Short Form 36: a valid and reliable measure of health-related quality of life. *Crit Care Med* 2000; 28: 3599-3605.
14. Hopkins RO, Weaver LK, Pope D, Orme JF, Bigler ED, Larson-Lohr V. Neuropsychological sequelae and impaired health status in survivors of severe acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1999; 160: 50-56.
15. Hopkins RO, Weaver LK, Collingridge D, Parkinson RB, Chan KJ, Orme JF Jr. Two-year cognitive, emotional, and quality-of-life outcomes in acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2005; 171: 340-347.
16. Hough CL, Curtis JR. Long-term sequelae of critical illness: memories and health-related quality of life. *Crit Care* 2005; 9: 145-146.
17. Jackson JC, Gordon SM, Ely EW, Burger C, Hopkins RO. Research issues in the evaluation of cognitive impairment in intensive care unit survivors. *Intensive Care Med* 2004; 30: 2009-2016.
18. Semmler A, Okulla T, Sastre M, Dumitrescu-Ozimek L, Heneka MT. Systemic inflammation induces apoptosis with variable vulnerability of different brain regions. *J Chem Neuroanat* 2005; 30: 144-157.
19. Ritter C, Andrades M, Frota Junior ML, Bonatto F, Pinho RA, Polydoro M, et al. Oxidative parameters and mortality in sepsis induced by cecal ligation and perforation. *Intensive Care Med* 2003; 29: 1782-1789.
20. Ritter C, Andrades ME, Reinke A, Menna-Barreto S, Moreira JC, Dal-Pizzol F. Treatment with N-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis. *Crit Care Med* 2004; 32: 342-349.
21. Barichello T, Martins MR, Reinke A, Feier G, Ritter C, Quevedo J, et al. Cognitive impairment in sepsis survivors from cecal ligation and perforation. *Crit Care Med* 2005; 33: 221-223.
22. Barichello T, Martins MR, Reinke A, Feier G, Ritter C, Quevedo J, et al. Long-term cognitive impairment in sepsis survivors. *Crit Care*

Med 2005; 33: 1671.
23. Shimizu I, Adachi N, Liu K, Lei B, Nagaro T, Arai T. Sepsis facilitates brain serotonin activity and impairs learning ability in rats. *Brain Res* 1999; 830: 94-100.
24. Rosa RM, Flores DG, Appelt HR, Braga AL, Henriques JA, Roesler R. Facilitation of long-term object recognition memory by pretraining administration of diphenyl diselenide in mice. *Neurosci Lett* 2003; 341: 217-220.
25. Schröder N, O'Dell SJ, Marshall JF. Neurotoxic methamphetamine regimen severely impairs recognition memory in rats. *Synapse* 2003; 49: 89-96.
26. Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 1985; 14: 149-167.
27. Rickels K, Schweizer EE. Current pharmacotherapy in anxiety and panic. In: Meltzer HY (Editor), *Psychopharmacology: The third generation of progress*. New York: Raven Press; 1987. p 1193-1203.
28. Porsolt RD. Animal model of depression. *Biomedicine* 1979; 30: 139-140.
29. Einat H, Clenet F, Shaldubina A, Belmaker RH, Bourin M. The antidepressant activity of inositol in the forced swim test involves 5-HT(2) receptors. *Behav Brain Res* 2001; 118: 77-83.
30. Einat H, Yuan P, Gould TD, Li J, Du J, Zhang L, et al. The role of the extracellular signal-regulated kinase signaling pathway in mood modulation. *J Neurosci* 2003; 23: 7311-7316.
31. Ennaceur A, Delacour J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behav Brain Res* 1988; 31: 47-59.
32. Baker KB, Kim JJ. Effects of stress and hippocampal NMDA receptor antagonism on recognition memory in rats. *Learn Mem* 2002; 9: 58-65.
33. Okuda S, Roozendaal B, McGaugh JL. Glucocorticoid effects on object recognition memory require training-associated emotional arousal. *Proc Natl Acad Sci U S A* 2004; 101: 853-858.
34. Rampon C, Tang YP, Goodhouse J, Shimizu E, Kyin M, Tsien JZ. Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice. *Nat Neurosci* 2000; 3: 238-244.
35. Tang YP, Shimizu E, Dube GR, Rampon C, Kerchner GA, Zhuo M, et al. Genetic enhancement of learning and memory in mice. *Nature* 1999; 401: 63-69.
36. Weiss J, Kilts C. Animal models of depression and schizophrenia. In: Nemeroff C, Schatzberg AS (Editors), *Textbook of psychopharmacology*. New York: American Psychiatric Association Press; 1998. p 88-123.

Capítulo 3.

Long-Term Cognitive Impairment in Sepsis Survivors

Tatiana Barichello, Márcio R. Martins, Adalisa Reinke, Gustavo Feier, Cristiane Ritter, João Quevedo, Felipe Dal-Pizzol

Critical Care Medicine (2005) 33:1671

Would Patients with More Subtle Signs of Coagulopathy Have Benefited from Treatment with Activated Protein C?

To the Editor:

We read with great interest the article by Dhainaut and colleagues (1) published in the February issue of *Critical Care Medicine*. In this study including 840 patients from the placebo arm of the PROWESS trial (2), the authors nicely showed that abnormalities in coagulation parameters occurring early in the course of sepsis are associated with an increased risk of organ dysfunction in the following days and with higher 28-day mortality, regardless of the severity of illness as assessed by the Acute Physiology and Chronic Health Evaluation (APACHE) II score. Moreover, the authors found a linear correlation between some coagulation parameters, such as prothrombin time and D-dimer levels, and 28-day mortality, suggesting that subtle change in these parameters, even within the normal ranges, already may identify patients who are at risk of developing subsequent organ dysfunction. Dhainaut et al. subsequently classified their patient population into three groups according to increasing severity of septic coagulopathy by using a Composite Coagulopathy Score based upon factors identified by logistic regression, with notable outcome differences between the groups. Since the authors wanted to assess the natural evolution over time between coagulation parameters within the 24 hrs of sepsis and the subsequent development of organ dysfunction in the following days, unfortunately only the placebo arm of the PROWESS trial was included in this study. It would have been interesting to assess the efficacy of activated protein C treatment across the different groups with increasing coagulopathy score. In particular, it would have been relevant to know whether patients with the more subtle signs of coagulopathy have benefited from this treatment.

Recombinant activated protein C has been approved for the treatment of sepsis in patients with two or more organ dysfunctions in Europe and an APACHE II score of more than 25 in the United States. Considering that activated protein C exerts its effect through an anticoagulopathic rather than antiinflammatory effect, septic patients with one organ failure and coagulopathy may be more likely to benefit from such a treatment than, for example, pneumonia patients with progressive renal dysfunction and hypotension (e.g., because of dehydration and/or a low cardiac output) without measurable coagulopathy. This is also supported by data coming from a subgroup analysis of the PROWESS trial (3), which revealed that activated protein C was only efficacious in the subset of patients with overt diffuse intravascular coagulopathy. Mortality in the treatment vs. placebo arm was 32% vs. 46.2% ($p < .05$) among patients with overt diffuse intravascular coagulopathy (n = 378) and 22.6% versus 26.5% ($p > .05$) in patients without it (n = 1,312). However, the definition of diffuse intravascular coagulopathy used in this study is less sensitive (4) than the Composite Coagulopathy Score identified by Dhainaut et al. (1). Should septic patients with minor or intermediately severe coagulopathy benefit from activated protein C, or should its use be restricted to patients with major coagulopathy or all patients with more than two organ failures, regardless of the severity of coagulopathy? By including the treatment arm of the PROWESS trial in their analysis, Dhainaut et al. might have been able to address this intriguing question.

Dominique D. Benoit, Pieter O. Depuydt, Jan J. De Waele, Eric A. Hoste, Kirsten E. Colpaert, Vanessa D. Van Hende, Johan M. Decruyenaere, Department of Intensive Care Medicine, Ghent University Hospital, Belgium

REFERENCES

1. Dhainaut JF, Shorr AF, Macias WL, et al: Dynamic evolution of coagulopathy in the first day of severe sepsis: Relationship with mortality and organ failure. *Crit Care Med* 2005; 33:341–347
2. Bernard GR, Vincent JL, Laterre PF, et al: Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med* 2001; 344:699–709
3. Ely EW, Laterre PF, Angus DC, et al: Drotrecogin alfa (activated) administration across clinically important subgroups of patients with severe sepsis. *Crit Care Med* 2003; 31:12–19
4. Taylor FB Jr, Toh CH, Hoots WK, et al: Scientific and standardization committee communications: Towards a definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. 2001. Available online at: http://www.med.unc.edu/isth/dicdef.htm. Accessed January 15, 2002

DOI: 10.1097/01.CCM.0000166875.23234.95

The authors reply:

We appreciate the comments of Dr. Benoit and colleagues regarding our study. Our goal in studying the placebo population of PROWESS was to examine the natural history of severe sepsis without the influence of rhAPC. Specifically, we wanted to explore the potential contributions of sepsis-induced coagulopathy on organ dysfunction and mortality.

Dr. Benoit and associates request information regarding the PROWESS groups treated with recombinant human activated protein C (rhAPC) and their mortality in relation to increasing coagulopathy score. First of all, we need to point out that performing such an analysis is not as straightforward as evaluating subgroups based solely on baseline characteristics. This difficulty arises because the Composite Coagulopathy Score utilizes three biomarkers (antithrombin, D-dimer, prothrombin time) measured at baseline and on the first day post-baseline. Therefore, a comparison of the rhAPC- versus placebo-treated patients is confounded by the use of post-baseline measures. Nonetheless, there is a trend toward lower Composite Coagulopathy Scores (Mantel-Haenszel chi-square test, $p = .10$) in rhAPC patients, indicating a trend for improving coagulopathy. This improvement was observed even though rhAPC patients tend to have an increase in prothrombin time (1), a pharmacodynamic effect of the drug. There was no significant evidence of a differential survival benefit attributable to rhAPC among the three coagulopathy classes (Breslow-Day test, $p = .70$).

Dr. Benoit and colleagues suggest that rhAPC was efficacious in PROWESS only among the patients with DIC. However, we are underpowered to make conclusions about the interaction of Xigris and baseline DIC on the basis of PROWESS. The information that they reference (2) indicates that the treatment effects of both DIC and non-DIC patients were generally consistent with the overall treatment effect, in that both of their 95% confidence intervals for the treatment effect encompass the overall PROWESS treatment effect. In addition, a recent article summarizes the findings with

Table 1. Data on PROWESS placebo versus rhAPC-treated groups in relation to mortality and increasing coagulopathy score

CCS	PLC *n* (% of PLC)	rhAPC *n* (% of rhAPC)	28-Day Mortality PLC, %	28-Day Mortality rhAPC, %	Relative Risk Reduction, %
0–1	168 (24.9)	215 (29.9)	14.3	12.6	12
2	327 (48.4)	323 (44.9)	26.9	22.6	16
3–4	180 (26.7)	181 (25.2)	45.6	35.4	22
	Mantel-Haenszel $p = .10$		Breslow-Day $p = .70$		

CCS, composite coagulopathy score; PLC, placebo; rhAPC, recombinant human activated protein C.

an improved definition of DIC for the PROWESS study (3). This publication indicates a 19% relative risk reduction with rhAPC for non-DIC patients and a 29% relative risk reduction for DIC patients. The Breslow-Day test indicates no statistical evidence that the odds ratios differ ($p = .26$). However, as with patients having higher Composite Coagulopathy Scores, the absolute mortality reduction is higher among DIC patients, because the placebo mortality rate is higher among them, whereas the relative risk reductions are similar between patients with and without DIC.

Jean-Francois Dhainaut, MD, PhD, Department of Intensive Care, Cochin Hospital, AP-HP, Cochin Institute, Cochin Port-Royal Medical School, Paris V University, Paris, France; William L. Macias, MD, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN; David R. Nelson, MS, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN

REFERENCES

1. Dhainaut JF, Yan SB, Margolis BD, et al: Drotrecogin alfa (activated) (recombinant human activated protein C) reduces host coagulopathy response in patients with severe sepsis. *Thromb Haemost* 2003; 90:642–653
2. Ely EW, Laterre PF, Angus DC, et al: Drotrecogin alfa (activated) administration across clinically important subgroups of patients with severe sepsis. *Crit Care Med* 2003; 31:12–19
3. Dhainaut J-F, Yan SB, Joyce DE, et al: Treatment effects of drotrecogin alfa (activated) in patients with severe sepsis with or without overt disseminated intravascular coagulation. *J Thromb Haemost* 2004; 2:1924–1933

DOI: 10.1097/01.CCM.0000166889.11063.07

Long-Term Cognitive Impairment in Sepsis Survivors

To the Editor:

In the January issue of *Critical Care Medicine*, Dr. Soriano wrote an editorial (1) regarding our published article (2) on cognitive deficits of survivors from cecal ligation and perforation (CLP). Dr. Soriano (1) stresses the major bias associated with our results: the time after sepsis chosen to perform the cognitive parameters. As Dr. Soriano (1) stated, "The 10 days after CLP reported in the study may not be a sufficiently long enough period for the animals to be free from the systemic septic alterations." After 10 days, the animals seemed to be free from infection. We performed blood cultures that were all negative in this period. The animals recovered their weight and grooming habits, blood counts returned to control levels, and reactive protein C values were negative. Because we believe that the described model will be an important tool in the study of these cognitive deficits, we extended the results to a later time point after CLP. Using the same model described in the article on which Dr. Soriano commented (2), we demonstrate in Figure 1*A* that rats 30 days after the CLP presented a significantly decreased step-down latency in the inhibitory avoidance task in the test session. No differences between groups were demonstrated in the inhibitory avoidance training session (data not shown). In addition, 30 days after the CLP, the continuous multiple-trials step-down inhibitory avoidance showed a significant increase in the number of training trials required to reach the acquisition criterion (50 secs on the platform) in the sepsis group compared with the sham group (Fig. 1*B*). In the retention test, as demonstrated previously (2), there was no difference between groups (data not shown). These results indicate that the memory and learning impairment demonstrated 10 days after CLP (2) persist 30 days after CLP. The persistency of the alterations 30 days after CLP reinforces that this model could be an important tool in the study of the late sequela of sepsis survivors. We hope that with these extended results our work can be a reference to the development of several studies designed to understand the factors and possible treatment options to the long-term sequela of sepsis survivors.

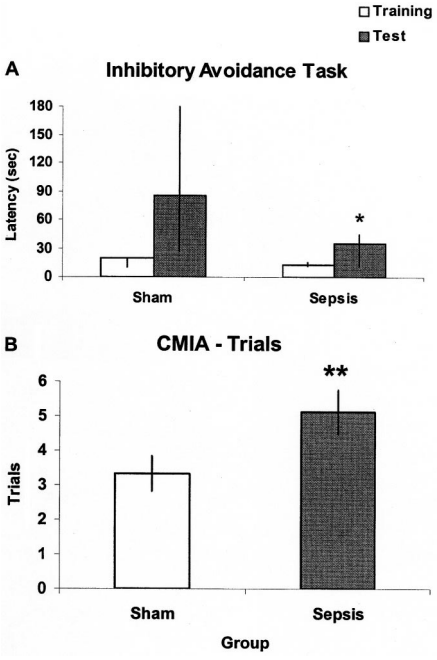


Figure 1. *A*, inhibitory avoidance task. Data are expressed as median (interquartile ranges) training and test session latencies, in seconds. *Significant difference compared with sham group (Mann-Whitney *U* test, $p < .01$). Both groups (n = 14 in the sham group and n = 13 in the sepsis group) showed significant training test differences (Wilcoxon's test, $p < .01$). *B*, continuous multiple-trials step-down inhibitory avoidance. Data are expressed as mean ± SEM of the number of training trials required to reach acquisition criterion (50 secs on the platform). *Significant difference between groups (Student's *t*-test, $p < .001$).

Tatiana Barichello, PharmD, MSc, Márcio R. Martins, BSc, Adalisa Reinke, BSc, Gustavo Feier, Bsc, Cristiane Ritter, MD, MSc, João Quevedo, MD, PhD, Felipe Dal-Pizzol, MD, PhD, Laboratório de Fisiopatologia Experimental (TB, AR, CR, FD-P) and the Laboratório de Neurociência (MRM, GF, JQ), Universidade do Extremo Sul Catarinense, Criciúma, Brazil

REFERENCES

1. Soriano FG: Quality of life: Late sequela in sepsis. *Crit Care Med* 2005; 33:262–263
2. Barichello T, Martins MR, Reinke A, et al: Cognitive impairment in sepsis survivors from cecal ligation and perforation. *Crit Care Med* 2005; 33:221–223

DOI: 10.1097/01.CCM.0000170192.54682.C1

Elevated Body Temperature in the Neuroscience Intensive Care Unit

To the Editor:

Elevated body temperature is a commonly encountered phenomenon in the neuroscience intensive care unit and is associated with worsened outcome. The treatment of these temperature elevations is frequently suboptimal; thus, I read with great interest the recent article by Mayer and colleagues (1) regarding the use of the Arctic Sun water-circulating system for treatment of fever in these patients. The authors are to be commended for recognizing and attempting to address this clinically significant and often time-consuming problem. The authors concluded that that the Arctic Sun system was found to be "superior to conventional water-circulating cooling blanket methodology for reducing fever burden in febrile neurocritical care patients."

However, these results need to be interpreted with caution. As pointed out in the accompanying commentary (2), the "conventional methodology" used as the standard for comparison was not evidence-based practice. As water-circulating cooling blankets rely on conduction for heat exchange, the increased surface area provided by two blankets provides increased contact (3) and is used in most institutions as the standard of care. In addition, the authors report that they set the SubZero water blanket temperature at the lowest possible temperature of 4°C. This is of significant concern as Caruso et al. (3) compared four different water blanket temperatures from 7 to 24°C in reducing fever and promoting patient comfort. They reported that the warmer water blanket temperatures were as effective in reducing temperature as colder temperatures and were better tolerated by the patients. Unfortunately, the use of lower water blanket temperatures to manage fever is a frequent misconception among clinicians and in patient care protocols. Due to the increased temperature gradient at lower blanket temperatures, patients are more likely to shiver (4), leading to ineffective therapy. Therefore, I must wonder if the lack of shivering reported (and also the lack of effect) of the standard therapy group could be related to use of the single overlay blanket.

The authors are to be praised for their systematic shivering protocol as this aerobic activity can have a high metabolic toll on the neurologically vulnerable patient. However, their protocol could have gone a step further as there has long been evidence supporting the use of protective wraps on the hands and feet for prevention of shivering (5), not just an intervention to manage it when using conductive cooling. In addition, although the use of meperidine for control of shivering/rigors is well established (5), its use in this patient population is of particular concern due to the need for accurate serial neurologic examinations and the increasing numbers of older adults presenting with neurologic insults. Lastly, the authors point to lack of set-point elevation due to neurogenic hyperthermia as a potential cause of the lack of shivering seen in some patients. However, the pioneering work of Ackerman and Rudy (6) previously found experimental neurogenic hyperthermia to be a highly regulated temperature response, which included thermogenesis via shivering; thus, active cooling in these patients should result in shivering.

The authors can conclude that the Arctic Sun demonstrated efficacy in reducing fever burden in the neurologically critically ill patient; however, they did not demonstrate its superiority to conventional therapy as the comparison protocol was flawed.

Hilaire J. Thompson, PhD, RN, University of Washington, Seattle, WA

REFERENCES

1. Mayer SA, Kowalski RG, Presciutti M, et al: Clinical trial of a novel surface cooling system for fever control in neurocritical care patients. *Crit Care Med* 2004; 32:2508–2515
2. Polderman KH: Keeping a cool head: How to induce and maintain hypothermia. *Crit Care Med* 2004; 32:2558–2560
3. Caruso CC, Hadley BJ, Shukla R, et al: Cooling effects and comfort of four cooling blanket temperatures in humans with fever. *Nurs Res* 1992; 41:68–72
4. Sund-Levander M, Wahren LK: Assessment and prevention of shivering in patients with severe cerebral injury: A pilot study. *J Clin Nurs* 2000; 9:55–61
5. Holtzclaw B: The febrile response in critical care: State of the science. *Heart Lung* 1992; 21:482–501
6. Ackerman D, Rudy TA: Thermoregulatory characteristics of neurogenic hyperthermia in the rat. *J Physiol* 1980; 307:59–70

DOI: 10.1097/01.CCM.0000170193.61598.89

Chlorhexidine Body Washing Plus Topical Polymyxin/tobramycin

To the Editor:

We read with interest the article published in *Critical Care Medicine* by Camus and colleagues (1) reporting the benefit of a decontamination regimen associating nasal mupirocin with chlorhexidine body washing plus topical polymyxin/tobramycin on acquired infections. However, the potential risk of both immediate and delayed hypersensitivity reactions due to chlorhexidine should be kept in mind.

The occurrence of life-threatening immediate hypersensitivity reactions has been described with chlorhexidine after topical application, intraurethral use, exposure to a disinfectant spray containing chlorhexidine, and after insertion of central catheters impregnated with chlorhexidine. In the UK during 1965–1996, the Committee on Safety of Medicines received 182 reported reactions to products containing chlorhexidine, most of these being cutaneous or mucosal eruptions (2). In Japan, between 1967 and 1989, 15 reported cases of anaphylactic shock were related to chlorhexidine, of which 13 cases followed application of the drug to mucous membranes. In 1984, the Japanese Ministry of Welfare recommended a prohibition on the use of chlorhexidine on mucous membranes. Taken together, more than 60 published case reports have confirmed the diagnosis of anaphylaxis due to chlorhexidine (3). In these reported cases, diagnosis of anaphylaxis was supported by the onset delay of the reaction (generally within a few minutes after the exposure), by the clinical symptoms observed (which were mainly severe, with hypotension, cardiovascular collapse, or circulatory arrest sometimes associated to a bronchospasm), and by the positivity of the skin tests with chlorhexidine. The severity of the reported or published cases due to the topical or intra-urethral use of chlorhexidine or with chlorhexidine-impregnated catheters prompted the United States Food and Drug Administration to issue in 1998 an alert to the medical community about the potential for serious hypersensitivity reactions to chlorhexidine. This alert stated that because of reports of anaphylaxis (including one potentially associated death), chlo-

rhexidine-impregnated medical devices had been banned in Japan (4).

