Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Curso de Pós-Graduação em Ciências Biológicas: Bioquímica

Estresse oxidativo e desenvolvimento de doenças inflamatórias agudas: possível papel terapêutico de antioxidantes

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PORTO ALEGRE 2007

“Quando a febre é contínua, a superfície externa do corpo está fria, e existe internamente uma grande sensação de calor e sede, a afecção é mortal”
(Hipócrates, 400 A.C.)**”**

Agradecimentos

Em especial ao meu marido pelo seu amor e pelos seus conhecimentos. Você é parte fundamental na minha vida, sem você não teria chegado até aqui.

Aos meus filhos maravilhosos, que são o motivo de meu empenho, que aceitam a minha ausência sempre que a mamãe sai para trabalhar.

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Aos meus pais pelos seus ensinamentos e por me proporcionarem condições para eu chegar até aqui.

Lista de abreviaturas

CARS – compensatory antiinflammatory response syndrome

CAT – catalase

CCl4 – tetracloreto de carbono

CLP – ligação cecal e perfuração

CuZnSOD – cobre/zinco superóxido dismutase

DFX – deferoxamina

DHL – desidrogenase lática

DNA – ácido desoxiribonucleico

EAO – espécies ativas de oxigênio

Fe^{2+} - ferro II

Fe^{3+} - ferro III

GMPc – ganilil monofosfato cíclico

GPx – glutationa peroxidase

GSH – glutationa reduzida

GSSG – glutationa oxidada

H^+ - próton

H_2O – água

H_2O_2 - peróxido de hidrogênio

IkB – inibidor do fator nuclear kB

IL – interleucina

iNOS – óxido nítrico sintase

LPA – lesão pulmonar aguda

LPS – lipopolissacarídeo

MnSOD – manganês superóxido dismutase

NAC – N-acetilcisteína

Na/K ATPase – sódio/potássio ATPase

NF-kB – fator nuclear kB

NO – óxido nítrico

O_2 – oxigênio molecular

O_2^- - ánion superóxido

OH^- - anion hidroxila

•OH - radical hidroxil

PaO2/FiO2 – relação entre pressão arterial de oxigênio e fração inspirada de oxigênio

PARS - poli-ADP ribose polimerase

ROOH – peróxido orgânico

SARA – síndrome da angústia respiratória aguda

SIRS – síndrome da resposta inflamatória sistêmica

SNC – sistema nervoso central

SOD – superóxido dismutase

TBARS – substâncias reativas ao ácido tiobarbitúrico

TNF – fator de necrose tumoral

Resumo

O estresse oxidativo tem um papel importante no desenvolvimento de diferentes doenças inflamatórias agudas, mas intervenções que diminuem a geração ou os efeitos das espécies reativas de oxigênio têm papel controvérsio em modelos animais e humanos. Neste trabalho avaliamos os efeitos de uma combinação de antioxidantes (N-acetilcisteína e deferoxamina) em diferentes modelos animais de doenças inflamatórias agudas: sepse induzida por ligação cecal e perfuração (CLP), insuficiência hepática aguda e lesão pulmonar aguda. No modelo de CLP os ratos tratados com antioxidantes apresentaram uma significativa redução na atividade de mieloperoxidase (como marcador de infiltração neutrofílica) e uma menor quantidade de espécies reativas ao ácido tiobarbitúrico em diferentes órgãos envolvidos na resposta à sepse. A produção mitocondrial de superóxido foi significativamente diminuída pelo tratamento antixodante. Além disto o tratamento melhora o equilíbrio entre superóxido dismutase e catalase e melhora a sobrevida dos animais submetidos a CLP. No modelo de insuficiência hepática aguda o tratamento com antioxidantes significativamente diminui o dano oxidativo hepático e no sistema nervoso central, atenua marcadores de insuficiência hepática e melhora a sobrevida dos animais. No modelo de lesão pulmonar aguda o tratamento com N-acetilcisteína e deferoxamina diminui o conteúdo de proteínas do lavado bronco-alveolar, assim como o influxo de células inflamatórias, marcadores de dano oxidativo e citocinas inflamatórias. Em conclusão, nossos dados demonstram pela primeira vez que o tratamento com N-acetilcisteína e deferoxamina é superior ao uso isolado destes antioxidantes em diferentes modelos animais de doenças inflamatórias agudas. Este efeitos é parcialmente

secundário a uma diminuição do estresse oxidativo, disfunção mitocondrial e resposta inflamatória no rato.