Furthermore, chlorhexidine is also known to elicit contact dermatitis, occurring predominantly after prolonged or repeated application (5). A few cases have been published, and diagnosis in these cases was supported by the positivity of the skin tests with chlorhexidine.

Therefore, the prescription of chlorhexidine should include consideration of the potential threat of an anaphylactic reaction and potential event of delayed hypersensitivity reaction.

Pascale Dewachter, MD, PhD, Claudie Mouton-Faivre, MD, Paul Michel Mertes, MD, PhD, CHU Hôpital Central, Nancy, France

REFERENCES

1. Camus C, Bellissant E, Sebille V, et al: Prevention of acquired infections in intubated patients with the combination of two decontamination regimens. *Crit Care Med* 2005; 33: 307–314
2. Chisholm DG, Calder I, Peterson D, et al: Intranasal chlorhexidine resulting in anaphylactic circulatory arrest. *BMJ* 1997; 315:785
3. Dewachter P, Mouton-Faivre C, Mertes PM: Preventing complications of central venous catheterization. *N Engl J Med* 2003; 348: 2684–2686
4. FDA Public Health Notice: Potential Hypersensitivity Reactions to Chlorhexidine-Impregnated Medical Devices. March 11, 1998. US Food and Drug Administration, Center for Devices and Radiological Health, Department of Health and Human Services. Available at: http://www.fda.gov/cdrh/chlorhex.html. Accessed May 16, 2005
5. Ebo DG, Stevens WJ, Bridts CH, et al: Contact allergic dermatitis and life-threatening anaphylaxis to chlorhexidine. *J Allergy Clin Immunol* 1998; 101:128–129

DOI: 10.1097/01.CCM.0000170194.67542.1C

The authors reply:

Dr. Dewachter and colleagues emphasize the potential risk of delayed and immediate hypersensitivity reactions due to chlorhexidine, especially anaphylaxis.

No case of anaphylaxis was reported during the study (1). The toilet of patients was made by aid-nurses and consisted of normally washing, then carefully rinsing with water and wiping, the uncovered skin and genitalia. Most reported cases of chlorhexidine-associated anaphylaxis occurred after usual disinfection of wounds, burns, mucous membranes, or normal skin before surgery, which does not include rinsing. The risk of this very rare but potentially life-threatening complication must be weighed against the much greater risk of severe acquired infections, which are also life-threatening. Clearly, the overall benefit achieved by the combination of polymyxin/tobramycin and mupirocin/chlorhexidine favors the use of this procedure.

Contact sensitivity to chlorhexidine is less exceptional and is usually a mild adverse event. Irritant contact dermatitis is a common adverse reaction to chlorhexidine in medical staff. A sensitization rate of about 2% has been reported in other studies (2). This rate is consistent with the 1.9% rate of allergy reported in the 259 patients who were washed with chlorhexidine (Hibiscrub) in our study but was not different from the 1.2% rate of allergy in the 256 patients who were washed with the conventional liquid soap without chlorhexidine ($p = .72$). Body washing with the study medication was discontinued in all these cases. Chlorhexidine should not be used for body washing in patients with a history of sensitization to chlorhexidine. We agree with Dr. Dewachter and colleagues that all clinicians should keep in mind the possibility of delayed or immediate hypersensitivity reactions after skin application of chlorhexidine and remove the drug in any case of allergy with no other obvious cause.

Christophe Camus, MD, Rémi Thomas, MD, Service de Maladies Infectieuses et Réanimation Médicale, Hôpital Pontchaillou, Rennes, France

REFERENCES

1. Camus C, Bellissant E, Sébille V, et al: Prevention of acquired infections in intubated patients with the combination of two decontamination regimens. *Crit Care Med* 2005; 33: 307–314
2. Krautheim AB, Jerman TH, Bircher AJ: Chlorhexidine anaphylaxis: Case report and review of the literature. *Contact Dermatitis* 2004; 50:113–116

DOI: 10.1097/01.CCM.0000170184.33302.AC

Simpson's Paradox

To the Editor:

The data reported by Topeli and colleagues (1) in the February 2005 issue of *Critical Care Medicine* provides a classic example of the statistical phenomenon known as Simpson's paradox. In their study, the institution of a closed intensive care unit system, run by an intensivist, decreased hospital mortality for intubated patients and likewise decreased hospital mortality for nonintubated patients. When these samples are combined, however, the remarkable result is that the outcome is reversed, with an apparent *increase* in mortality with the closed intensive care unit system (Table 1, adapted from original article). The explanation of this apparent paradox lies in examination of the unequal sample sizes within the subgroups.

In the open period, aggregate mortality is weighted toward the large sample of patients not receiving mechanical ventilation, a group with lower expected mortality to begin with. In contrast, during the late closed period, aggregate mortality is weighted toward the large sample of patients treated with mechanical ventilation, a group with higher expected mortality. These unequal sample sizes result in aggregate data essentially comparing mortality between the mechanically ventilated patients and the nonmechanically ventilated patients. Thus, although each subgroup experiences improved mortality, data aggregation obscures this trend and, in fact, completely reverses it, with a highly significant $p < .001$ comparing the open period with the late closed period.

Table 1. Combined results showing an apparent increase in mortality with the closed intensive care unit system

	Open Period	Early Closed Period	Late Closed Period	Conclusion
Hospital mortality if mechanically ventilated (%)	19/21 (90.5)	38/67 (56.7)	79/134 (59.0)	Closed better
Hospital mortality if not mechanically ventilated (%)	32/179 (17.9)	7/82 (8.5)	10/76 (13.2)	Closed better
Combined total (%)	51/200 (25.5)	45/149 (30.2)	89/210 (42.4)	Closed worse

Summary table adapted from Topeli et al (1).

This statistical phenomenon, although recognized for many years, was brought to widespread attention by E. H. Simpson in 1951 (2) and is thus popularly referred to as Simpson's paradox. It occurs in the setting of unequal group sizes and an unaccounted (or lurking) variable in the aggregate data—in this case, intubation status. Once the authors identified this important variable and analyzed their data by subgroups, they arrived at the correct conclusion—implementation of an intensivist-run, closed intensive care unit system improved patient survival in this study—and thus avoided falling victim to Simpson's paradox.

Ken Kunisaki, MD, University of Minnesota, Minneapolis, MN

REFERENCES

1. Topeli A, Laghi F, Tobin MJ: Effect of closed unit policy and appointing an intensivist in a developing country. *Crit Care Med* 2005; 33: 299–306
2. Simpson EH: The interpretation of interaction in contingency tables. *J R Stat Soc B* 1951; 13:238–241

DOI: 10.1097/01.CCM.0000170197.42215.F6

The authors reply:

We thank Dr. Kunisaki for taking the time to respond to our article (1). He has taken into consideration an important, yet often overlooked, methodologic issue and has comprehensively discussed its potential effects on study results.

In our study, the association between the intensive care unit system (open or closed) and intensive care unit mortality was found to be different among subgroups when we stratified for use of mechanical ventilation. Unequal proportions for use of mechanical ventilation in closed and open systems led to different mortality rates in the overall populations. We agree that data aggregation obscures the "real" association between intensive care unit system and mortality. It even reverses it, and it leads to a paradox when ventilator use is overlooked.

The mixing of the effect of an extraneous variable with the effects of exposure (or disease) of interest is termed confounding (2). Confounding is not an all-or-none property of an extraneous variable, and it may occur to varying degrees in different studies. Sometimes, it is possible that the confounding variable changes the direction of an association. This situation is termed Simpson's paradox (3). As expressed by Rothman (4), Simpson's paradox is not really a paradox but the logical consequence of failing to recognize the presence of confounding variables. A confounding variable, if expected before a study, can be considered in designing a study and controlled for by either a) restricting the sample to limited levels of the potential confounder or b) by matching the confounder variable (as in a matched case-control study). In a situation in which the confounder is detected in analysis, as was the case in our study, confounding can be controlled for by a) stratification (Table 3 of our original study (1): the association between the system and mortality was studied for different subgroups of mechanical ventilation) or b) multivariate analysis techniques (Table 2 of our original study (1): mechanical ventilation was included in the system, and controlled for as a potential confounder), in order to obtain valid conclusions (4, 5).

As pointed out by Dr. Kunisaki, use of stratified analysis in evaluating the effect of the intensive care unit system on mortality prevented our study from falling victim to Simpson's paradox.

Arzu Topeli, Medical Intensive Care Unit, Hacettepe University Faculty of Medicine, Ankara, Turkey; Franco Laghi, Division of Pulmonary and Critical Care Medicine, Edward Hines Jr. Veterans Administration Hospital, and Loyola University of Chicago Stritch School of Medicine, Hines, IL; Banu Cakir, MD, PhD, Department of Public Health, Hacettepe University Faculty of Medicine, Ankara, Turkey; Martin J. Tobin, Division of Pulmonary and Critical Care Medicine, Edward Hines Jr. Veterans Administration Hospital, and Loyola University of Chicago Stritch School of Medicine, Hines, IL

REFERENCES

1. Topeli A, Laghi F, Tobin MJ: Effect of closed unit policy and appointing an intensivist in a developing country. *Crit Care Med* 2005; 33: 299–306
2. Greenberg RS, Daniels SR, Flanders WD, et al: Medical Epidemiology. Third Edition. Stamford, CT, Lange Medical Books, McGraw-Hill, 2001, pp 149–150
3. Last J (Ed): A Dictionary of Epidemiology. Fourth Edition. New York, Oxford University Press, 2001, p 168
4. Rothman KJ: A pictorial presentation of confounding in epidemiologic studies. *J Chronic Dis* 1975; 28:101–108
5. Rothman KJ, Greenland S: Modern Epidemiology. Second Edition. Philadelphia, Lippincott-Raven Publishers, 1998, pp 253–255

DOI: 10.1097/01.CCM.0000170185.05101.BE

Critical Illness Polyneuropathy

To the Editor:

We read with interest the article by Garnacho-Montero and colleagues (1) that added significantly to the understanding of critical illness polyneuropathy (CIP). This study found that patients with CIP (diagnosed by neurophysiologic studies) spent more time on the ventilator in the liberation phase—the time after recovery of the acute insult leading to respiratory failure. Because management of mechanical ventilation is a complex process, we believe that several questions remain to properly interpret the conclusions of the study. It is unclear what practices were in place in the primary ICU to evaluate patients for ventilator liberation. Although the general criteria provided for being eligible for ventilator liberation are consistent with consensus guidelines (2), it is unclear how uniformly these were applied in practice. For example, those with CIP reached candidacy for "weaning" 5 days later than those without CIP. This might be due to differences in the severity of illness between the groups, a delay in recovery due to CIP, or differences in management strategies. It is unclear if the clinicians making ventilator management decisions remained blinded to the results of the neurodiagnostic testing. Being alert to the presence of CIP could alter the aggressiveness of ventilator management and discontinuation by practitioners because of a perceived risk of failed ventilator liberation. It might also prompt earlier or more frequent consideration of tracheostomy. We also wonder if ICU readmission rates were different between patients with CIP and those without. More than half of hospital deaths occurred outside of the ICU and were more common in the CIP patients. If ICU readmission rates were similar between the groups, then the increased mortality of CIP patients in the post-ICU period might reflect a bias toward less aggressive care (e.g., less likely to readmit to the ICU) in these patients.

In addition to questions about processes of care, we wonder about the relative utility of routine neurophysiologic studies compared with a neuro-

muscular physical examination. Detection of subclinical neurologic dysfunction may explain why Garnacho-Montero and colleagues (1) reported a prevalence of CIP (53%) twice that of ICU-acquired weakness (25%) observed by De Jonghe et al. (3) when using bedside neuromuscular exam as the clinical discriminator. Some studies suggest that ICU-acquired weakness is often a combined pathology involving both muscle and nerve (4). If a thorough physical examination provides data as useful as the more complex testing employed by Garnacho-Montero and colleagues (1), it would allow clinicians to be attentive to ICU-acquired weakness without requiring routine neurophysiologic testing in all patients with sepsis.

In conclusion, we feel this study raises important questions about the natural history of ICU-acquired weakness that deserve further clarification. Advancing our understanding of the long-term consequences of sepsis is critical for us to properly plan for patients' return to their home and workplaces.

Naeem A. Ali, MD, James O'Brien, MD, Stephen Hoffmann, MD, The Ohio State University Medical Center, Columbus, OH

REFERENCES

1. Garnacho-Montero J, Amaya-Villar R, Garcia-Garmendia JL, et al: Effect of critical illness polyneuropathy on the withdrawal from mechanical ventilation and the length of stay in septic patients. *Crit Care Med* 2005; 33:349–354
2. MacIntyre NR, Cook DJ, Ely EW Jr, et al: Evidence-based guidelines for weaning and discontinuing ventilatory support: a collective task force facilitated by the American College of Chest Physicians; the American Association for Respiratory Care; and the American College of Critical Care Medicine. *Chest* 2001; 120(6 Suppl):375S–395S
3. De Jonghe B, Sharshar T, Lefaucheur JP, et al: Paresis acquired in the intensive care unit: A prospective multicenter study. Groupe de Reflexion et d'Etude des Neuromyopathies en Reanimation. *JAMA* 2002; 288:2859–2867
4. Bednarik J, Lukas Z, Vondracek P: Critical illness polyneuromyopathy: The electrophysiological components of a complex entity. *Intensive Care Med* 2003; 29:1505–1514

DOI: 10.1097/01.CCM.0000170196.25242.E6

The authors reply:

We appreciate the interesting comments expressed by Dr. Ali and colleagues regarding our recent contribution to *Critical Care Medicine*. The main conclusion of our study is that critical illness polyneuropathy (CIP) significantly lengthens the duration of mechanical ventilation and is an independent risk factor for weaning failure in a cohort of critically ill septic patients (1). This finding is in agreement with another recent publication that reported similar results in a heterogeneous cohort of mechanically ventilated patients (2).

Apart from the presence of CIP, prolonged mechanical ventilation might be due to other factors, such as the severity of illness, the presence of co-morbidities, or the total doses of sedatives administered to the patients. These variables were analyzed in our study, and we found no differences between patients with CIP and without CIP or between patients with and without weaning failure according to our reported definition. It is worth noting that these variables were not recorded in clinical trials comparing different weaning modes, adding an extraordinary value to our results. Obviously, prolonged mechanical ventilation can be explained by the fact that the physicians in charge of the patients were not blinded to the results of the neurophysiologic evaluation (the investigator who performed all these evaluations was unaware of the patient's medical condition). Nevertheless, daily, the attending physicians assessed patient readiness for liberation from mechanical ventilation following an updated protocol as it is done in clinical trials evaluating different weaning approaches (3, 4).

It is true that more than half of the deaths occurred after discharge from the intensive care unit. This may be explained by the fact that these patients are usually elderly patients with severe weakness, which makes them extremely vulnerable in the post–intensive care unit period (5). Interestingly, there is a lack of information available in the medical literature about the evolution of patients with CIP after being discharged from the intensive care unit.

Finally, a very high rate of CIP has been reported in adults with sepsis and multiple organ dysfunction syndrome. Witt et al. (6) carried out the first prospective study in a cohort of 43 patients with sepsis and multiple organ dysfunction syndrome, and 70% of these patients were diagnosed with CIP. Subsequent prospective studies have reported a wide prevalence (0–85%), depending on the group of critically ill patients evaluated, the timing of the electrophysiologic investigation, and the definitions used for identifying neuropathy (7). Very recently, clinically relevant paresis was found in 60% of the patients in the recovery of an episode of acute respiratory distress syndrome. The neurophysiologic evaluation was consistent with CIP in all except two of these patients (8).

To summarize, there is compelling evidence that CIP can influence the management and course of critically ill patients. In fact, mechanical ventilation is prolonged by the development of this neurologic complication. Because of the harmful consequences that this may cause, further studies are warranted to assess various interventions (different weaning strategies, early tracheostomy, early use of noninvasive after extubation) that could help to improve patient outcome.

Jose Garnacho-Montero, MD, PhD, Rosario Amaya-Villar, MD, Carlos Ortiz-Leyba, MD, PhD, Intensive Care Unit, Hospital Universitario Virgen del Rocío, Sevilla, Spain

REFERENCES

1. Garnacho-Montero J, Amaya-Villar R, García-Garmendía JL, et al: Effect of critical illness polyneuropathy on the withdrawal from mechanical ventilation and the length of stay in septic patients. *Crit Care Med* 2005; 33:349–354
2. De Jonghr B, Bastuji-Garin S, Sharshar T, et al: Does ICU-acquired paresis lengthen weaning from mechanical ventilation. *Intensive Care Med* 2004; 30:1117–1121
3. Esteban A, Frutos F, Tobin MJ, et al: A comparison of four methods of weaning patients from mechanical ventilation. *N Engl J Med* 1995; 332:345–350
4. Esteban A, Alia I, Tobin MJ, et al: Effect of spontaneous breathing trial duration on outcome of attempts to discontinue mechanical ventilation. *Am J Respir Crit Care Med* 1999; 159:512–518
5. Garnacho-Montero J, Madrazo-Osuna J, García Garmendia JL, et al: Critical illness polyneuropathy: Risk factors and clinical consequences. A cohort study in septic patients. *Intensive Care Med* 2001; 27:1288–1296
6. Witt NJ, Zochodne DW, Bolton CF, et al: Peripheral nerve function in sepsis and multiple organ failure. *Chest* 1991; 99:176–184
7. Garnacho-Montero J, Madrazo-Osuna J, García Garmendia JL, et al: Neuromuscular disorders of the critically ill patient. *Clin Pulm Med* 2001; 8:354–359
8. Bercker S, Weber-Carstens S, Deja M, et al: Critical illness polyneuropathy and myopathy in patients with acute respiratory syndrome. *Crit Care Med* 2005; 33:711–715

DOI: 10.1097/01.CCM.0000170407.44717.89

Mixing Up Old Data

To the Editor:

In their secondary analysis of an international study of mechanical ventilation practices, Ferguson and colleagues (1) demonstrated considerable interpatient variability in tidal volumes. Indeed, mean tidal volume was 8.8 mL/kg measured body weight with an SD of 2.2 mL/kg measured body weight. Several comments can and must be made regarding the usefulness and adequacy of this new analysis of old data.

First, it is questionable whether all practitioners were aware of the role of tidal volume settings in ventilator-induced lung injury at the moment of collecting data. Indeed, almost all studies on lower tidal volumes have been published at or directly after that time (2–5). It would be far more interesting to see how tidal volumes are set nowadays, several years after the landmark study by the Acute Respiratory Distress Syndrome Network (6). Several studies (7–9) show that tidal volumes are presently still too large.

Second, concerns arise on the absence of data on height of the studied subjects, making it only possible to express tidal volumes in milliliters per kilogram of actual body weight. Although actual body weight exceeded predicted body weight by approximately 20% in one study (6), it is not correct to simply state that "mean tidal volume of 8.8 mL/kg actual body weight might be equivalent to 10–11 mL/kg predicted body weight" in the present analysis because weight was not normally distributed among the several groups. Indeed, significantly higher weight values were registered in patients mechanically ventilated with lower tidal volumes, whereas weight values were significantly lower in those mechanically ventilated with higher tidal volumes. Did actual body weight exceed predicted body weight evenly in all groups?

This also raises a third concern. Were the heavier patients just taller, or did they have a higher body mass index? High body mass index is associated with mortality (10). Interestingly, from the present analysis, one might also conclude that patients with a higher risk of death (those that were heavier) were mechanically ventilated with a more lung-protective strategy than patients with a lower risk of death.

Thus, although the investigators were able to examine patterns of tidal volume use, it is hard to draw firm conclusions because important information (height) is missing.

Marcus J. Schultz, MD, PhD, Esther K. Wolthuis, MD, MSc, Rogier M. Determann, MD, MSc, Department of Intensive Care Medicine, Academic Medical Center, Amsterdam, The Netherlands

REFERENCES

1. Ferguson ND, Frutos-Vivar F, Esteban A, et al: Airway pressures, tidal volumes, and mortality in patients with acute respiratory distress syndrome. *Crit Care Med* 2005; 33: 21–30
2. Brochard L, Roudot-Thoraval F, Roupie E, et al: Tidal volume reduction for prevention of ventilator-induced lung injury in acute respiratory distress syndrome: The Multicenter Trial Group on Tidal Volume reduction in ARDS. *Am J Respir Crit Care Med* 1998; 158:1831–1838
3. Stewart TE, Meade MO, Cook DJ, et al: Evaluation of a ventilation strategy to prevent barotrauma in patients at high risk for acute respiratory distress syndrome: Pressure- and Volume-Limited Ventilation Strategy Group. *N Engl J Med* 1998; 338:355–361
4. Amato MB, Barbas CS, Medeiros DM, et al: Effect of a protective-ventilation strategy on mortality in the acute respiratory distress syndrome. *N Engl J Med* 1998; 338:347–354
5. Brower RG, Shanholtz CB, Fessler HE, et al: Prospective, randomized, controlled clinical trial comparing traditional versus reduced tidal volume ventilation in acute respiratory distress syndrome patients. *Crit Care Med* 1999; 27:1492–1498
6. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome: The Acute Respiratory Distress Syndrome Network. *N Engl J Med* 2000; 342: 1301–1308
7. Weinert CR, Gross CR, Marinelli WA: Impact of randomized trial results on acute lung injury ventilator therapy in teaching hospitals. *Am J Respir Crit Care Med* 2003; 167: 1304–1309
8. Young MP, Manning HL, Wilson DL, et al: Ventilation of patients with acute lung injury and acute respiratory distress syndrome: Has new evidence changed clinical practice? *Crit Care Med* 2004; 32:1260–1265
9. Wolthuis EK, Korevaar JC, Spronk P, et al: Feedback and education improve physician compliance in use of lung-protective mechanical ventilation. *Intensive Care Med* 2005; 31:540–546
10. Goulenok C, Monchi M, Chiche JD, et al: Influence of overweight on ICU mortality: A Prospective study. *Chest* 2004; 125:1441–1445

DOI: 10.1097/01.CCM.0000170198.14613.F4

The authors reply:

We thank Dr. Schultz and colleagues for their interest in our article that examined ventilation practices and outcome associations in patients with acute respiratory distress syndrome (1). They point out that most of the clinical studies of lung-protective ventilatory strategies were published at or after the time the data for this report were collected. We agree wholeheartedly that determining whether there has been a temporal change in tidal volume prescriptions during the intervening 7-yr period is an interesting and important future research question. Our data from the current publication may serve as a reference point against which to judge this potential change.

We also agree that it is difficult to draw firm conclusions about the effect of different tidal volumes without the ability to standardize these for height. This is why we instead focused on the observed variability in tidal volume. We would point out that when we stated that 8.8 mL/kg actual body weight might be equivalent to 10–11 mL/kg predicted body weight, we qualified that this extrapolation should be done cautiously. In addition, we were referring to the global mean tidal volume for all patients, in which the distribution was approximately normal (Fig. 1 in the original article), not to individual tidal volume groups as Dr. Schultz and colleagues imply.

Finally, in the absence of height data, any statements regarding the influence of body mass index are purely speculative. Now that height-based predicted body weights have become widely used (which was not the case in 1998), this will be an important variable to consider in future studies.

Again, we thank Dr. Schultz and colleagues for the opportunity to clarify the issues above and to reassert the conclusions we drew from this work: 1) tidal volumes varied considerably during the study period, 2) late-onset acute respiratory distress syndrome and low levels of positive end-expiratory pressure were independently associated with increased mortality, and 3) no evidence of increased mortality was observed at lower inspiratory pressures.

Niall D. Ferguson, MD, FRCPC, MSc, Thomas E. Stewart, MD, FRCPC, Interdepartmental Division of Critical Care Medicine, University Health Net-

work and Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada; Fernando Frutos Vivar, MD, Andrés Esteban, MD, PhD, Unidad de Cuidados Intensivos, Hospital Universitario de Getafe, Madrid, Spain

REFERENCES

1. Ferguson ND, Frutos Vivar F, Esteban A, et al: Airway pressures, tidal volumes, and mortality in patients with the acute respiratory distress syndrome. *Crit Care Med* 2005; 33:21–30

DOI: 10.1097/01.CCM.0000170945.82570.FF

Clinical Relevance of the LRINEC (Laboratory Risk Indicator for Necrotizing Fasciitis) Score for Assessment of Early Necrotizing Fasciitis

To the Editor:

Early diagnosis of necrotizing fasciitis with subsequent operative debridement has been shown in many studies to improve survival (1, 2). However, delayed diagnosis is frequently seen because early in the evolution of this disease, it is often clinically indistinguishable from other more benign soft-tissue infections such as cellulitis. We developed the Laboratory Risk Indicator for Necrotizing Fasciitis (LRINEC) score (3) with the hope that routine laboratory parameters for the assessment of severe soft-tissue infection may yield diagnostic clues of the presence of necrotizing soft-tissue infection, even early in the evolution of the disease when clinical findings were nondiagnostic.

The LRINEC score was developed retrospectively by comparing the laboratory parameters (complete blood count, electrolytes, erythrocyte sedimentation rate, and C-reactive protein) of patients with proven necrotizing fasciitis vs. patients with severe soft-tissue infections. On validation with our own data, at a cutoff of a LRINEC score of ≥ 6, the score has a positive predictive value of 92.0% and negative predictive value of 96.0% (3). One weakness of this study we acknowledge is its retrospective nature that predisposes the study to selection bias (4). Prospective validation of the score is needed before routine application can be recommended.

Wang and Hung (5) recently performed a prospective study using tissue saturation monitoring for diagnosing necrotizing fasciitis. A total of 234 consecutive patients who fulfilled the United States Centers for Disease Control and Prevention criteria of soft-tissue infections were enrolled into the study. Of these 234 patients, 19 were later confirmed to have necrotizing fasciitis and 215 patients had cellulitis. Routine parameters for evaluation of soft-tissue infection were taken at admission. In this study, the majority of cases were early necrotizing fasciitis. Early diagnosis was possible because of a rigorous protocol that combined tissue oxygen saturation monitoring, computed tomographic scanning, and tissue biopsy. As a secondary analysis, Dr. Wang (6) used the LRINEC score in his study subjects and found a positive predictive value of 40% and a negative predictive value of 95%.

The performance of the LRINEC score in this prospective model shows a high specificity but a low sensitivity. The implications are two-fold. First, the majority Dr. Wang's patients had early necrotizing fasciitis. A negative predictive value of 95% (a low false-negative rate) shows that the LRINEC score will not miss these cases (early necrotizing fasciitis). Second, from a clinical standpoint, specificity is more important than sensitivity. To this end, the LRINEC score seems to fulfil the purpose for which it was originally devised. When the score is <6, necrotizing fasciitis is quite unlikely. From Dr. Wang's data of a positive predictive value of 40%, a high false-positive rate is expected for early necrotizing fasciitis. For patients classified as high risk based on the LRINEC score, urgent further evaluation is needed. The use of additional tools, such as computed tomography, magnetic resonance imaging, tissue biopsy, or tissue oxygen monitoring can exclude or confirm necrotizing fasciitis. The LRINEC score therefore functions as tool to limit and more importantly target the use of these expensive (but more sensitive) modalities to such high-risk patients. We hope that early diagnosis and reduction in mortality is possible with such an approach.

Chin-Ho Wong, MD, MRCS(Ed), Department of Plastic, Reconstructive and Aesthetic Surgery, Singapore General Hospital, Singapore; Lay-Wai Khin,MD, MSC, Clinical Trials and Epidemiology Research Unit, National Medical Research Council (NMRC), Ministry of Health, Singapore

REFERENCES

1. Wong CH, Chang HC, Pasupathy S, et al: Necrotizing fasciitis: Clinical presentation, microbiology and determinants of mortality. *J Bone Joint Surg* (*Am*) 2003; 85-A:1454–1460
2. McHenry CR, Piotrowski JJ, Petrinic D, et al: Determinants of mortality in necrotizing soft tissue infections. *Ann Surg* 1995; 221: 558–563
3. Wong CH, Khin LW, Heng KS, et al: The LRINEC (Laboratory Risk Indicator for Necrotizing Fasciitis) score: A tool for distinguishing soft tissue infection from other soft tissue infections. *Crit Care Med* 2004; 32:1535–1541
4. Barie PS: The laboratory risk indicator for necrotizing fasciitis (LRINEC) score: Useful tool or paralysis by analysis? *Crit Care Med* 2004; 32:1618–1619
5. Wang TL, Hung CR: Role of tissue oxygen saturation monitoring in diagnosing necrotizing fasciitis of the lower limbs. *Ann Emerg Med* 2004; 44:222–228
6. Wang TL: Tissue oxygen saturation monitoring in the diagnosing necrotizing fasciitis: A valuable tool but only for a select few. Reply. *Ann Emerg Med* 2005; 45:462–463

DOI: 10.1097/01.CCM.0000170199.43624.B8

Capítulo 4.

Oxidative variables in the rat brain after sepsis induced by cecal ligation and perforation

Tatiana Barichello, Jucélia J. Fortunato, Ângeles M. Vitali, Gustavo Feier, Adalisa Reinke, José Cláudio F. Moreira, João Quevedo, Felipe Dal-Pizzol

Critical Care Med (2006) 34:886-889

Oxidative variables in the rat brain after sepsis induced by cecal ligation and perforation

Tatiana Barichello, PharmD, MSc; Jucélia J. Fortunato, MSc; Ângeles M. Vitali, BSc; Gustavo Feier, BSc; Adalisa Reinke, BSc; José Cláudio F. Moreira, PhD; João Quevedo, MD, PhD; Felipe Dal-Pizzol, MD, PhD

Objective: **The underlying mechanisms of the changes in mental status, septic encephalopathy, and long-term cognitive symptoms in sepsis survivors have only been defined in part. The present study was undertaken to assess different variables of oxidative stress in several brain structures after cecal ligation and perforation in the rat.**

Design: **Prospective animal study.**

Setting: **Animal basic science laboratory.**

Subjects: **Male Wistar rats, weighing 250–350 g.**

Interventions: **Rats were subjected to cecal ligation and perforation (sepsis group) with saline resuscitation (at 50 mL/kg immediately and 12 hrs after cecal ligation and perforation) or sham operation (control group).**

Measurements and Main Results: **Oxidative damage, assessed by the thiobarbituric acid reactive species and the protein carbonyl assays, occurred early (after 6 hrs) in the course of sepsis development in the hippocampus, cerebellum, and cortex. At longer times after sepsis induction (12–96 hrs), there was no evidence of oxidative damage in all analyzed structures. Except for the striatum, earlier in sepsis development (6 hrs) we demonstrated an increase in superoxide dismutase activity without a proportional increase in catalase activity with a consequent increase in the relation of superoxide dismutase/catalase. The balance between these enzymes was restored in the studied structures 12–96 hrs after sepsis induction.**

Conclusions: **The short-term oxidative damage demonstrated here could participate in the development of central nervous system symptoms during sepsis development, or even septic encephalopathy. The alterations in the superoxide dismutase/catalase relation were temporally related to the occurrence or not of oxidative damage in the central nervous system. (Crit Care Med 2006; 34:886–889)**

KEY WORDS: septic shock; oxidative stress; free radicals; rat brain; septic encephalopathy

Sepsis and septic shock have become some of the most frequent causes of morbidity and mortality in intensive care units. Neurologic abnormalities during sepsis development include agitation, confusion, disorientation, lethargy, and coma, which are early characteristic findings in these patients (1). The concept of septic encephalopathy as an entity that cannot be explained by hepatic or renal dysfunction, hypotension, or hypoxia is relatively new and has been reported to occur in a range of 8–70% of septic patients, usually associated with a poor outcome (1). In addition, sepsis survivors present long-term cognitive deficits that could be secondary to central nervous system alterations during sepsis development (2). The underlying mechanisms of the changes in mental status, septic encephalopathy, and long-term cognitive symptoms in sepsis survivors have only been defined in part (1).

The systemic inflammation resulting from infection or other causes appears to be the cause of septic encephalopathy (1). Inflammatory mediators released by leukocytes (such as tumor necrosis factor and reactive oxygen species) in sepsis have profound effects on endothelial cells, astrocytes, and neurons; damage to these cells results in impaired central nervous system function (1).

Studies of sepsis in humans are difficult because the seriousness of the disease mandates immediate intervention and because the heterogeneity of patients' presentations imposes substantial limitations on clinical trials. Thus, animal models have been used extensively to explore the pathogenesis of sepsis and to generate preclinical data for therapeutic interventions. In this way we recently demonstrated that oxidative stress might have a major role in the development of sepsis and severe hepatic failure (3, 4). In addition, we demonstrated that oxidative damage in the rat brain might be relevant to the outcome after hepatic failure in an animal model (4). To the best of our knowledge there is no report that describes oxidative variables in different brain regions after sepsis induction.

Thus, the aim of this study was to investigate the temporal variation of different oxidative variables in several brain structures after sepsis induction in rats.

MATERIALS AND METHODS

In vivo studies were performed in accordance with National Institutes of Health guidelines and approved by the Ethics Committee of Universidade do Extremo Sul Catarinense.

Cecal Ligation Puncture (CLP) Model. Male Wistar rats 2–3 months old, weighing 250–350 g, were subjected to CLP (14-gauge

From Laboratório de Neurociências (TB, JJF, AMV, GF, JQ) and Laboratório de Fisiopatologia Experimental (AR, JCFM, FD-P), Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil; and Centro de Estudos em Estresse Oxidativo (JCFM), Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

Supported, in part, by grants from UNESC, CNPq, and FUNCITEC.

Address requests for reprints to: Felipe Dal Pizzol, MD, PhD, Laboratório de Fisiopatologia Experimental, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil, Avenida Universitária, 1105, 88006-000. E-mail: piz@unesc.net

DOI: 10.1097/01.CCM.0000201880.50116.12

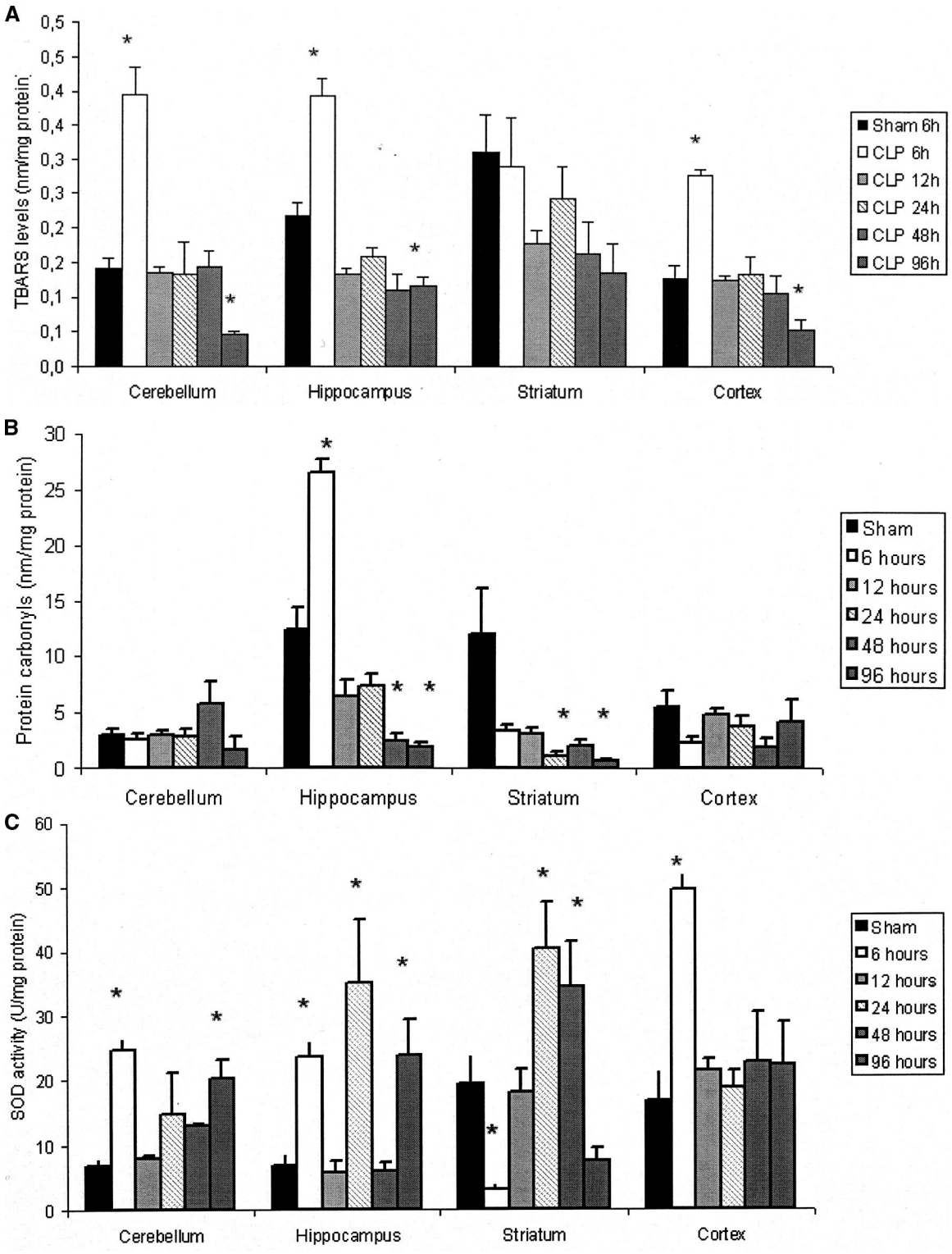


Figure 1. Oxidative variables in the rat brain during sepsis development. Rats were submitted to cecal ligation and perforation (*CLP*) (14-gauge puncture) or sham operation. Animals were killed, and brain structures (hippocampus, striatum, cortex, and cerebellum) were isolated 0 (immediately after), 6, 12, 24, 48, and 96 hrs after CLP to determine (*A*) thiobarbituric acid reactive species (*TBARS*), (*B*) protein carbonyls, (*C*) superoxide dismutase (*SOD*) activity, and (*D*) catalase (*CAT*) activity as described in "Material and Methods." Values are expressed as mean ± SD (n = 5 for each group). Oxidative variables were not statistically different between the sham and 0-hr group (data not shown). Oxidative variables were not statistically different between sham groups when comparing all time point analyzed; thus we presented in the graphs only one sham group. *Different from sham operated ($p < .05$).

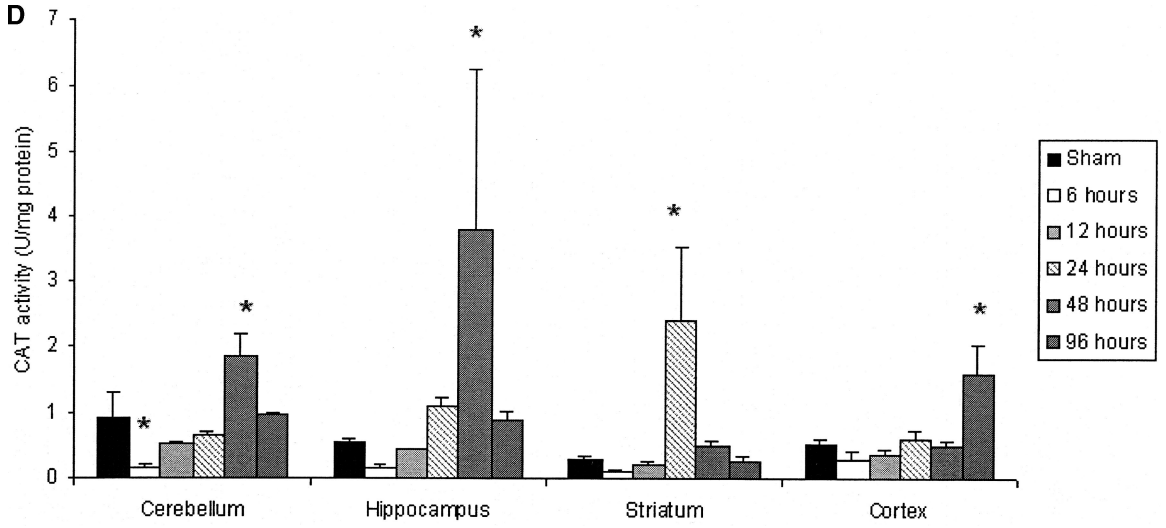


Figure 1. Continued.

needle) as previously described (3). A sham operation (laparotomy and cecal exposure without any more manipulation) was performed as control. The sham and CLP groups were allocated randomly during the procedure. To avoid hypotension, animals were resuscitated immediately after CLP with normal saline (50 mL/kg of body weight subcutaneously, bolus injection every 12 hrs in the first day) (3). Several times (0, 6, 12, 24, 48, and 96 hrs) after CLP five rats were killed by decapitation, and brain structures (cerebellum, hippocampus, striatum, and cortex) were immediately isolated and stored at −80°C for posterior analyses. Sham animals (n = 5) were killed at the same times after operation. Fourteen animals died during the 96 hrs and were excluded from the oxidative variable analyses. All animals presented signs of encephalopathy at 6 hrs after sepsis (lethargy, mild ataxia, lack of spontaneous movement, loss of righting reflex). Animals gradually returned to their normal awake status 24–36 hrs after CLP, but some remained lethargic up to 96 hrs.

Measurement of Thiobarbituric Acid Reactive Species (TBARS). As an index of lipid peroxidation, we used the formation of TBARS during an acid-heating reaction as previously described (3). Briefly, the samples were mixed with 1 mL of trichloroacetic acid 10% and 1 mL of thiobarbituric acid 0.67% and then heated in a boiling water bath for 15 mins. TBARS were determined by the absorbance at 535 nm.

Measurement of Protein Carbonyls. The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhidrazine as previously described (4). Briefly, proteins were precipitated by the addition of 20% trichloroacetic acid and redissolved in dinitrophenylhidrazine, and the absorbance was read at 370 nm.

Table 1. Superoxide dismutase/catalase relation in the rat brain during sepsis development

	Cerebellum	Hippocampus	Striatum	Cortex
Sham	7.50 + 1.2	12.39 + 2.3	62.94 + 5.4	31.62 + 3.8
6 hrs	147.63 + 17[a]	133.18 + 23[a]	24.28 + 4.7	172.42 + 28[a]
12 hrs	14.88 + 1.8	13.60 + 2.3	83.71 + 6.9	56.87 + 9.8
24 hrs	23.09 + 3.9	31.82 + 3.8	16.94 + 1.3[a]	30.65 + 4.5
48 hrs	6.97 + 2.1	1.57 + 0.12[a]	69.75 + 6.3	44.47 + 5.7
96 hrs	21.19 + 6.7	26.47 + 4.5	28.22 + 4.8	13.99 + 4.6

[a]Different from sham ($p < .05$).