Abstract

Oxidative stress plays an important role in the development of acute inflammatory diseases, but interventions that reduce the generation or the effects of reactive oxygen species exert controversial effects in animal models and in humans. Here we have evaluated the effects of a combination of antioxidants (*N*-acetylcysteine plus deferoxamine) in a murine model of polymicrobial sepsis induced by cecal ligation and puncture (CLP), in a murine model of acute hepatic failure and in a murine model of acute lung injury. In the CLP model of sepsis rats treated with antioxidants had significantly lower myeloperoxidase activity (as an index of neutrophil infiltration) and thiobarbituric acid reactive species formation in several organs involved in septic response. Mitochondrial superoxide production was significantly reduced by antioxidant treatment. Furthermore, antioxidants significantly improved the balance between catalase and superoxide dismutase activities, and improved survival to 66%. In the acute hepatic failure model, *N*-acetylcysteine plus deferoxamine treatment significantly attenuated hepatic and central nervous system oxidative damage, attenuated markers of hepatic failure and improves survival to 80%. In the acute lung injury model, N-acetylcysteine plus deferoxamine decreased bronchoalveolar lavage fluid protein, inflammatory cells, oxidative damage variables, and proinflammatory cytokines. In conclusion, our data provide the first experimental demonstration that N-acetylcysteine plus deferoxamine is superior to each antioxidant used alone in different animal models of acute inflammatory diseases. This effect is partially secondary to a decrease in oxidative stress, mitochondrial dysfunction and inflammatory response in the rat.

Sumário

1- INTRODUÇÃO

1.1- Espécies ativas de oxigênio:

O oxigênio é ao mesmo tempo necessário e tóxico aos organismos. Em organismos aeróbios o oxigênio é necessário para a respiração celular por ser o aceptor final da cadeia de transporte de elétrons, sendo então reduzido à água. A característica do elemento oxigênio é a presença de dois elétrons não pareados com spins paralelos (Halliwell e Gutteridge, 2007).

A molécula de oxigênio pode aceitar um total de quatro elétrons para ser reduzida a duas moléculas de água, o que requer uma alta energia de ativação, porque para que isso ocorra é necessário que um dos elétrons do oxigênio ou do substrato inverta seu spin. Entretanto, a estrutura eletrônica do oxigênio permite sua redução em “passos de um único elétron”, assim, sendo reduzido em um elétron de cada vez, a reação não fica sujeita a essa barreira cinética, mas leva à geração das espécies ativas de oxigênio (EAO), conforme demonstrado na figura 1.

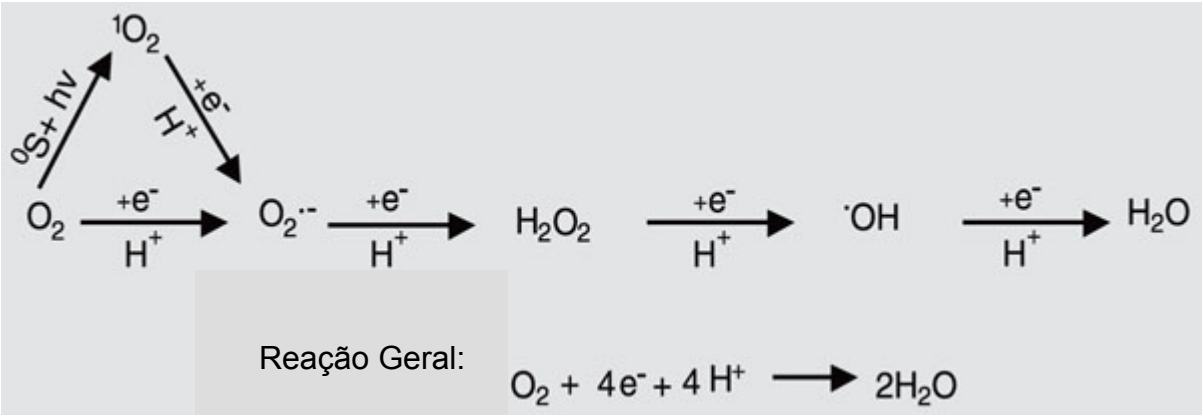


Figura 1 – A redução univalente do oxigênio para água leva a geração de diversos intermediários denomidados espécies ativas de oxigênio.