Measurement of Catalase (CAT) and Superoxide Dismutase (SOD) Activity. To determine CAT activity, samples were sonicated in 50 mM phosphate buffer, and the resulting suspension was centrifuged at 3000 × *g* for 10 mins. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm (3). SOD activity was assayed by measuring the inhibition of adrenaline autooxidation, as previously described (3).

Protein Measurements. All the results were normalized by protein concentration measured by the Lowry assay.

Reagents. Thiobarbituric acid, catalase, superoxide dismutase, dinitrophenylhidrazine, adrenaline, hydrogen peroxide, luminal, and succinate were purchased from Sigma Chemical (St. Louis, MO). We purchased 2,2'-azobis (2-28 methylpropionamidine) dihydrochloride from Aldrich Chemical (Milwaukee, WI).

Statistical Analysis. All data are presented as mean ± SD. Data were analyzed by two-way analysis of variance, and multiple comparisons were performed by Newman-Keuls' test. To determine whether data were normally distributed, we performed Bartlett's test for homogeneity of variance. We considered $p < .05$ to be significant.

RESULTS

We demonstrate in Figure 1, *A* and *B*, that oxidative damage, assessed by TBARS and the protein carbonyl assays, occurred early in the course of sepsis development in several brain regions. The TBARS levels were elevated in the hippocampus, the cortex, and the cerebellum 6 hrs after sepsis induction ($p < .05$; Fig. 1*A*). In contrast, carbonyl levels were increased only in the hippocampus 6 hrs after sepsis induction ($p < .05$; Fig. 1*B*). These suggest that oxidative damage was more consistent in lipids compared with proteins. In longer times after sepsis induction (12–96 hrs), there was no evidence of oxidative damage in all analyzed structures. In contrast, both TBARS and protein carbonyls levels tended to be lower from 12 to 96 hrs after sepsis induction.

To determine enzymatic antioxidant status during sepsis development in the rat brain, we determined the activity of the main central nervous system antioxidant enzymes (CAT and SOD). We had

The short-term oxidative damage demonstrated here could participate in the development of central nervous system symptoms during sepsis development, or even septic encephalopathy.

previously described that an imbalance between SOD and CAT activities in heart, kidney, lung, and diaphragm is, in part, responsible for the oxidative damage and outcome in the CLP model (3). Except for the striatum, earlier in sepsis development (6 hrs) we demonstrated an increase in SOD activity without a proportional increase in CAT activity ($p < .05$; Fig. 1*C* and 1*D*) with consequent increase in the relation of SOD and CAT (Table 1). Thus, an imbalance in SOD and CAT activity occurred at the same time and in the same structures in which we demonstrated oxidative damage. This early oxidative damage and imbalance in antioxidant enzymes seemed not to be secondary to renal or hepatic failure since in this early time we had no alterations in plasmatic markers of renal and hepatic function (data not shown). In addition, it seemed not to be secondary to hypotension since the study design with fluid resuscitation was not associated with hypotension at 6 hrs after sepsis induction.

DISCUSSION

We demonstrate for the first time that, differently from other organs involved in septic response, central nervous system oxidative stress is restricted to earlier times after sepsis induction. These results are intriguing since oxidative damage occurs in lung, liver, heart, and kidney for longer times after sepsis induction (3). Abd El-Gawad and Khalifa (5) demonstrated that as early as 2 hrs after lipopolysaccharide administration there was an increase in free radicals generation in the rat brain, but these authors did not analyze oxidative variables at longer times after lipopolysaccharide administration. In contrast, it was demonstrated that oxidative stress occurred until 48 hrs after sepsis induction the rat brain (6, 7). However, in these reports fluid resuscitation was administered only once, and this could induce late hypotension (6, 7). Supporting our results, Messaris et al. (8) recently demonstrated mitochondrial-mediated apoptosis in the rat brain early (6–12 hrs), but not later, in sepsis development. These mitochondrial alterations could be one of the mechanisms associated with brain oxidative stress. In addition, the impairment of astrocytic clearance of dehydroascorbic acid from the extracellular fluid and consequent decrease of intracellular ascorbate concentration could be associated with central nervous system oxidative stress (9). This idea is reinforced by the decrease in cerebrospinal fluid ascorbate levels in patients with septic encephalopathy (10). Furthermore, sepsis induces inducible nitric oxide synthase and inhibits glutamate uptake by astrocytes through mechanisms that can be modulated by intracellular ascorbate (9). The protection of the central nervous system from oxidative damage seems to be an adaptation to minimize dysfunction and could be related to central nervous system antioxidant defenses adaptation, diminution of central nervous system metabolism, or several other unstudied factors.

We believe that, as we demonstrated for other organs (3), an imbalance between SOD and CAT could be responsible, in part, for the occurrence of oxidative damage in the rat brain. The balance between these enzymes was restored in the studied structures 12–96 hrs after sepsis induction. In these times we demonstrated that both SOD and CAT activity did not change or increase proportionally or that the increase in CAT activity was higher than SOD activity increase (Fig. 1*C* and 1*D*). In these later times we could not demonstrate an increase in the relation of SOD and CAT (Table 1) and oxidative damage in all analyzed structures. Thus, the alterations in the SOD/CAT relation were temporally related to the occurrence or not of oxidative damage in the central nervous system. To the best of our knowledge there are no reports in the literature that describe SOD and CAT modulation in the rat brain after sepsis induction.

We cannot ascertain that the short-term oxidative damage demonstrated here can lead to central nervous system symptoms during sepsis development, or even septic encephalopathy. Further studies must determine the relation between the demonstrated short-term oxidative damage and the development of septic encephalopathy or long-term cognitive impairments in sepsis survivors.

REFERENCES

1. Papadopoulos MC, Davies DC, Moss RF, et al: Pathophysiology of septic encephalopathy: A review. *Crit Care Med* 2000; 28:3019–3024
2. Barichello T, Martins MR, Reinke A, et al: Cognitive impairment in sepsis survivors from cecal ligation and perforation. *Crit Care Med* 2005; 33:221–223
3. Ritter C, Andrades ME, Reinke A, et al: Treatment with N-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis. *Crit Care Med* 2004; 32:342–349
4. Ritter C, Reinke A, Andrades M, et al: Protective effect of N-acetylcysteine and deferoxamine on carbon tetrachloride-induced acute hepatic failure in rats. *Crit Care Med* 2004; 32:2079–2083
5. Abd El-Gawad HM, Khalifa AE: Quercetin, coenzyme Q10, and L-canavanine as protective agents against lipid peroxidation and nitric oxide generation in endotoxin-induced shock in rat brain. *Pharmacol Res* 2001; 43: 257–263
6. Sener G, Toklu H, Kapucu C, et al: Melatonin protects against oxidative organ injury in a rat model of sepsis. *Surg Today* 2005; 35: 52–59
7. Czapski GA, Cakala M, Kopczuk D, et al: Inhibition of nitric oxide synthase prevents energy failure and oxidative damage evoked in the brain by lipopolysaccharide. *Pol J Pharmacol* 2004; 56:643–646
8. Messaris E, Memos N, Chatzigianni E, et al: Time-dependent mitochondrial-mediated programmed neuronal cell death prolongs survival in sepsis. *Crit Care Med* 2004; 32: 1764–1770
9. Korcok J, Wu F, Tyml K, et al: Sepsis inhibits reduction of dehydroascorbic acid and accumulation of ascorbate in astroglial cultures: Intracellular ascorbate depletion increases nitric oxide synthase induction and glutamate uptake inhibition. *J Neurochem* 2002; 81:185–193
10. Voigt K, Kontush A, Stuerenburg HJ, et al: Decreased plasma and cerebrospinal fluid ascorbate levels in patients with septic encephalopathy. *Free Radic Res* 2002; 36: 735–739

Capítulo 5.

Antioxidant treatment reverses late cognitive impairment in an animal model of sepsis

Tatiana Barichello, Roberta A. Machado, Larissa Constantino, Samira S. Valvassori, Gislaine Z. Reus, Fabricia Petronilho, Cristiane Ritter, João Quevedo, Felipe Dal-Pizzol

Critical Care Med (2007) no prelo

Critical Care Medicine
The Intensive Care Professionals

Antioxidant treatment reverses late cognitive impairment in an animal model of sepsis

Journal:	*Critical Care Medicine*
Manuscript ID:	CCM-00016-2007
Manuscript Type:	Original Articles - Laboratory Investigations
Date Submitted by the Author:	04-Jan-2007
Complete List of Authors:	Barichello, Tatiana; UNESC Machado, Roberta; UNESC Constantino, Larissa; UNESC Valvassori, Samira; UNESC Reus, Gislaine; UNESC Petronilho, Fabricia; UNESC Ritter, Cristiane; UNESC Quevedo, Joao; UNESC Dal-Pizzol, Felipe; Universidad do Extremo Sul Catarinese, Department of Medicine
Key Words:	septic shock, oxidative stress, memory, central nervous system

Antioxidant treatment reverses late cognitive impairment in an animal model of sepsis

Tatiana Barichello[1],PharmD, MSc, Roberta Albino Machado[1],BSc,

Larissa Constantino[1],BSc, Samira S. Valvassori[2],BSc, Gislaine Z. Réus[2], BSc,

Fabricia Petronilho[1],MSc, Cristiane Ritter[1] MD, MSc,

João Quevedo[2], MD, PhD Felipe Dal-Pizzol[1], MD, PhD

[1] Laboratório de Fisiopatologia Experimental, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil.

[2] Laboratório de Neurociências, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil.

Address for reprints: Felipe Dal-Pizzol. Laboratório de Fisiopatologia Experimental, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil. Avenida Universitária, 1105, 88806-000. phone: 55 48 4312641, fax: 55 48 431 2750, e-mail:piz@unesc.net

This work was supported by grants from UNESC and CNPq.

Key Words: septic shock, oxidative stress, memory, central nervous system

Abstract

*Objective: A*ssess the effect of antioxidant treatment on late cognitive impairment and early hippocampus oxidative stress after cecal ligation and perforation (CLP) in the rat.

Design: Prospective, controlled experiment.

Setting: Animal basic science laboratory.

Subjects: Male Wistar rats, weighing 300–350 g.

Interventions: Rats subjected to CLP were treated with either *N*-acetylcysteine (NAC) plus deferoxamine (DFX) or vehicle with or without "basic support" (saline at 50 mL/kg immediately and 12 hrs after CLP plus ceftriaxone at 30 mg/kg and clindamycin 25 mg/kg every 6 hrs).

Measurements and Main Results: Ten and thirty days after surgery, the animals underwent three behavioral tasks: a) inhibitory avoidance task; b) habituation to an open field; and c) continuous multiple trials step-down inhibitory avoidance task. The sepsis group showed significantly decreased performance in latency retention compared with the sham group in inhibitory avoidance. In the open-field task the sepsis group presented memory impairment after sepsis. In the continuous multiple trials step-down inhibitory avoidance task, the sepsis group showed a significant increase in the number of training trials required to reach the acquisition criterion. All these cognitive impairments were reversed by NAC plus DFX treatment, but not its isolate use. In addition, the combined use of antioxidants attenuated oxidative damage in hippocampus 6 hours after sepsis induction.

Conclusions: antioxidant treatment could significantly attenuate late cognitive deficits in sepsis survivors from cecal ligation and perforation. This was associated with attenuation on hippocampus oxidative damage in early periods of sepsis development.

Introduction

Advances in critical care medicine have led to improved survival rates among those patients admitted to the ICU. Critical illness often results in multiple system organ dysfunctions, including neurological dysfunction, and is associated with poor neurological outcomes (1). Investigations of the effects of critical illness on neurological dysfunction have been relatively neglected compared to the effects on other organ systems, mainly neurocognitive outcomes in survivors of critical illness (2-6).

The mechanisms associated to neuropsychological and intellectual impairments secondary to direct brain injury is better understood when compared to the neurocognitive impairment secondary to the general critical illness. It is well characterized the participation of inflammatory and apoptotic pathways in neuronal damage secondary to brain injury (7-9). But, to the best of our knowledge there is no published study describing the mechanisms associated to the long-term cognitive deficit in acute inflammatory diseases.

We had previously described oxidative damage in different brain regions in two different models of acute inflammatory disease (10,11), and it is well known that oxidative stress could participate in the development of several central nervous system diseases (12). Thus we here determine the role of hippocampal oxidative stress in the development of long-term cognitive impairment in sepsis survivors, using an animal model recently described by our group (13,14).

Methods

In vivo studies were performed in accordance with National Institutes of Health guidelines and approved by the Ethics Committee of Universidade do Extremo Sul Catarinense.

Animal model of long-term congnitive impairment: this model was described in detail elsewhere (13,14). Briefly, after sepsis induction using the cecal ligation and perforation procedure, the sepsis group received "basic support" (saline at 50 mL/kg immediately and 12 hrs after CLP plus ceftriaxone at 30 mg/kg and clindamycin at 25 mg/kg every 6 hrs over a total of 3 days). The sham-operated group received only saline, 50 mL/kg, immediately and 12 hrs after surgery, and the volume of saline corresponded to antibiotic administration. Survival in the sham group was 100%, and in the sepsis group was 40%. The number of survivals is in accordance with our previous reports (13,14). Ten or 30 days after surgery, different cohorts of animals each time and each task underwent three behavioral tasks: a) the step-down inhibitory avoidance task (single-training); b) continuous multiple-trials step-down inhibitory avoidance task; and c) the open-field task. The behavioral tests were performed by the same person who was blinded to the experimental group (sham or CLP). All experimental procedures involving animals were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and with the approval of the local ethics committee.

The step-down inhibitory avoidance apparatus and procedures have been described in previous reports (15). Briefly, the training apparatus was a 50x25x25-cm acrylic box (Albarsch, Porto Alegre, Brazil) whose floor consisted of parallel caliber stainless steel bars (1-mm diameter) spaced 1 cm apart. A 7-cmwide, 2.5-cm-high platform was placed on the floor of the box against the left wall. In the training trial, animals were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, the animals received a 0.4-mA, 2.0-sed foot shock and returned to their home cage. A retention test trial was performed 24 hrs after training. The retention test trial was procedurally identical to training, except that no foot shock was presented. The retention test step-down latency (maximum, 180 seconds) was used as a measure of

inhibitory avoidance retention. Habituation to an open field was carried out in a 40x60-cm open field surrounded by 50-cm high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. Animals were gently placed on the left rear quadrant, and left to explore the arena for 5 min (training session). Immediately following this, the animals were taken back to their home cage, and 24 hrs later submitted again to a similar open-field session (test session). Crossing of the black lines and rearing performed in both sessions were counted. The decrease in the number of crossings and rearings between the two sessions was taken as a measure of the retention of habituation (16). Continuous multiple-trials step-down inhibitory avoidance task was performed in the same step-down inhibitory avoidance apparatus described above. However, in the training session, the animal was placed on the platform and immediately after stepping down on the grid, received a 0.3-mA, 2.0-sec foot shock. This procedure continued until the rat remained on the platform for 50 seconds. The animal was then returned to the home cage. The number of training trials required to reach the acquisition criterion of 50 seconds on the platform was recorded. The retention test was performed 24 hrs later.

Antioxidant effects on the development of long-term cognitive impairment in sepsis survivors: in the above described model 10 days after sepsis animals presented deficits in the three cognitive skills (step-down inhibitory avoidance, open field and continuous multiple-trials step-down inhibitory avoidance) (13), and 30 days in two of three cognitive skills (step-down inhibitory avoidance and continuous multiple-trials step-down inhibitory avoidance) (14). In addition we had previously determined that early after sepsis development oxidative damage occurs, mainly in the hippocampus (11). Thus, to assess the participation of oxidative damage in the observed cognitive deficits, we determine the effects of early antioxidant administration on long-term cognitive tasks and on early markers of oxidative damage in the hippocampus. Using an antioxidant

treatment protocol described in detail by our group elsewhere (17) we determined the effects of the administration of N-acetylcysteine (NAC, administered 20 mg/kg, 3 hrs, 6 hrs, 12 hrs, 18 hrs, and 24 hrs after CLP, subcutaneously), deferoxamine (DFX, 20 mg/kg, 3 hrs and 24 hrs after CLP, subcutaneously) or both in the above described parameters. To asses the effects of antioxidant administration on long-term cognitive deficits in sepsis survivors we divided animals in six different groups: 1) sham-operated; 2) CLP - received antibiotics and fluid resuscitation as described above; 3) CLP plus NAC - as the CLP group with the administration of NAC as described above; 4) CLP plus DFX - as the CLP group with the administration of DFX as described above; 5) CLP plus NAC and DFX - as the CLP group with the administration of both NAC and DFX as described above; 6) NAC plus DFX - septic animals that did not receive antibiotics, but only NAC and DFX as described above. To assess the participation of early hippocampal oxidative damage on the long-term cognitive deficits we used, in a separate cohort of animals, these six groups. Six hours after sepsis induction animals were sacrificed by decapitation, hippocampus was removed to the determination of thiobarbituric acid reactive substances (18) and protein carbonyls (19) as described in detail elsewhere (11).

Data from the inhibitory avoidance task and retention test latencies from continuous multiple-trials step-down inhibitory avoidance were expressed as median and interquartile ranges. Statistical significances were determined by the Mann-Whitney U test, p <.05. Data from the open-field task and the number of training trials from continuous multiple trials step-down inhibitory avoidance were expressed as mean $\pm$ S.D.. Statistical significances were determined by paired samples Student's *t*-test, p < .05. Data from oxidative damage parameters are presented as means $\pm$ S.D. Statistical significances were determined by 2-way ANOVA and multiple comparisons were performed by a Newman-Keuls test, $p < .05$.

Results

Effects of early antioxidant administration on the inhibitory avoidance test: we had previously described that in the CLP model sepsis survivors presented alterations in the inhibitory avoidance test 10 and 30 days after sepsis induction. No differences between groups were demonstrated in the inhibitory avoidance training session. In the test session, the step-down latency was significantly decreased in the sepsis group compared to the sham group, and this alteration was not reversed with the isolate use of NAC or DFX 10 (Figure 1A) or 30 (Figure 1B) days after sepsis induction. In contrast, both 10 and 30 days after sepsis the combined use of NAC and DFX, with or without antibiotics reversed the observed alteration (Figures 1A and 1B).

Effects of early antioxidant administration on the continuous multiple-trials step-down inhibitory avoidance test: As demonstrated previously, sepsis survivors presented an increase in the number of training trials required to reach the acquisition criterion (50 secs on the plataform) in the continuous multiple-trials step-down inhibitory avoidance test compared to the sham group 10 (Figure 2*A*) and 30 (Figure 2B) days after sepsis. As demonstrated previously, in the retention test, there was no difference between groups (data not shown). Nor NAC or DFX could reverse the observed deficit in both times tested (Figures 2A and 2B), but as demonstrated to the inhibitory avoidance the combined use of NAC and DFX reversed the observed alteration (Figures 2A and 2B).

Effects of early antioxidant administration on the open-field task: In the open-field task, there were no differences in the number of crossings and rearings between groups in the habituation to the open-field training session, demonstrating no difference in motor and exploratory activity between groups (Figure 3A and 3B), as we previously demonstrated (13). As we previously demonstrated, the sepsis group did not show a

difference in crossings and rearings between training test sessions, demonstrating memory impairment in this group (Figure 3A and 3B). The use of NAC plus DFX, but not its isolated use, reversed the memory impairment in the open-field task 10 days after sepsis induction. We did not investigate the effects of antioxidant administration 30 days after sepsis in the open-field task since we demonstrated previously that at this period sepsis survivors did not differ significantly from sham animals in this task (14).

Effects of antioxidant administration on hippocampus oxidative damage parameters after sepsis: We demonstrated previously the occurrence of oxidative damage 6 hours after sepsis induction in this model of rodent sepsis (11). Since hippocampus is a major SNC structure responsible to memory formation we determined if the reversal of late cognitive deficits in sepsis survivors was associated to the attenuation of early oxidative damage. We demonstrated in figure 4A and 4B that NAC plus DFX, but no other treatment reversed oxidative damage assessed by two different oxidative damage parameters, and this correlates to an improve in cognitive performance in sepsis survivors.

Discussion

We demonstrated for the first time that antioxidant treatment could significantly attenuate late cognitive deficits in sepsis survivors from cecal ligation and perforation. This was associated with attenuation on hippocampus oxidative damage in early periods of sepsis development.

The evidence from 10 ongoing cohorts suggests that 25 to 78% of ICU survivors experience cognitive impairments (for an excellent review see 20). In these patients generally memory is the most frequently observed deficit, followed by executive function and attention deficit (20). The mechanisms underlying these alterations are still obscure. There is probably not a single cause of these impairments, but rather a number of

several factors that interact dynamically with premorbid variables and result in adverse outcomes (20). Several methodological issues limit the clear distinction between these factors in the clinical setting. Thus, using a clinically relevant animal model of sepsis we demonstrated that early hippocampus oxidative damage seems to be important in the development of late cognitive deficits in sepsis survivors. We supposed that this early alteration initiate a complex cascade of events that leads ultimately to SNC dysfunction and late cognitive deficits observed in CLP survivors.

Some few studies were published in the intent to determine the molecular mechanisms associated with late cognitive deficits in the context of critical care medicine. Using an animal model of meningitis based on *S. pneumoniae* Irazuzta et al. (21) reported that the dexamethasone treatment of rats with bacterial meningitis leads to decreased activation of caspase 3 but not of caspase 1 and these was associated with a preservation of neurobehavioral performance, but we need to be very cautious when evaluating learning and memory performance in subjects treated with dexamethasone (22). In addition, the administration of hydrocortisone during septic shock in a dosage similar to the endogenous production rate was associated with a lower incidence of the so-called posttraumatic stress disorder in long-term survivors (23). Irazuzta group demonstrated that although hypothermia can reduce the inflammatory response and biomarkers of brain injury it fails to improve neurobehavioral performance in an animal model of bacterial meningitis (24). It is well known that neuronal and cerebral endothelial cells undergone apoptosis via activation of toll-like receptor 2 (25,26), but its relation to acute and chronic neurologic deficits is unknown. In the same way, LPS could trigger CNS inflammation and neuronal death during chronic neurodegeneration (27), but the relevance of this to chronic neurological deficits was never studied.

Since oxidative stress is associated to the development of neurodegenerative disease (12) and is important to the development of multiple organ dysfunction

syndrome during sepsis (28) is reasonable suppose that it could contribute to long-term cognitive deficits in sepsis survivors. Oxidative stress was demonstrated previously in different animal models of critical care disease. We had demonstrated the occurrence of oxidative damage in an animal model of sepsis (11) and acute hepatic failure (10). Sener et al described oxidative damage and reduce antioxidant content in a murine model of sepsis and determined an antioxidant effect to melatonin in this setting (29). None of these previous studies correlate brain oxidative stress and long-term cognitive deficits in survivors from critical care diseases.

The evaluation of molecular mechanisms and signaling cascades which underlie the events that ultimately lead to brain damage during sepsis will improve our pathophysiological knowledge of this disease. More importantly, it will also promote the development of new and more efficient therapeutic strategies, especially to prevent neurological sequelae. In this context our results suggested, for the first time, a role to early SNC oxidative damage in the development of long-term cognitive deficits. In addition we demonstrated a new role to antioxidant treatment in an animal model of sepsis.

References

1 - Vincent JL, Moreno R, Takala J, et al. The SOFA (Sepsis related Organ Failure Assessment) score to describe organ dysfunction/failure: on behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. *Intensive Care Med* 1996; 22:707– 710.

2 - Angus DC, Musthafa AA, Clermont G, Griffin MF, Linde-Zwirble WT, Dremsizov TT, Pinsky M. Quality-adjusted survival in the first year after the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2001; 163:1389–1394.

3 - Herridge MS, Cheung AM, Tansey CM, Matte-Martyn A, Diaz-Granados N, Ali-Saidi F, Cooper AB, Guest CB, Mazer CD, Mehta S, Stewart TE, Barr A, Cook D, Slutsky AS. One-year outcomes in survivors of the acute respiratory distress syndrome. *N Engl J Med* 2003; 348:683–693.

4 - Jackson JC, Hart RP, Gordon SM, Shintani A, Truman B, may L, Ely EW. Six-month neuropsychological outcome of medical intensive care unit patients. *Crit Care Med* 2003; 31:1226–1234.

5 - Orme J Jr., Romney JS, Hopkins RO, Pope D, Chan KJ, Thomsen G, Crapo RO, Weaver LK. Pulmonary function and health-related quality of life in survivors of acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2003; 167:690–694.

6 - Weinert CR, Gross CR, Kangas JR, Bury CL, Marinelli WA. Health-related quality of life after acute lung injury. *Am J Respir Crit Care Med* 1997; 156:1120–1128.

7 - Leib SL, Chow LL, Kim YS, Sheldon RA, Tauber MG. Reactive oxygen intermediates contribute to necrotic and apoptotic neuronal injury in an infant rat model of bacterial meningitis due to group B streptococci. *J Clin Invest* 1996; 98:2632–2639.

8 - Koedel U, Pfister HW. Oxidative stress in bacterial meningitis. *Brain Pathol* 1999; 9:57–67.

9 - Nau R, Bruck W, Soto A. Apoptosis of neurons in the dentate gyrus in humans suffering from bacterial meningitis. *J Neuropathol Exp Neurol* 1999; 58:3265–3274.

10 - Ritter C, Reinke A, Andrades M, Martins MR, Rocha J, Menna-Barreto S, Quevedo J, Moreira JCF, Dal-Pizzol F. Protective effect of N-acetylcysteine and deferoxamine on carbon tetrachloride-induced acute hepatic failure in rats. *Crit Care Med* 2004; 32:2079 – 2084

11 - Barichello T, Fortunato JJ, Meller A, Feier G, Reinke A, Moreira JCF, Quevedo J, Dal-Pizzol F. Oxidative variables in the rat brain after sepsis induced by cecal ligation and perforation. *Crit Care Med* 2006; 34: 886-889.

12 - Halliwell B. Oxidative stress and neurodegeneration: where are we now? *J Neurochem* 2006; 97:1634-1658.

13 - Barichello T, Martins MR, Reinke A, Ritter C, Quevedo J, Dal-Pizzol F. Cognitive impairment in sepsis survivors from cecal ligation and perforation. *Crit Care Med* 2005; 33:221–223.

14 - Barichello T, Martins MR, Reinke A, Feier G, Ritter C, Quevedo J, Dal-Pizzol F. Long-term cognitive impairment in sepsis survivors. *Crit Care Med* 2005; 33: 1671-1672.

15 - Quevedo J, Vianna MR, Roesler R, de-Paris F, Izquierdo I, Rose SP. Two time windows of anisomycin-induced amnesia for inhibitory avoidance training in rats: Protection from amnesia by pretraining but not pre-exposure to the task apparatus. *Learn Mem* 1999; 6:600–607.

16 - Vianna MR, Alonso M, Viola H, Quevedo J, de Paris F, Furman M, de Stein ML, Medina JH, Izquierdo I. Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat. *Learn Mem* 2000; 7:333–340.

17 - Ritter C, Andrades ME, Reinke A, Menna-Barreto S, Moreira JCF, Dal-Pizzol F. Treatment with N-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis. *Crit Care Med* 2004; 32:342–349.

18 - Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol* 1990; 186: 421.

19. Levine RL, Garland D, Oliver CN. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 1990; 186: 464.

20 - Hopkins RO, Jackson JC. Long-term Neurocognitive Function After Critical Illness. *Chest* 2006; 130:869–878.

21 - Irazuzta J, Pretzlaff RK, Courten-Myers G, Zemlan F, Zingarelli B. Dexamethasone decreases neurological sequelae and caspase activity. *Intensive Care Med* 2005; 31:146–150.

22 - Martins MR, Barichello T, Ritter C, Reinke A, Quevedo J, Dal-Pizzol F. Dexamethasone therapy and memory performance. *Intensive Care Med* 2005; 31:1001.

23 - Schelling G, Briegel J, Roozendaal B, Stoll C, Rothenhausler HB, Kapfhammer HP. The effect of stress doses of hydrocortisone during septic shock on posttraumatic stress disorder in survivors. *Biol Psychiatry* 2001; 50:978–985.

24 - Irazuzta J, Pretzlaff R, Xue V, Zemlan F, Zingarelli B. Modulation of NF-kB activation and decreased markers of neurological injury associated with hypothermic therapy in experimental bacterial meningitis. *Crit Care Med* 2002; 30:2553–2559.

25 - Lehnardt S, Henneke P, Lien E, Kasper DL, Volpe JJ, Bechmann I, Nitsch R, Weber JR, Golenbock DT, Vartanian T. A mechanism for neurodegeneration induced by group B streptococci through activation of the TLR2/MyD88 pathway in microglia. *J Immunol* 2006;177:583-52.

26 - Bermpohl D, Halle A, Freyer D, Dagand E, Braun JS, Bechmann I, Schordcer NW, Weber JR. Bacterial programmed cell death of cerebral endothelial cells involves dual death pathways. *J Clin Invest* 2005;115:1607-1615.

27 - Cunningham C, Wilcockson DC, Campion S, Lunnon K, Perry VH. Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration. *J Neurosci* 2005; 25:9275-9284.

28 - Salvemini D, Cuzzocrea S. Oxidative stress in septic shock and disseminated intravascular coagulation. *Free Radic Biol Med* 2002; 33:1173-1185.

29 - Sener G, Toklu H, Kapucu C, Ercan F, Erkanli G, Kacmaz A, Tilki M, Yegen BC. Melatonin protects against oxidative organ injury in a rat model of sepsis. *Surg Today* 2005; 35:52-59.

Figure Legends

Figure 1 - Inhibitory avoidance task. Rats were sham-operated or submitted to cecal ligation and puncture (*CLP*). CLP animals were assigned to receive basic support (Sup), Sup plus *N*-acetylcysteine (*NAC*), Sup plus deferoxamine (*DFX*), Sup plus NAC and DFX or only NAC and DFX as described in Materials and Methods. Survivors from sepsis were submitted to the inhibitory avoidance task **A.** 10 days or **B.** 30 days after sepsis. Data are expressed as median (interquartile ranges) training and test session latencies, in seconds. n = 15 each group. *Significant difference compared with Sham group (Mann-Whitney U test, $p < .01$). Sup = basic support, NAC = N-acetylcysteine, DFX = Deferoxamine

Figure 2 - Continuous multiple-trials step-down inhibitory avoidance. Rats were sham-operated or submitted to cecal ligation and puncture (*CLP*). CLP animals were assigned to receive basic support (Sup), Sup plus *N*-acetylcysteine (*NAC*), Sup plus deferoxamine (*DFX*), Sup plus NAC and DFX or only NAC and DFX as described in Materials and Methods. Survivors from sepsis were submitted to the continuous multiple-trials step-down inhibitory avoidance task **A.** 10 days or **B.** 30 days after sepsis. Data are expressed as mean $\pm$ SD of the number of training trials required to reach acquisition criterion (50 secs on the platform). n = 15 each group *Significant difference compared with Sham groups (Student's *t*-test, $p< .01$). Sup = basic support, NAC = N-acetylcysteine, DFX = Deferoxamine

Figure 3 - Open-field task. Rats were sham-operated or submitted to cecal ligation and puncture (*CLP*). CLP animals were assigned to receive basic support (Sup), Sup plus *N*-acetylcysteine (*NAC*), Sup plus deferoxamine (*DFX*), Sup plus NAC and DFX or only NAC and DFX as described in Materials and Methods. Survivors from sepsis were submitted to the open-field task 10 days after sepsis. Data are expressed as mean $\pm$ SD of **A.** crossings and **B.** rearings of training (*white columns*) and test (*gray columns*)

session. n = 15 each group. *Significant difference between training test (paired samples Student's *t*-test, $p < .01$). # Significant difference between groups in training and test sessions (Student's *t*-test, $p < .01$). *Significant difference compared with Sham group Sup = basic support, NAC = N-acetylcysteine, DFX = Deferoxamine

Figure 4 - Early oxidative damage in the hippocampus from septic rats. CLP animals were assigned to receive basic support (Sup), Sup plus *N*-acetylcysteine (*NAC*), Sup plus deferoxamine (*DFX*), Sup plus NAC and DFX or only NAC and DFX as described in Materials and Methods. Six hours after sepsis induction animals were sacrificed and hippocampus isolated to the determination of **A.** thiobarbituric acid reactive substances and **B.** protein carbonyls. Data are expressed as mean $\pm$ SD n = 5 each group *Significant difference compared with Sham groups (ANOVA followed by Newman-Keuls test, $p< .01$). Sup = basic support, NAC = N-acetylcysteine, DFX = Deferoxamine

Figure 1A

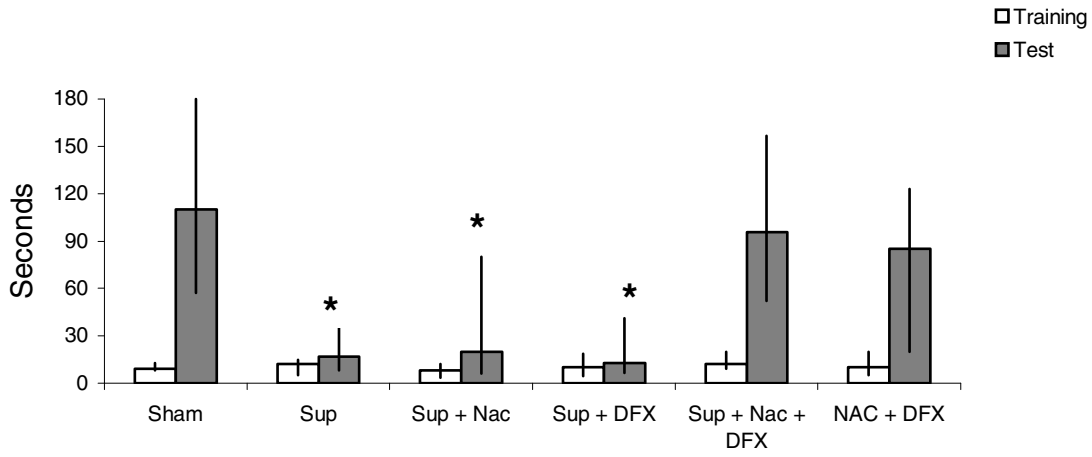


Figure 1B

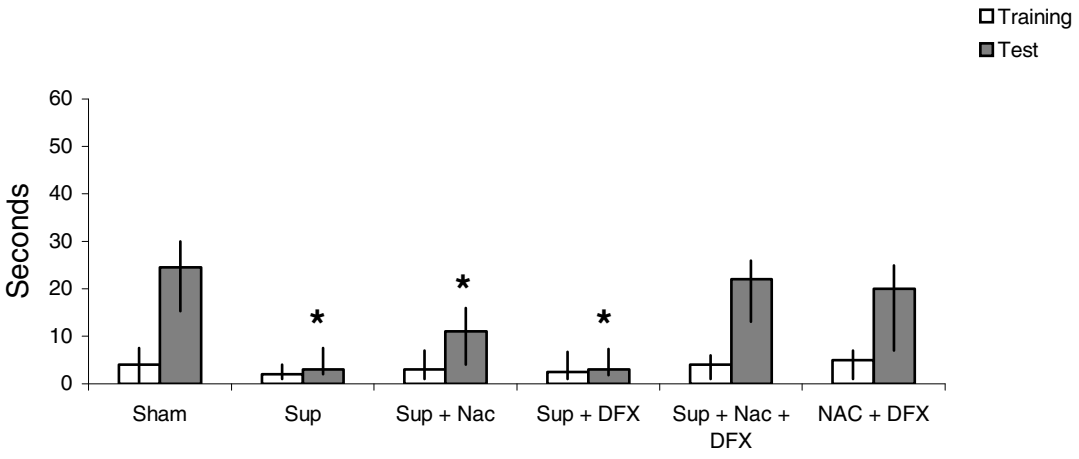


Figure 2A

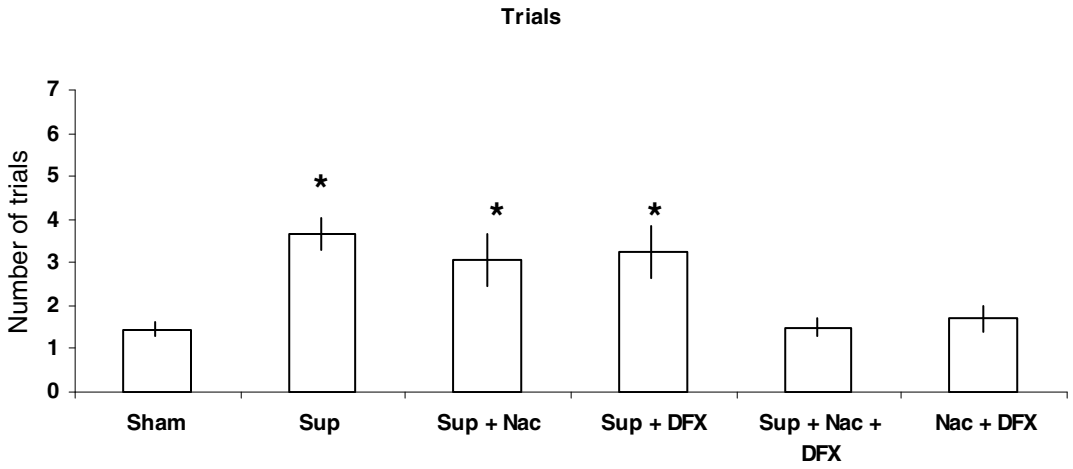


Figure 2B

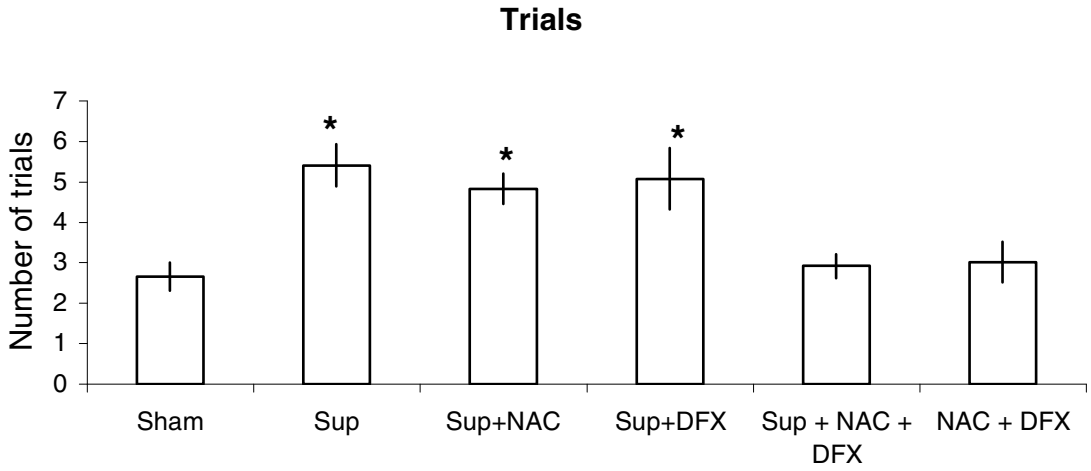


Figure 3A

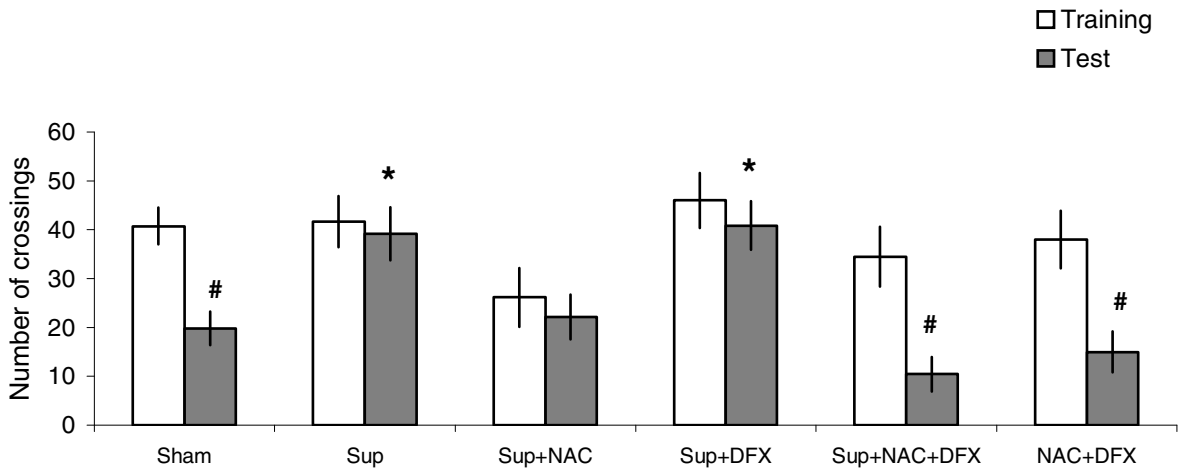


Figure 3B

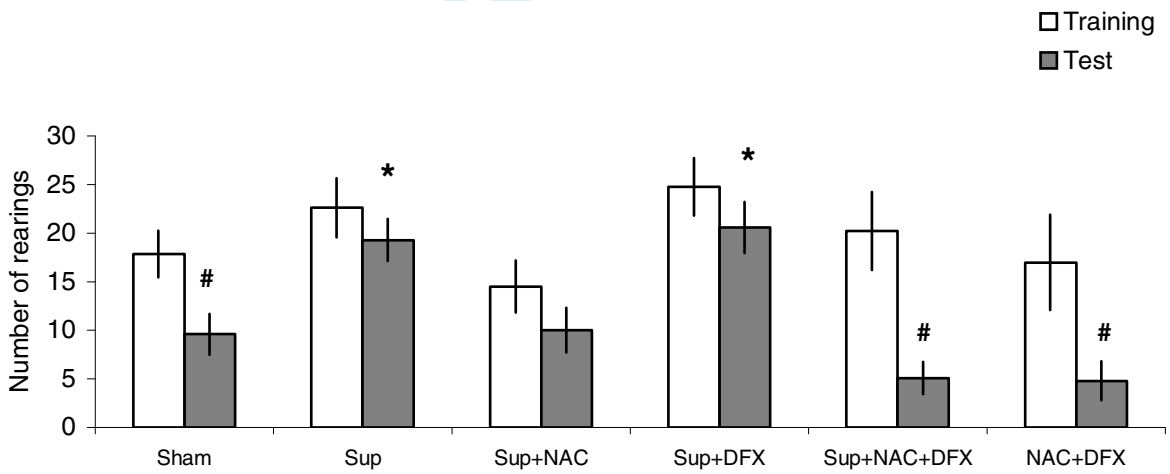


Figure 4A

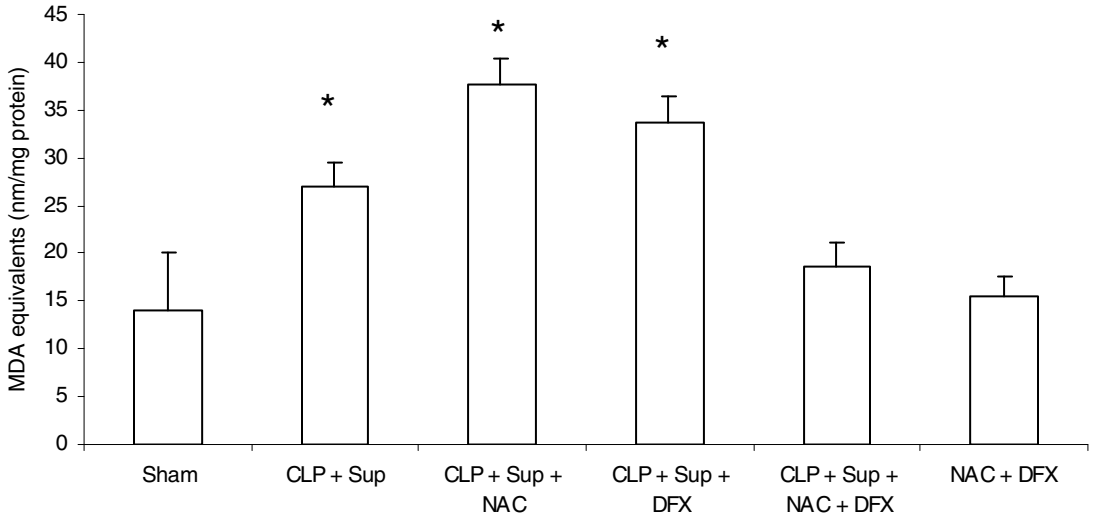
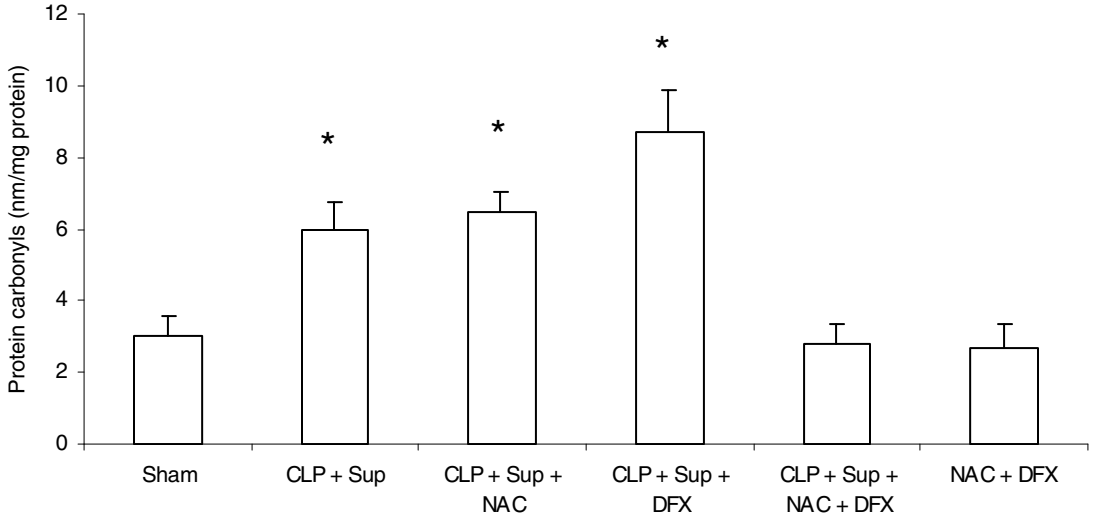


Figure 4B



DISCUSSÃO

Dano cognitivo em ratos sobreviventes a sepse

Os resultados dos experimentos do **Capítulo 1 e 2** demonstraram que ratos sobreviventes a sepse após 10 dias da indução nos testes de habituação ao campo aberto, esquiva inibitória, esquiva inibitória de múltiplos treinos, reconhecimento de objeto, labirinto de cruz elevada e natação forçada apresentaram incapacidade na aprendizagem, na memória, e sintomas de depressão.

No Teste de Habituação ao Campo Aberto, os ratos submetidos à sepse não apresentaram alteração na atividade locomotora e exploratória quando comparados ao grupo sham, porém demonstraram incapacidade na habituação ao local, sugerindo uma incapacidade na memória e aprendizagem.

No teste de esquiva inibitória o tempo de latência foi significativamente menor no grupo sepse, demonstrando que estes possuem uma incapacidade na memória aversiva. O teste de esquiva inibitória envolve vários estímulos, incluindo percepção espacial e visual, sensibilidade à dor, acompanhados de um componente emocional amplamente modulado por hormônios relacionados ao estresse (Gold, 1986). Os ratos submetidos á sepse que foram submetidos ao teste de esquiva inibitória de múltiplos treinos mostraram um significativo aumento no número de treinamentos requeridos para alcançar o critério de aquisição (50 segundos na plataforma) e no teste de retenção não houve diferença entre os grupos. Os resultados desta tarefa mostraram que o grupo sepse requereu aproximadamente duas vezes mais estímulos para alcançar o critério da aquisição quando comparado

com o grupo sham, indicando uma incapacidade de aprendizagem (aquisição de novo conhecimento).

A tarefa de reconhecimento de objetos, desenvolvida originalmente por Ennaceur e por Delacour (Ennaceur e Delacour, 1988), é baseada na tendência dos roedores explorarem o objeto novo. Nenhuma estimulação, recompensa ou aversividade são utilizadas durante o treinamento, a aprendizagem ocorre sob circunstâncias de estresse ou de medo relativamente baixo (Ennaceur e Delacour 1988). Os dados encontrados mostraram que os ratos sobreviventes a sepse apresentaram incapacidade significativa da memória de reconhecimento do objeto familiar. Estes achados são relevantes desde que à tarefa de reconhecimento do objeto familiar nos roedores seja um teste não espacial e não aversivo para memória. Além disso, a tarefa do reconhecimento do objeto está sendo usada também cada vez mais como uma ferramenta experimental poderosa para avaliar os efeitos de droga na memória e investigar a aprendizagem dos mecanismos neurais e memórias subjacentes (Rosa *et al*, 2003; Schröder *et al*, 2003; Baker e Kim, 2002; Okuda *et al*, 2004; Rampon *et al*, 2000).

No teste do labirinto de cruz elevada, um teste validado para avaliar comportamento ansioso (Pellow *et al*, 1985), nenhuma diferença foi demonstrada entre os grupos, indicando que os sobreviventes de sepse não apresentam sintomas de ansiedade após a recuperação da doença.

Os sobreviventes de sepse apresentaram sintomas ligados à depressão avaliados no teste forçado da natação. O tempo de imobilidade foi significativamente mais longa no grupo dos sobreviventes de sepse. A origem do teste de natação forçado relatada por Porsolt (1979) era aquela de um modelo da depressão com as características similares àquelas do modelo instruído do comportamento

desamparado, mas tecnicamente mais fácil de reproduzir. O estado afetivo interno dos roedores após a exposição ao nado inicial no teste de natação forçada é caracterizado como comportamento de desespero. O teste preliminar ao procedimento da indução da natação foi proposto para ser similar à sessão inicial que induz o comportamento desesperado instruído para expor os ratos ao estresse de não poder escapar daquela situação. A Indução do comportamento de desespero produz um largo aspecto comportamental afetando a cognição, o sono e o desempenho motor que se assemelham muitos dos sintomas de depressão (Weiss e Kilts, 1998). Adicionalmente, como descrito acima, o grupo de sobreviventes de sepse não apresentou incapacidade motora, suportando a idéia que o longo tempo de imobilidade no grupo séptico foi relacionado aos sintomas ligados a depressão. Estes resultados estão de acordo com estudos clínicos sobre sintomas depressivos nos quais sobreviventes de doenças severas tais como o sepse e choque séptico apresentam (Angus *et al*, 2001; Granja *et al*, 2004; Granja *et al*, 2005; Heyland *et al*, 2000 ; Hopkins *et al*, 2005).

Quando realizado os testes de esquiva inibitória, esquiva inibitória de múltiplos treinos e habituação ao campo aberto após 30 dias, ratos sobreviventes a sepse ainda demonstraram incapacidade de aprendizagem e memória, estes resultados são apresentados no **Capítulo 3**

Estes resultados indicam que a incapacidade de memória e aprendizagem demonstrada 10 dias após a indução da sepse, persistem após 30 dias do CLP. A persistência das alterações 30 dias após CLP reforça a idéia que este modelo poderia ser uma ferramenta importante no estudo das seqüelas cognitivas em sobreviventes de sepse.

Os dados deste estudo fornecem a primeira demonstração experimental em um modelo animal de sepse clinicamente relevante para demonstrar a incapacidade na aprendizagem e memória. Neste contexto, Shimizu e colaboradores (1999) demonstraram que 24 horas após CLP, os animais mostraram incapacidade de aprendizagem na esquiva inibitória. Os resultados deste estudo podem ter um significado maior, pois foram realizados os experimentos 10 dias e 30 dias após CLP, e não 24 horas. Os animais após 10 dias da cirurgia estão recuperados inteiramente, sem nenhum sinal de infecção ou alterações motoras (Ritter *et al*, 2004; Ritter *et al*, 2003). Além disso, este estudo utilizou reposição hídrica e antibióticos para replicar o mais próximo a terapia de suporte executada no ajuste clínico, quando comparado com o estudo de Shimizu e colaboradores (1999).

Em um modelo animal de sepse induzido por LPS, foi encontrado mecanismos conhecidos de apoptoses no hipocampo após 24 horas após a indução, sendo assim, o mais severamente afetado após a indução do sepsis (Semmler *et al.,* 2005).

Semmler e colaboradores (2007) mostraram que ratos submetidos à sepse pelo modelo de LPS (injeções de polissacarídeos) apresentaram após 3 meses, alterações comportamentais, dano neuronal em sub-regiões hipocampais e redução da inervação colinérgica em áreas corticais.

As incapacidades cognitivas parecem estar relacionadas diretamente aos processos dependentes do hipocampo, danificando a memória (Squire e Zola-Morgan, 1991; Jarrard, 1995). Similarmente, como indicou Semmler e colaboradores (2007) em seu estudo, caracterizado por uma redução significativa na densidade dos neurônios CA1/CA2 do hipocampo. Adicionalmente foi encontrado uma densidade reduzida de células neuronais no córtex pré-frontal, onde as lesões podem conduzir

aos distúrbios de orientação e aos problemas amenésicos em tarefas complexas (Heidbreder e Groenewegen, 2003; Mogensen *et al*., 2005). Além disso, foi demonstrado uma densidade diminuída de VAChT após a indução da sepse experimental, indicando uma inervação colinérgica diminuída nas áreas citadas acima no estudo de Semmler e colaboradores (2007) , provavelmente relacionado também as incapacidades cognitivas observadas, como mudanças na densidade parietal de VAChT com incapacidades na memória foram encontrados também 1 ano após a indução experimental de trauma cerebral (Dixon et al., 1999).

Entretanto, ainda citando o estudo de Semmler e colaboradores (2007) a VAChT é expressada excepcionalmente nos neurônios colinérgicos e concentrado em seus terminais axônicos (Gilmor *et al*., 1996; Arvidsson *et al*., 1997), a redução da imunoreatividade de VAChT dentro o córtex parietal junto com uma densidade inalterada dos neurônios do córtex parietal indica reduzida inervação colinérfica nessa área. Além disso, a maioria da inervação colinérgica hipocampal e cortical são originadas fora do hipocampo e do córtex (Mesulam *et al*., 1983). Isto sugere que a diminuição da imunoreatividade de VAChT reflete principalmente a retirada da fibra colinérgica dos neurônios, como os neurônios colinérgicos representam 5- 10% dos neurônios corticais e hipocampais (Gage *et al*., 1983; Eckenstein *et al*., 1988). Os neurônios gabaérgicos e glutamatérgicos, a somatostatina e o neuropeptideo Y são mais representativos nestas áreas.

As incapacidades observadas neste estudo, pelo menos parte delas, como as alterações cognitivas são observadas em sobreviventes de sepse, particularmente a incapacidade na memória. Nesta maneira, o modelo de CLP para indução da sepse pode ajudar a investigar os mecanismos biológicos envolvidos na incapacidade cognitiva associada à sepse e para determinar ações terapêuticas

para este problema. Estudos precedentes que descreveram os sintomas de pacientes sobreviventes de sepse na unidade de terapia intensiva demonstram que a maioria destes pacientes apresentou alguma incapacidade cognitiva na alta hospitalar (Hopkins *et al*, 1999; Angus *et al*, 2001; Granja *et al*, 2004; Heyland *et al*, 2000). Após 1 ano, a maioria dos pacientes mostrou uma melhoria na função cognitiva total; entretanto, algumas habilidades cognitivas, como a memória, não melhorou completamente (Hopkins *et al*, 1999; Angus *et al*, 2001; Heyland *et al*, 2000). Entretanto os mecanismos envolvidos na incapacidade cognitiva ainda permanecem obscuros.

Medidas de estresse oxidativo no cérebro de ratos sobreviventes a sepse

No **Capítulo 4** foi realizada a mensuração do estresse oxidativo em ratos submetidos à sepse para tentar esclarecer os mecanismos neurobiológicos envolvidos na incapacidade cognitiva.

Foi demonstrado que, os danos oxidativos, avaliados pelo TBARS e carbonil, e ocorrem precocemente no desenvolvimento do curso da sepse em diversas regiões do cérebro. Os níveis de TBARS foram elevados no hipocampo, córtex, e cerebelo 6 horas após a indução da sepse. Em contraste, níveis de carbonil foram elevados somente no hipocampo 6 horas após a indução da sepse. Estes sugerem danos oxidativos mais consistentes nos lipídios em comparação com proteínas.

Para determinar a atividade enzimática antioxidante durante o desenvolvimento da sepse no cérebro dos ratos, foi determinada a atividade antioxidante enzimática do sistema nervoso central (CAT e SOD). À exceção do

estriado, foi demonstrado um aumento na atividade de SOD sem um proporcional aumento na atividade de CAT, com aumento conseqüente na relação de SOD e CAT. Assim, um desequilíbrio na atividade de SOD e CAT ocorrido ao mesmo tempo e dentro das mesmas estruturas em que foi demonstrado o dano oxidativo. Foi descrito previamente por Ritter e colaboradores (2004) que um desequilíbrio entre as atividades de SOD e CAT no coração, fígado, pulmão, e diafragma são, em parte, responsável pelos danos oxidativos em um modelo de. Os danos oxidativos e desequilíbrio nas enzimas antioxidantes no início do curso da sepse pareceram não ser secundários à falha renal ou hepática, pois no início não foi relatada nenhuma alteração em marcadores plasmáticos da função renal e hepática. Além disso, o dano não parece ser secundário a hipotensão, pois foi utilizado líquido de ressuscitação não foi associado com a hipotensão em 6 horas após a indução de sepse.

Foi demonstramos pela primeira vez que, diferentemente de outros órgãos envolvidos na resposta séptica, no sistema nervoso central o estresse oxidativo é restringido a períodos mais iniciais após a indução da sepse. Estes resultados são intrigantes, pois os danos oxidativos no pulmão, no fígado, no coração, e no rim ocorrem em períodos mais distantes após a indução da sepse (Ritter *et al*, 2004). Abd El-Gawad e Khalifa (2001) demonstraram que 2 horas após a administração de lipopolisacarideos havia um aumento na geração de radicais livres no cérebro de ratos, porém estes autores não analisaram variáveis oxidativas em períodos mais distantes após a administração de lipopolisacarideos. Em contraste, demonstrou-se que o estresse oxidativo ocorreu até 48 horas após a indução da sepse no cérebro dos ratos (Sener *et al*, 2005; Czapski *et al*, 2004). Entretanto, nestes estudos o fluído para ressuscitação foi administrado somente uma

vez, e isto poderia induzir posteriormente a uma hipotensão (Sener et al, 2005; Czapski *et al*, 2004). Suportando os dados apresentados neste estudo, Messaris e e colaboradores (2004) demonstraram recentemente a apoptose mediada pela mitocôndria no cérebro de ratos em períodos iniciais após a indução da sepse (6-12 horas), porém não em períodos mais tardis. Estas alterações mitocondriais poderiam ser um dos mecanismos associados ao estresse oxidativo no cérebro.

Estudos do grupo do Dr Boveris demonstraram que a produção de NO durante a sepse leva a inativação transitória da atividade da cadeia de transporte de elétrons, provavelmente complexo II e IV seriam os alvos principais. Esta inativação levaria a produção de superóxido mitocondrial e, conseqüentemente, de peroxinitrito que inativaria irreversivelmente a cadeia de transporte de elétrons levando efeito em cascata (Boczkowski *et al* 1999).

Outra possibilidade é a de que os diferentes mediadores da resposta inflamatória da sepse possam levar à disfunção mitocondrial e esta a liberação de espécies reativas de oxigênio. Entre estes, o TNF-α tem capacidade de levar as alterações celulares, inclusive morte, mediadas por vias de sinalização intracelulares dependente de ativação do seu receptor. A ativação de receptores de TNF ativa esfingomielinases, levando a um aumento intracelular de ceramida e fosfocolina. A ceramida pode levar a formação do poro de permeabilidade transitória mitocondrial (MPT), com conseqüente liberação de citocromo C (Siskind *et al* 2002), ativação de caspase-3 (Von Haefen *et al* 2002) e apoptose. Em concentrações de milimolar ceramida inibe a fosforilação oxidativa e leva a produção de EAO (Garcia-Ruiz *et al* 1997).

Além disso, a incapacidade da limpeza dos astrócitos de ácido desidroascórbico do líquido extracelular e da diminuição conseqüente do ascorbato

intracelular, a concentração poderia ser associada com o estresse oxidativo do sistema nervoso central (Korcok *et al*, 2002). Esta idéia é reforçada pela diminuição do ascorbato nos níveis do fluído cerebrospinal nos pacientes com encefalopatia séptica (Voigt *et al*, 2002). Entretanto, a sepse induz a óxido nítrico sintase e inibe a recaptação de glutamato pelos astrocitos através dos mecanismos que podem ser modulados pelo ascorbato intracelular (Korcok *et al*, 2002).

A proteção do sistema nervoso central aos danos oxidativos parece ser a adaptação para minimizar a disfunção e também poderia estar relacionado a adaptação das defesas antioxidantes do sistema nervoso central, diminuição do metabolismo do sistema nervoso central, ou outros diversos fatores ainda não esclarecidos. Os dados deste estudo demonstram que, como em outros órgãos (Ritter *et al*, 2004), um desequilíbrio no meio SOD e CAT poderia ser responsáveis, em parte, pela ocorrência de danos oxidativos no cérebro dos ratos. Em contrapartida estas enzimas normalizaram-se nas estruturas estudadas 12-96 horas após a indução da sepse. Neste período (12-96 horas após a indução da sepse) foi demonstrado que a atividade da SOD e da CAT não mudou nem aumentou proporcionalmente ou que, o aumento na atividade da CAT foi mais elevada do que o aumento da atividade da SOD. Assim, as alterações na relação de SOD/CAT foram relacionadas temporariamente à ocorrência ou não de danos oxidativos dentro do sistema nervoso central. Em relação ao exposto acima, não há nenhum estudo que descreve a modulação da SOD e da CAT no cérebro de ratos após a indução da sepse. Porém, não foi possível verificar em curto prazo os danos oxidativos demonstrados neste estudo se podem conduzir ao sistema nervoso central sintomas durante o desenvolvimento da sepse, ou mesmo a encefalopatia séptica.

Tratamento com antioxidantes e dano cognitivo

Baseado nos resultados apresentados no **Capítulo 1, 2 e 4**, foi realizada a avaliação dos efeitos do tratamento com antioxidantes NAC e DFX na reversão da incapacidade cognitiva demonstradas nos testes de habituação ao campo aberto, esquiva inibitória, esquiva inibitória de múltiplos treinos e no estresse oxidativo em hipocampo após a indução de sepse em ratos sobreviventes para tentar esclarecer os possíveis mecanismos neurobiológicos que levam ao dano cognitivo.

Nos testes comportamentais citados acima, após 10 e 30 dias da indução de sepse, DFX e NAC quando administrados concomitantemente reverteram à incapacidade cognitiva apresentada nos **Capítulo 1 e 3**.

Foi demonstrada previamente no **Capítulo 4**, a ocorrência de dano oxidativo 6 horas após a indução da sepse no hipocampo de cérebros de ratos sépticos. O hipocampo é a estrutura principal do SNC responsável pela formação da memória, devido a isso, a reversão dos danos cognitivos tardios em sobreviventes de sepse poderia estar associada à atenuação dos danos oxidativos precoces. Foi demonstrado que NAC e DFX administrados concomitantemente, mas não com o seu uso isolado, reverteram os danos oxidativos avaliados por dois parâmetros oxidativos diferentes de danos. Foi demonstrado pela primeira vez que o tratamento com antioxidantes pode significativamente reverter danos cognitivos tardios em sobreviventes de sepse em um modelo animal de CLP. Isto foi associado com a atenuação nos danos oxidativos do hipocampo em períodos iniciais do desenvolvimento da sepse.

A grande maioria dos estudos em animais (Andrades *et al* 2005, Jao *et al* 2005, Koksal *et al* 2004) e em humanos (Goode *et al* 1995, Cighetti *et al* 2005,

Mishra *et al* 2005, Winterbourn et al 2000) demonstram uma relação entre estresse oxidativo e disfunção de múltiplos órgãos durante a sepse, mas não em SNC. Neste sentido, diversos estudos utilizam antioxidantes para o tratamento de sepse em animais (Victor *et al* 2003, Matejovic *et al* 2005, Supinski *et al* 2006, Carlson *et al* 2006) e humanos (Spapen *et al* 2005, Emet *et al* 2004, Hein *et al* 2004, Heller *et al* 2001, Rank *et al* 2000, Ortolani *et al* 2000, Spapen *et al* 1998, Angstwurm *et al* 2007). Tanto a NAC quanto a DFX já foram testados isoladamente para o tratamento de sepse em animais. A NAC é efetiva em diferentes modelos de sepse, incluindo endotoxemia (Hsu *et al* 2006) e CLP (Ozdulger *et al* 2003). Em modelo de CLP, Messaris e colaboradores (2004) demonstraram que o uso profilático de DFX reduz apoptose e mortalidade.

As evidências de 10 coortes sugerem que 25 a 78% dos sobreviventes de centros de terapia intensiva têm experiências de danos cognitivos (Hopkins e Jackson, 2006). Nestes pacientes geralmente a memória é o dano mais freqüentemente observado, seguido pela função executiva e o dano na atenção (Hopkins e Jackson, 2006).

Os mecanismos subjacentes a estas alterações ainda são obscuros. Há provavelmente várias causa para estas incapacidades, mas sim diversos de fatores que interagem dinamicamente com variáveis e resultados de pré morbidagem (Hopkins e Jackson, 2006). Assim, usando um modelo animal clinicamente relevante de sepse foi possível demonstrar que os danos oxidativos do hipocampo em períodos inicias parecem ser importantes no desenvolvimento das incapacidades cognitivas tardias em sobreviventes de sepse.

Este estudo supõe que haja uma alteração em cascatas de complexos eventos que conduzam finalmente a disfunção do SNC e danos cognitivos tardios

observados em ratos sobreviventes de CLP. Alguns poucos estudos foram publicados na intenção de determinar os mecanismos moleculares associados os danos cognitivos tardios no contexto da medicina do cuidado crítico. Usando um modelo animal de meningite baseado em *S. pneumoniae* Irazuzta e colaboradores (2005) relataram que o tratamento com dexametasona em ratos com meningite bacteriana conduz a menor ativação da caspase 3 mas não da caspase 1 e estes foram associados com a preservação do desempenho neurocomportamental, porém neste estudo necessita-se ser muito cauteloso quando o desempenho da avaliação da aprendizagem e da memória tratados com dexametasona (Martins *et al*, 2005). Porém, a administração de hidrocortisona durante o choque séptico em uma dosagem similar à taxa de produção endogenosa foi associada com uma menor incidência de alterações pós-traumáticas de estresse em sobreviventes em longo prazo (Schelling *et al*, 2001).

O Grupo de Irazuzta tem demonstrado isto, embora a hipotermia possa estar reduzindo a resposta inflamatoria e biomarcadores de ferimento do cérebro não melhoram o desempenho neurocomportamental em um animal modelo de meningite bacteriana (Irazuzta *et al*, 2002).

Desde que o estresse oxidativo é associado ao desenvolvimento de doenças neurodegenerativas (Halliwell, 2006) sendo importante para o desenvolvimento da síndrome da disfunção dos múltiplos órgãos durante a sepse (Salvemini e Cuzzocrea, 2002) e suponha-se que poderia contribuir a longo prazo para os danos cognitivos em sobreviventes de sepse. O estresse Oxidativo foi demonstrado previamente dentro de modelos animais diferentes de doença crítica. Sener e colaboradores (2005) relataram que os danos oxidativos descritos reduzem o índice antioxidante em um modelo animal de sepse e determinando um efeito

antioxidante no ajuste. Nenhum estudo precedente correlacionam o estresse oxidativo do cérebro e danos cognitivos a longo prazo de sobreviventes de doenças críticas. A avaliação dos mecanismos e das cascatas moleculares sinalizadoras dos quais induzem eventos que conduzem finalmente aos danos no cérebro durante a sepse aperfeiçoaram os conhecimentos sobre a fisiopatologia da sepse e, mais importante, promoverá também o desenvolvimento de estratégias terapêuticas mais eficientes, para impedir as seqüelas neurológicas.

Os antioxidantes utilizados podem interferir com NO e seus metabólitos ou com espécies reativas de oxigênio que devem ter ligação com a disfunção mitocondrial da sepse. NAC pode atuar como *scavenger* direto de NO e peroxinitrito (Halliwell e Gutteridge 2007), além de o uso de NAC e DFX, como descrito anteriormente, pode interferir na geração de EAO e com isto diminuir o estresse oxidativo e preservar a função mitocondrial.

O uso de antioxidantes pode apresentar uma dupla face na sepse. Por um lado pode reduzir a resposta inflamatória e minimizar o dano oxidativo, mas a resposta inflamatória e necessária para a erradicação do patógeno, reparo dos tecidos envolvidos e ativação do sistema imune adquirido. Neste sentido cada vez mais se caracteriza e determina a importância da resposta antiinflamatória na sepse (Hotchkiss RS *et al* 2003). Por isso o uso de antioxidantes não pode ser visto como isento de risco. Reduzir a resposta inflamatória através de estratégias farmacológicas específicas já se mostrou ineficaz em estudos clínicos (Eichacker *et al* 2002). Portanto o tempo e a dose da administração de antioxidantes devem ser fundamentais na eficácia desta terapia, para que exista redução do dano oxidativo e mitocondrial e atenuação da resposta inflamatória, sem prejuízo da erradicação dos patógenos.

Neste contexto os resultados deste estudo sugerem uma incapacidade cognitiva em curto e longo prazo incluindo alterações do aprendizado e memória, além de sintomas ligados a depressão e, pela primeira vez, um papel dos danos oxidativos em períodos iniciais no SNC no desenvolvimento do dano cognitivo em curto e longo prazo. A associação de NAC e DFX pode ter um papel terapêutico para o dano oxidativo em hipocampo. Entretanto, é difícil com nossos dados determinar o exato mecanismo que leva ao estresse oxidativo e ao tratamento adequado com antioxidantes para reverter e prevenir este processo.

Limitações do estudo e perspectivas futuras

Os modelos de doenças inflamatórias empregados apresentam algumas limitações. O modelo de sepse, CLP, certamente é o mais empregado na literatura por mimetizar sepse abdominal de humanos, ainda mais quando se utiliza reposição volêmica e antibióticos, além de administrar as drogas de estudo após o desenvolvimento de sepse como em nosso estudo. Entretanto, diferente da prática clínica, em nosso modelo não removemos o foco infeccioso cirurgicamente por dificuldades na realização de tal procedimento nestes animais. Existe modelo recentemente desenvolvido, peritonite fecal por cateterização do cólon ascendente, que facilita a remoção cirúrgica do foco infeccioso (Lustig *et al* 2007). Entretanto, este modelo ainda não é bem estabelecido e o perfil de citocinas parece ser diferente do CLP (Maier *et al* 2004), sendo uma das possibilidades de expansão de nossos resultados no futuro.

A compreensão, mesmo que ainda não definitiva, dos fenômenos neurobiológicos envolvidos no dano cognitivo em sobreviventes a sepse, evoluiu

sobremaneira nesta última década. Os conceitos, contudo, não foram alterados; ocorreu, de fato, um aprofundamento do entendimento de seu substrato biológico. Os resultados compilados nesta tese compreendem uma pequena fração desse corpo de novos conhecimentos sobre os mecanismos que levam a incapacidade cognitiva. O novo desafio é compreender os mecanismos que levam ao dano cognitivo em sobreviventes a sepse e o surgimento de novas terapias que possam impedir este dano a curto e longo-prazo.

REFERÊNCIAS BIBLIOGRÁFICAS

Agranoff BW, Davis RE, Brink JJ. Memory fixation in the goldfish. Proc Natl Acad Sci USA 1965 54:788-93.

Al-Saidi F, McAndrews MP, Cheung AM, Tansey CM, Matte-Martyn A, Diaz-Granados N, Herridge M. Neuropsychological sequelae in ARDS survivors. Am J Respir Crit Care Med 2003 167:A737.

Alvarez S, Evelson PA. Nitric oxide and oxygen metabolism in inflammatory conditions: sepsis and exposition to polluted ambients. Front Biosci 2007 12: 964-74.

Ames BN, Shigenaga MK. Hagem, TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Science 1993 90:7915–7922.

Andrades M, Ritter C, Moreira JC, Dal-Pizzol F. Oxidative parameters differences during non-lethal and lethal sepsis development. J Surg Res 2005 125:68-72.

Andreazza AC, Soares DG, Kehl LF, Lima Borella ML, Salvador M. Transtornos neuropsiquiátricos e estresse oxidativo. in: Izquierdo I, Quevedo J, Kapczinski F. Bases Biológicas dos Transtornos Psiquiátricos. 1ed. Porto Alegre: Artmed, 2004. 489-496.

Angus DC, Musthafa AA, Clermont G, Griffin MF, Linde-Zwirble WT, Dremsizov TT, Pinsky MR. Quality-adjusted survival in the first year after the acute respiratory distress syndrome. American Journal of Respiratory and Critical Care Medicine 2001 163:1389-1394.

Arvidsson, U., Riedl, M., Elde, R., Meister, B. Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. J. Comp. Neurol 1997 378, 454–467.

Atis S, Nayci A, Ozge A, Comelekoglu U, Gunes S, Bagdatoglu O. N-acetylcysteine protects the rats against phrenic nerve dysfunction in sepsis. Shock 2006 25:30-5.

Azevedo LC, Janiszewski M, Soriano FG, Laurindo FR. Redox mechanisms of vascular cell dysfunction in sepsis. Endocr Metab Immune Disord Drug Targets 2006 6:159-64

Baker SK, Tarnoplski MA. Targeting cellular energy production in neurological disorders, Expert. Opin Investig Drugs 2003 12-1655-1679.

Basler T, Meier-Hellmann A, Bredle D, Reinhart K. Amino-acid imbalance early in septic encephalopathy. Intensive Care Med 2002 28:293–8.

Basu S, Eriksson M. Oxidative injury and survival during endotoxemia. FEBS Lett 1998 438:159-160

Beishuizen A, Thijs LG. Endotoxin and the hypothalamo-pituitary-adrenal (HPA axix). J Endotoxin Res 2003 9:3–24.

Bermpohl D, Halle A, Freyer D, Dagand E, Braun JS, Bechmann I, Schordcer NW, Weber JR. Bacterial programmed cell death of cerebral endothelial cells involves dual death pathways. J Clin Invest 2005115:1607-1615.

Beutler, E. Nutritional and Metabolic aspects of Glutathione. Annual Review of Nutrition 1989 9:287-302.

Bleck TP, Smith MC, Pierre-Louis SJ, Jares JJ, Murray J, Hansen CA. Neurologic complications of critical medical illnesses. Critical Care Medicine 1993 21:98–103.

Bone RC. Modulators of coagulation. A critical appraisal of their role in sepsis J Am Med Assoc 1992, 268:3452–3455.

Bone RC, Grodzin CJ, Balk RA. Sepsis: a new hypothesis for pathogenesis oh the disease process. Chest 1997 112:235-243

Booke M, Westphal M, Hinder F, Traber LD, Traber DL. Cerebral blood flow is not altered in sheep with Pseudomonas aeruginosa sepsis treated with norepinephrine or nitric oxide synthase inhibit. Anesth Analg 2003 96:1122–8.

Bowie RA, O'connor PJ, Mahajan RP. Cerebrovascular reactivity to carbon dioxide in sepsis syndrome. Anaesthesia 2003 58:261–79.

Bowton DL, Bertels NH, Prough DS, Stump DA. Cerebral blood flow is reduced in patients with sepsis syndrome. Crit Care Med 1989 17:399–403.

Bromont C, Mareie C, Bralet J. Increased lipid peroxidation in vulnerable brains regions after transient forebrain ischemia. Stroke 1989 20:918-924.

Brun-Buisson C. The epidemiology of the systemic inflammatory response. Intensive Care Medicine 2000, 26:S64–S74.

Calabrese V, Scapagnini G, Colombrita C, Ravagna A, Pennisi G, Giuffrida SAM, Galli F, Butterfield DA. Redox regulation of heat shock protein expression in aging neurodegenerative disorders associated with oxidative stress: nutritional approach. Amino Acids 2003 25:437-444.

Callahan LA, Nethery D, Stofan D, Dimarco A, Supinski G. Free radical induced contractile protein dysfunction in endotoxin induced sepse. Am J Respir Cell Mol Biol 2001 24: 210- 217.

Chrousos GP. The hypothalamic-pituitary-adrenal-axis and immune-mediated inflammation. N Engl J Med 1995, 332:1351-1362.

Chrousos GP, Gold PW. The concepts of stress and stress system disorders. Overview of physical and behavorial homeostasis. JAMA 1992 267:1244-1252.

Cohen J. The immunopathogenesis of sepsis. Nature 2002 420:885–891.

Cohen MV. Free radicals in ischemic and reperfusion myocardial injury: is this time for clinical trials?.Ann. International Med 1989 111:918-931.

Coyle JT, Puttfarcken P. Oxidative stress, glutamate and neurodegenerative disorders. Science 1993 262:689-695.

Cunningham C, Wilcockson DC, Campion S, Lunnon K, Perry VH. Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration. J Neurosci 2005 25:9275-9284

Cuzzocrea S, Rilley DP, Caputi AP, Salvenini A. Antioxidant theraphy: A new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. The American Society for Pharmacology and Experimental Therapeutics 2001 53:135-159.

Cuzzocrea S, Mazzon E, Di Paola R, Esposito E, Macarthur H, Matuschak GM, Salvemini D. A role for nitric oxide-mediated peroxynitrite formation in a model of endotoxin-induced shock. J Pharmacol Exp Ther 2006 319:73-81.

Czapski GA, Cakala M, Kopczuk D, Kaminska M, Strosznajder JB. Inhibition of nitric oxide synthase prevents energy failure and oxidative damage evoked in the brain by lipopolysaccharide. Pol J Pharmacol 2004 56:643–646

Dal-Pizzol F, Klamt F, Vianna MMR, Schroder N, Quevedo J, Benfato MS, Moreira JCF, Walz R. Lipid peroxidation in hippocampus early and late after status epilepticus induced by pilocarpine or kainic acid in Wistar rats. Neurosci Lett 2002 91:179-182.

Dal-Pizzol F, Klamt F, Benfato MS, Bernanrd EA, Moreira JCF. Retinol supplementation induces oxidative stress and modulates antioxidant enzyme activities in rat Sertoli cells. Free Radic Res 2001 34: 395-404.

Dantzer R, Bluthe RM, Gheusi G, Cremona S, Laye S, Parnet P, Kelley KW. Molecular basis of sickness behavior. Ann NY Acad Sci 1998 856:132–138.

Descamps L, Coisne C, Dehouck B, Cecchelli R, Torpier G. Protective effect of glial cells against lipopolysaccharide-mediated blood barrier injury. Glia 2003 42:46–58.

Desidere A, Falconi M. Prokaryotic Cu, Zn Superoxide dismutases. Biochem Soc Trans 2003 31:1322-1325.

Dinarello CA. Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. J Endotoxin Res 2004 10:201–222.

Dix TA, Hess KM, Medina MA, Sullivan RW, Tilly SL, Webb LL. Mechanism of site-selective DNA nicking by the hydrodioxyl (perhydroxyl) radical. Biochemistry 1996 35:4578-4583.

Dixon, C.E., Kochanek, P.M., Yan, H.Q., Schiding, J.K., Griffith, R.G., Baum, E., Marion, D.W., DeKosky, S.T. One-year study of spatial memory performance, brain morphology, and cholinergic markers after moderate controlled cortical impact in rats. J. Neurotrauma 1999 16, 109–122.

Durant R, Klouche K, Delbosc S, Morena M, Amigues L, Beraud JJ, Canaud B, Cristol JP. Superoxide anion overproduction in sepsis: effects of vitamin e and simvastatin. Shock 2004 22:34-9.

Eaton JW. Catalases and peroxidases and glutathione and hydrogen peroxide: mysteries of the bestiary. Journal Lab. Clin. Méd 1991 118:.3-4.

Ferreira ALA, Matsubara LS. Radicais livres: conceitos doenças relacionadas, sistema de defesa e estresse oxidativo. Revista da Associação Medica Brasileira 1997 43:61-68.

Eckenstein, F.P., Baughman, R.W., Quinn, J. An anatomical study of cholinergic innervation in rat cerebral cortex. Neuroscience 1988 25: 457–474.

Eichacker PQ, Parent C, Kalil A, Espósito C, Cui X, Banks SM, Gerstenberger EP, Fitz Y, Danner RL, Natanson C. Risk and the Efficacy of Antiinflammatory Agents:

Retrospective and Confirmatory Studies of Sepsis Am. J. Respir. Crit. Care Med 2002 166:1197-1205.

Emet S, Memis D, Pamukcu Z. The influence of N-acetyl-L-cystein infusion on cytokine levels and gastric intramucosal pH during severe sepsis. Crit Care 2004 8:R172-9

Fernandes D, da Silva-Santos JE, Duma D, Villela CG, Barja-Fidalgo C, Assreuy J. Nitric oxide-dependent reduction in soluble guanylate cyclase functionality accounts for early lipopolysaccharide-induced changes in vascular reactivity. Mol Pharmacol 2006 69:983-90

Floyd RA. Antioxidants, oxidative stress, and degenerative neurological disorders. Proc. Soc. Exp. Biol. Med 1999 222:236-245.

Floyd RA, Hensley K. Oxidative stress in brain aging implications for therapeutics of neurodegenerative diseases, Neurobiology of Aging 2002 23:795-807.

Freund HR, Muggia-Sullam M, Peiser J, Melamed E. Brain neurotransmitter profiled is deranged during sepsis and septic encephalopathy in the rat. J Surg Res 1985 38:267–71.

Frey U, Schollmeier K, Reymann KG, Seidenbecher T. Asymptotic hippocampal long-term potentiation in rats does not preclude additional potentiation at later phases. Neuroscience 1995 67:799-807.

Friedman G, Silva E, Vincent JL. Has the mortality of septic shock changed with time? Critical Care Medicine1998 26:2078–2086.

Fijimura N, Sumita S, Aimono M, Masuda Y, Schichinoche Y, Narimatsu E, Namiki A. Effect of free radical scavengers on diaphragmatic contractility in septic peritonitis. Am J Respir Crit Care Med 2000 162: 2159-2165.

Gage, F.H., Bjorklund, A., Stenevi, U. Reinnervation of the partially deafferented hippocampus by compensatory collateral sprouting from spared cholinergic and noradrenergic afferents. Brain Res 1983 268, 27–37.

Garcia-Ruiz C, Colell A, Mari M, Morales A, Fernandez-Checa JC. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. J Biol Chem 1997 272:11369–77.

Gevich V, Ma XM, Jirikowski G, Verbalis J, Agulera G. Lipopolysaccharide endotoxin potentiates the effect of osmotic stimulation on vasopressin synthesis and secretion in the rat hypothalamus. J Neuroendocrinol 2003 15:141–9.

Cighetti G, Paroni R, Marzorati S, Borotto E, Giudici R, Magnanini G, Iapichino G. Evaluation of oxidative stress in serum of critically ill patients by a commercial assay and gas chromatography-mass spectrometry. Clin Chem 2005 51:1515-7.

Gilgum-Sherki Y, Rosenbaum Z, Melamed E, Offen D. Antioxidant theraphy in acute central nervous injury: current state. Pharmacology Review 2002 54:271-284.

Gilmor, M.L., Nash, N.R., Roghani, A., Edwards, R.H., Yi, H., Hersch, S.M., Levey, A.I. Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. J. Neurosci 1996 16, 2179–2190.

Gold PE. The use of avoidance training is studies of modulation of memory storage. Behav Neural Biol 1986 46:87-98

Goode HF, Cowley HC, Walker BE, Howdle PD, Webster NR. Decreased antioxidant status and increased lipid peroxidation in patients with septic shock and secondary organ dysfunction. Crit Care Med 1995 23:646-51.

Gordon SM, Jackson JC, Ely EW, Burger C, Hopkins RO. Clinical identification of cognitive impairment in ICU survivors: insights for intensivists. Intensive Care Medicine 2004 30:1997-2008

Granja C, Dias C, Costa-Pereira A, Sarmento A. Quality of life of survivors from severe sepsis and septic shock may be similar to that of others who survive critical illness. Critical Care 2004 8:91-98

Granja C, Lopes A, Moreira S, Dias C, Costa-Pereira A, Carneiro A. JMIP Study Group: Patients' recollections of experiences in the intensive care unit may affect their quality of life. Critical Care 2005 9:96-109.

Halliwell B, Gutterigde JMC. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 1990 186:1-85.

Halliwell B, Gutterigde JMC, John MC. Free Radicals in Biology and Medicine. 2ed. New York: Oxford University Press 1999.

Halliwell B. Oxidative stress and neurodegeneration: where are we now? J Neurochem 2006, 97:1634-1658

Halliwell B, Gutteridge J. Free Radicals in Biology and Medicine. Oxford University Press. Fourth Edition, 2007.

Hart RP, Kwentus JA, Taylor JR, Harkins SW. Rate of forgetting in dementia and depression. J Consult Clin Psychol 1997 55:101–105.

Heidbreder, C.A., Groenewegen, H.J. The medial prefrontal cortex in the rat: evidence for a dorso-ventral distinction based upon functional and anatomical characteristics. Neurosci. Biobehav 2003 27, 555–579.

Hein OV, Ohring R, Schilling A, Oellerich M, Armstrong VW, Kox WJ, Spies C. N-acetylcysteine decreases lactate signal intensities in liver tissue and improves liver function in septic shock patients, as shown by magnetic resonance spectroscopy: extended case report. Crit Care 2004 8:R66-71.

Hensley K, Pye QN, Maidt ML, Stewart CA, Robinson KA, Jaffreyt F. Interaction of α-phenyl-N-tert-butylnitrone and alternative electron acceptors with complex 1 indicates

a substrate reduction site upstream from the rotenone binding site. Journal Neurochemistry 1998 71:2549-2557.

Hollenberg SM, Dumasius A, Easington C, Colilla SA, Neumann A, Parrillo JE. Characterization of a hyperdynamic murine model of resuscitated sepsis using echocardiography. Am J Respir Crit Care Med 2001 164:891-5.

Holub M .Sepsis: infection and systemic inflammatory response. Cas Lek Cesk, 2007 46:109-14.

Hopkins R.O, Weaver LK, Collingridge D, Parkinson RB, Chan KJ, Orme Jr JF. Two-year cognitive, emotional, and quality-of-life outcomes in acute respiratory distress syndrome. American Journal of Respiratory and Critical Care 2005 171:340-347.

Hopkins RO, Weaver LK, Pope D, Orme JF, Bigler ED, Larson-Lohr V. Neuropsychological sequelae and impaired health status in survivors of severe acute respiratory distress syndrome. Am J Respir Crit Care Med 1999 160:50–56.

Hopkins RO, Weaver LK, Chan KJ, Orme Jr JF. Quality of life, emotional, and cognitive function following acute respiratory distress syndrome. J Int Neuropsychol Soc 2004 10:1005– 1017.

Hopkins RO, Jackson JC. Long-term Neurocognitive Function After Critical Illness. Chest 2006a 130:869–878

Hopkins RO, Gale SD, Weaver LK. Brain atrophy and cognitive impairment in survivors of acute respiratory distress syndrome. Brain Inj 2006b; 20:263–271

Hough CL, CurtisJR. Long-term sequelae of critical illness: memories and health-related quality of life. Critical Care 2005 9:145-6.

Hotchkiss RS, Karl IK. The pathophysiology and treatment of sepsis. The New England Journal of Medicine 2003 348:138–150.

Hsu BG, Lee RP, Yang FL, Harn HJ, Chen HI. Post-treatment with N-acetylcysteine ameliorates endotoxin shock-induced organ damage in conscious rats. Life Sci 2006 79:2010-6

Irazuzta J, Pretzlaff R, Xue V, Zemlan F, Zingarelli B. Modulation of NF-kB activation and decreased markers of neurological injury associated with hypothermic therapy in experimental bacterial meningitis. Crit Care Med 2002 30:2553–2559.

Irazuzta J, Pretzlaff RK, Courten-Myers G, Zemlan F, Zingarelli B. Dexamethasone decreases neurological sequelae and caspase activity. Intensive Care Med 2005 31:146–150.

Iwata-Ichikawa E, Kondo Y, Miyazaki I, Asanuma M, Ogawa N . Glial cells protect neurons against oxidative stress via transcriptional up-regulation of glutathione synthesis. Journal Neurochemistry 19997 2:2344-2344.

Jackson AC, Gilbert JJ, Young GB, Bolton CF. The encephalopathy of sepsis. Can J Neurol Sci 1985 12:303–7.

Jackson JC, Gordon SM, Ely EW, Burger C, Hopkins RO. Research issues in the evaluation of cognitive impairment in intensive care unit survivors. Intensive Care Medicine 2004 30:2009-2016.

Jackson JC, Hart RP, Gordon SM, Shintani A, Truman B, May L, Ely EW . Six-month neuropsychological outcome of medical intensive care unit patients. Crit Care Med 2003a 31:1226–1234

Jackson JC, Gordon SM, Burger C, et al. Acute respiratory distress syndrome and long-term cognitive impairment: a case study. Arch Clin Neuropsychol 2003b; 18:688

Jao HC, Lin YT, Tsai LY, Wang CC, Liu HW, Hsu C. Early expression of heme oxygenase-1 in leukocytes correlates negatively with oxidative stress and predicts hepatic and renal dysfunction at late stage of sepsis. Shock 2005 23:464-9.

Jones RD, Tranel D, Benton A, Paulsen J. Differentiating dementia from pseudo-dementia early in the clinical course: utility of neuropsychological tests. Neuropsychology 1992 6:13–21.

Jarrard, L.E. What does the hippocampus really do? Behav. Brain Res 1995 71, 1–10.

Jones C, Griffiths RD, Slater T. Significant cognitive dysfunction in non-delirious patients identified during and persisting following critical illness. Intensive Care Med 2006 32:923–926

Kadoi Y, Saito S, Kunimoto F, Imai T, Fujita T. Impairment of the brain beta-adrenergic system during experimental endotoxemia. J Surg Res 1996 61:496–502.

Koksal GM, Sayilgan C, Aydin S, Oz H, Uzun H. Correlation of plasma and tissue oxidative stresses in intra-abdominal sepsis. J Surg Res 2004 122:180-3.

Kong CW, Tsai K, Chin JH, Chan WL, Hong CY. Magnolol attenuates peroxidative damage and improves survival of rats with sepsis. Shock 2000 13:24-8.

Komatsubara T, Kadoi Y, Saito S. Augmented sensitivity to benzodiazepine in septic shock rats. Can J Anesth 1995 42:937–43.

Kong CW, Tsai K, Chin JH, Chan WL, Hong CY. Magnolol attenuates peroxidative damage and improves survival of rats with sepsis. Shock 2000 13:24-8.

Korcok J, Wu F, Tyml K, Hammond RR, Wilson JX. Sepsis inhibits reduction of dehydroascorbic acid and accumulation of ascorbate in astroglial cultures: Intracellular ascorbate depletion increases nitric oxide synthase induction and glutamate uptake inhibition. J Neurochem 2002 81:185–193

Koutsilieri E, Scheller C, Tribl F, Riederer P. Degeneration of neuronal cells due to oxidative stress-microglial contribuition. Parkinsonism and Related Disorders 2002 8:401-406.

Larsson A, Lipcsey M, Sjolin J, Hansson LO, Eriksson MB. Slight increase of serum S-100B during porcine endotoxemic shock may indicate bloodbrain barrier damage. Anesth Analg 2005 101:1465–9.

Lehnardt S, Henneke P, Lien E, Kasper DL, Volpe JJ, Bechmann I, Nitsch R, Weber JR, Golenbock DT, Vartanian T. A mechanism for neurodegeneration induced by group B streptococci through activation of the TLR2/MyD88 pathway in microglia. J Immunol 2006, 177:583-52.

Licinio J, Wong ML. Interleukin 1 receptor antagonist gene expression in rat pituitary in the systemic inflammatory response syndrome: pathophysiological implications. Mol Psychiatry 1997 2:99–103.

Lissi E, Salim-Hanna M, Pascual C, Castillo MD. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. Free Radic Biol Med 1995 18:153-158.

Llesuy SF. Introducción y Especies Activas de Oxígeno. In Marrón N. P. Estresse Oxidativo e Antioxidantes. 1ed. Canoas: ULTRA 2002 21-32.

Lustig MK, Bac VH, Pavlovic D, Maier S, Grundling M, Grisk O, Wendt M, Heidecke CD, Lehmann C. Colon ascendes stent peritonitis - A model of sepsis adopted to the rat: physiological, microcirculatory and laboratory changes. Shock 2007 in press.

Macarthur H, Westffal TC Riley DP. Misko TP, Salvemini D. Inativaction of catecholamines by superoxide gives new insigts on the pathogenesis of septic shock. Proc. Natl. Acad. Science 2000 97:9753-9758.

Macarthur H, Westffal TC Riley DP. Misko TP, Salvemini D. Inactivation of catecholamines by superoxide gives new insights on the pathogenesis of septic shock. Proc Natl Acad Sci USA 2000 97:9753-9758.

Maier S, Traeger T, Entleutner M, Westerholt A, Kleist B, Huser N, Holzmann B, Stier A, Pfeffer K, Heidecke CD. Cecal ligation and puncture versus colon ascendens stent peritonitis: two distinct animal models for polymicrobial sepsis. Shock 2004 21:505-11.

Magistrelli Pierre. Brain Energy Metabolism, in: Squire LR. Bloom FE, McConnel SK. Fundamental Neuroscience 1999 2 ed., U.S.A, 339-360.

Maier SF, Goehler LE, Fleshner M, Watkins LR. The role of the vagus nerve in cytokine-to-brain communication. Ann NY Acad Sci 1998 840:289–300.

Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Méd 2005 348:1546–1554

Martins MR, Barichello T, Ritter C, Reinke A, Quevedo J, Dal-Pizzol F. Dexamethasone therapy and memory performance. Intensive Care Med 2005 31:1001.

Massman PJ, Delis DC, Butters N, Dupont RM, Gillin JC. The subcortical dysfunction model of memory deficits in depression: neuropsychological validation in a subgroup of patients. J Clin Exp Neuropsychol 1992 14:687–706.

Mastronardi CA, Yu WH, Rettori V, McCann S. Lipopolysaccharide-induced leptin release is not mediated by nitric oxide, but is blocked by dexamethasone. Neuroimmunomodulatio 2000 n 8:91–97

Mastronardi CA, Yu WH, McCann S. Lipopolysaccharide-induced tumor necrosis factor-alpha release is controlled by the central nervous system. Neuroimmunomodulation 2001 9:148– 156.

Mastronardi CA, Yu WH, Srivastava VK, Dees WL, McCann S. Lipopolysaccharide-induced leptin release is neurally controlled. Proc Natl Acad Sci USA 2001 98:14720–14725.

Mastronardi CA, Srivastava V, Yu WH, Les Dees W, McCann SM. Lipopolysaccharide-induced leptin synthesis and release are differentially controlled by alpha-melanocyte-stimulating hormone. Neuroimmunomodulation 2005, 12:182–188.

Matejovic M, Krouzecky A, Martinkova V, Rokyta R Jr, Radej J, Kralova H, Treska V, Radermacher P, Novak I. Effects of tempol, a free radical scavenger, on long-term hyperdynamic porcine bacteremia. Crit Care Med 2005 33:1057-63.

Matthies H. Plasticity in the nervous system: an approach to memory research. In: Ajmone-Marsan C, Matthies H, editors. Neuronal plasticity and memory formation. New York: Raven Press 1982 1982. p. 1-15.

Matthies H. In search of the cellular mechanisms of memory. Prog Neurobiol 1989 32:277-349.

McGlynn SM, Schacter DL. Unawareness of deficits in neuropsychological syndromes. J Clin Exp Neuropsychol 1989 11:143–205.

Mello Filho AC, Hoffman ME, Meneghini R. Cell Killing and DNA damage by hydrogen peroxide are mediate by intracellular iron. Journal Biochemistry 1993 218:273-275.

Mega MS, Cummings JL, Salloway S, Malloy P. The limbic system: an anatomic, phylogenetic, and clinical perspective. J Neuropsychiatry Clin Neurosci 1997 9:315-30.

Messaris E, Memos N, Chatzigianni E, Konstadoulakis MM, Menenakos E, Katsaragakis S, Voumvourakis C, Androulakis G.Time-dependent mitochondrial-mediated programmed neuronal cell death prolongs survival in sepsis. Crit Care Med 2004a 32:1764–1770.

Messaris E, Antonakis PT, Memos N, Chatzigianni E, Leandros E, Konstadoulakis MM. Deferoxamine administration in septic animals: improved survival and altered apoptotic gene expression. Int Immunopharmacol 2004b 4:455-9.

Mesulam, M.M., Mufson, E.J., Wainer, B.H., Levey, A.I., 1983. Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1–Ch6). Neuroscience 10, 1185–1201.

Mishra V, Baines M, Wenstone R, Shenkin A. Markers of oxidative damage, antioxidant status and clinical outcome in critically ill patients. Ann Clin Biochem 2005 42:269-76

Mogensen, J., Moustgaard, A., Khan, U., Wortwein, G., Nielsen, K.S., 2005. Egocentric spatial orientation in a water maze by rats subjected to transaction of the fimbria-fornix and/or ablation of the prefrontal cortex. Brain Res. Bull. 65, 41–58.

Moosmann B, Behl C. Antioxidants as treatment for neurodegenerative disorders. Expert. Opin Investig Drugs 2002 10:1407-1435.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM (2002).Neurobiology of depression. Neuron, 28:13-25

Oliver CN, Starke-Reed PE, Stadtman ER, Liu GJ, Carney JM, Floyd RA. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. Proc. Natl. Acad. Science USA 1990 87:5144-5157.

Orlikowski D, Sharshar T, Annane D. The brain in sepsis. Advances in sepsis 2003 3:4–8.

Ozdulger A, Cinel I, Koksel O, Cinel L, Avlan D, Unlu A, Okcu H, Dikmengil M, Oral U. The protective effect of N-acetylcysteine on apoptotic lung injury in cecal ligation and puncture-induced sepsis model. Shock 2003 19:366-72.

Pahan K, Sheikh FG, Namboodiri AM, Singh I. N-acetyl cysteine inhibits induction of no production by endotoxin or cytokine stimulated rat peritoneal macrophages, C6 glial cells and astrocytes. Free Radic Biol Med 1998 24:39-48.

Papadopoulos MC, Davies DC, Moss RF, Tighe D, Bennett E.D. Pathophysiology of septic encephalopathy: a review. Crit Care Med 2000 28:3019-3024.

Protti A, Singer M. Bench-to-bedside review: potential strategies to protect or reverse mitochondrial dysfunction in sepsis-induced organ failure. Crit Care 2006 10:228.

Rao AV, Balachandran B. Role of oxidative stress and antioxidants in neurodegenerative diseases. Nutr Neuroscience 2002 5:291-309.

Ravata JL, Berger M, Benard F, Langlois R, Ouellet R, Van Lier JE, Cadet J. Photochem. Photobiology 1992 55:809-814.

Rice ME, Russo-Mema I. Differential compartmentalization of brain ascorbate and glutathione between neurons and glia. Neuroscience 1998 82:1213-1222.

Richards PM, Ruff RM. Motivational effects on neuropsychological functioning: comparison of depressed versus nondepressed individuals. J Consult Clin Psychol 1989 57:396–402.

Ritter C, Andrades ME, Reinke A, Menna-Barreto S, MoreiraJC, Dal-Pizzol F. Treatment with N-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis. Crit Care Med 2004 32:342-349.

Salvemini D, Cuzzocrea S. Oxidative stress in septic shock and disseminated intravascular coagulation. Free Radic Biol Med 2002 33:1173-1185.

Saper CB, Breder CD. The neurologic basis of fever. N Engl J Méd 1994 330:1880-1886.

Salvemini D, Cuzzocrea S. Therapeutic potential of superoxide dismutase mimetics as therapeutic agents in critical care medicine. Crit Care Med 2003 31:S29-38.

Sammler A, Frisch C, Debeir T, Ramanathan, Okulla T, Klockgether, Heneka MT. Long-term cognitive impairment, neuronal loss and reduced cortical cholinergic innervation after recovery from sepsis in a rodent model. Experimental Neurology 2007 204: 233-240

Sasse KC, Nauenberg E, Long A, Anton B, Tucker HJ, Hu TW. Long-term survival after intensive care unit admission with sepsis Crit CareMed 1995 23:1040–1047.

Schelling G, Stoll C, Haller M, Briegel J, Manert W, Hummel T, Lenhart A, Heyduck M, Polasek J, Meier M, Preuss U, Bullinger M, Schuffel W, Peter K. Health-related quality of life and posttraumatic stress disorder in survivors of the acute respiratory distress syndrome. Crit Care Med 1998 26:651–659.

Schelling G, Briegel J, Roozendaal B, Stoll C, Rothenhausler HB, Kapfhammer HP. The effect of stress doses of hydrocortisone during septic shock on posttraumatic stress disorder in survivors. Biol Psychiatry 2001 50:978–985.

Scragg P, Jones A, Fauvel N. Psychological problems following ICU treatment. Anaesthesia 2001 56:9–14

Semmler, A., Okulla, T., Sastre, M., Dumitrescu-Ozimek, L., Heneka, M.T. Systemic inflammation induces apoptosis with variable vulnerability of different brain regions. J. Chem. Neuroanat 2005 30, 144–157.

Sener G, Toklu H, Kapucu C, Ercan F, Erkanli G, Kacmaz A, Tilki M, Yegen BC. Melatonin protects against oxidative organ injury in a rat model of sepsis. Surg Today 2005 35:52-59.

Sharshar T, Annane D, Grandmaison G de la, Brouland JP, Hopkinson NS, Gray F . The neuropathology of septic shock. Brain Pathol 2004 14:21-33.

Sharshar T, Gray F, Lorin de la Grandmaison G, Hopkinson NS, Ross E, Dorandeu A, Orlikowski D, Raphael J-C, Gajdos P, Annane D. Apoptosis of neurons in

cardiovascular autonomic centres triggered by inducible nitric oxide synthase after death from septic shock. Lancet 2003 362:1799-1805.

Siskind LJ, Kolesnick RN, Colombini M. Ceramide channels increase the permeability of the outer mitochondrial membrane to small proteins. J. Biol. Chem 277 2002 24:26796–803.

Skozol JW, Vender JS. Anxiety, delirium, and pain in an intensive care unit. Crit Care Clin 2001 17:821–842.

Soejima Y, Fujii Y, Ishikawa T, Takeshita H, Maekawa T. Local cerebral glucose utilization in septic rats. Crit Care Med 1990 18:423–7.

Squire, L.R., Zola-Morgan, S., 1991. The medial temporal lobe memory system. Science 253, 1380–1386.

Sprung CL, Peduzzi PN, Shatney CH, Schein RM, Wilson MF, Sheagren JN, Hinshaw LB. Impact of encephalopathy on mortality in the sepsis syndrome. The Veterans Administration Systemic Sepsis Cooperative Study Group. Critical Care Medicine 1990 18:801–806.

Sprong RC, Winkelhuyzen-Janssen AM, Aarsman CJ, van Oirschot JF, van der Bruggen T, van Asbeck BS. Low-dose N-acetylcysteine protects rats against endotoxin-mediated oxidative stress, but high-dose increases mortality. Am J Respir Crit Care Med 1998 157:1283-93.

Spyer KM. Neural mechanisms involved in cardiovascular control during affective behaviour. Trends Neurosci 1989 12:506-513.

Suchyta MR, Hopkins RO, White J, et al. The incidence of cognitive dysfunction after ARDS [abstract]. Am J Respir Crit Care Med 2004; 169:A18

Sukantarat KT, Burgess PW, Williamson RC, Brett SJ. Prolonged cognitive dysfunction in survivors of critical illness. Anaesthesia 2005 60:847–853.

Supinski G, Nethery D, DiMarco A. Effect of free radical scavengers on endotoxin-induced respiratory muscle dysfunction. Am Rev Respir Dis 1993 148:1318-24.

Supinski GS, Callahan LA. Polyethylene glycol-superoxide dismutase prevents endotoxin-induced cardiac dysfunction. Am J Respir Crit Care Med 2006 173:1240-7.

Takala A, Nupponen I, Kylanpaa-Back ML, Repo H Markers of inflammation in sepsis Ann Med 2002. 34:614–623.

Tran DD, Groeneveld AB, Van Der Meulen J, Nauta JJ, Strack RJ, Van Schijndel, Thijs LG. Age, chronic disease, sepsis, organ system failure, and mortality in a medical intensive care unit. Critical Care Medicine 1990 18:474–479.

Xiang L, Klintman D, Thorlacius H. Allopurinol inhibits CXC chemokine expression and leukocyte adhesion in endotoxemic liver injury. Inflamm Res 2003 52:353-8.

Young GB, Bolton CF, Austin TW, Archibald YM, Gonder J, Wells GA. The encephalopathy associated with septic illness. Clinical and Investigative Medicine 1990 13:297–304.

Weinert CR, Gross CR, Kangas JR. Health-related quality of life after acute lung injury. Am J Respir Crit Care Med 1997 156:1120–1128

Wheeler AP, Bernard GR. Treating patients with severe sepsis. N Engl J Méd 1999 340:207-214.

Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock--a review of laboratory models and a proposal. J Surg Res 1980 29:189-201.

Williams KJ, Licinio J, Tabas I. Acute systemic inflammation up-regulates secretory sphingomyelinase in vivo: a possible link between inflammatory cytokines and atherogenesis. Proc Natl Acad Sci US 2000 97:8681–8686.

Wong ML, Bongiorno PB, Rettori V, McCann SM, Licinio J. Interleukin (IL) 1beta, IL-1 receptor antagonist, IL-10, and IL-13 gene expression in the central nervous system

and anterior pituitary during systemic inflammation: pathophysiological implications. Proc Natl Acad Sci USA 1997 94:227–232.

Wong ML, Rettori V, al-Shekhlee A, Bongiorno PB, Canteros G, McCann SM, Gold PW, Licinio J. Inducible nitric oxide synthase gene expression in the brain during systemic inflammation. Nat Med 1996 2:581–584.

Wong ML, O'Kirwan F, Khan N, Hannestad J, Wu KH, Elashoff D, Lawson G, Gold PW, McCann SM, Licinio J. Identification, characterization, and gene expression profiling of endotoxin-induced myocarditis. Proc Natl Acad Sci USA 2003 100:14241–1424

Vandijck D, Decruyenaere JM, Blot SI. The value of sepsis definitions in daily ICU-practice. Acta Clin Belg 2006 5:220-6

Victor VM, Rocha M, De la Fuente M. N-acetylcysteine protects mice from lethal endotoxemia by regulating the redox state of immune cells. Free Radic Res 2003 37:919-29.

Voigt K, Kontush A, Stuerenburg HJ, Muench-Harrach D, Hansen HC, Kunze K. Decreased plasma and cerebrospinal fluid ascorbate levels in patients with septic encephalopathy. Free Radic Res 2002 36:735–9.

Von Haefen C, Weider T, Gillissen B, Starck L, Graupner V, Dorken B, Daniel PT. Ceramide induces mitochondrial activation and apoptosis via a Bax-dependent pathway in human carcinoma cells. Oncogene 2002 21:4009-19.

Zhang H, Slursky AS, Vincent JL. Oxygen free radicals in ARDS, septic shock and organ dysfunction. Intensive Care Med 2000 26:474 –476.