Normalmente a reação ocorre numa única etapa e a grande energia de ativação necessária é vencida com o auxílio da enzima citocromo oxidase. Mas apesar da grande eficiência da citocromo oxidase, aproximadamente 5% do oxigênio utilizado na respiração celular durante o metabolismo normal da célula é reduzido em um elétron de cada vez, formando as EAO. As EAO são capazes de reagir indiscrimidamente com qualquer tipo de molécula orgânica, extraindo elétrons e gerando novos radicais livres em reações em cadeia altamente citotóxicas. O radical hidroxil (•OH) é provavelmente a mais potente das EAO e o provável iniciador das reações em cadeia que formam os peróxidos de lipídeos e os radicais orgânicos.

Proteínas, lipídeos, carboidratos e ácidos nucléicos são alvos celulares dos danos oxidativos. Em proteínas, os aminoácidos prolina, histidina, arginina, cisteína e metionina são particularmente suscetíveis ao ataque por •OH. A oxidação de aminoácidos leva a fragmentação, carbonilação, cross-linking e agregação protéica com conseqüente perda de função e proteólise. A peroxidação lipídica invariavelmente leva a danos na estrutura molecular dos lipídeos. Quando estes fazem parte de membranas biológicas, o arranjo em bicamadas e sua organização estrutural geralmente são perdidos. As EAO também constituem uma grande fonte de dano ao DNA. Aproximadamente 20 diferentes tipos de moléculas de DNA modificadas foram até hoje identificadas, muitas delas reconhecidamente indicativos de mutações.

Existe um equilíbrio entre a produção de EAO e sua detoxificação na célula normal. Quando ocorre um aumento na produção ou uma diminuição das defesas antioxidantes, existe uma condição chamada de estresse oxidativo.

1.2- Ferro e espécies ativas de oxigênio:

Dentre todas as formas de geração de estresse oxidativo, a produção de peróxido de hidrogênio parece ter um papel central na produção e manutenção desta condição. Apesar do peróxido de hidrogênio (H_2O_2) ser considerado um oxidante relativamente estável, assume seus efeitos deletérios através da sua interação com íons ferro e conseqüente geração de hidroxil (•OH) - reação de Fenton (**$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$**). Além do ferro, íons cobre também são capazes, *in vitro*, de gerar •OH, mas, provavelmente, devido a maior disponibilidade intracelular*, in vivo*, o ferro parece ser o metal envolvido na síntese de •OH a partir de peróxido de hidrogênio (H_2O_2) (Meneghini 1997).

Não apenas o ferro livre que pode participar na oxidação do peróxido de hidrogênio. O grupamento heme e certas “heme-proteínas” podem reagir com peróxidos de lipídeos e com peróxido de hidrogênio causando dano celular. Por isso, organismos superiores desenvolveram estratégias para diminuir a quantidade de ferro reativo, ao mesmo tempo em que mantém níveis de ferro para as necessidades metabólicas (Meneghini 1997).

1.3- Estresse oxidativo e defesas antioxidantes:

Para se proteger contra os efeitos danosos das EAO, as células apresentam pequenas moléculas, scavengers de EAO, ou enzimas específicas que atacam diretamente as EAO, formando produtos menos agressivos.

Existem três enzimas antioxidantes clássicas em eucariontes superiores: superóxido dismutase, catalase e glutationa peroxidase:

$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (superóxido dismutase)

$2H_2O_2 \rightarrow O_2 + 2H_2O$ (catalase)

$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$ (glutationa peroxidase)

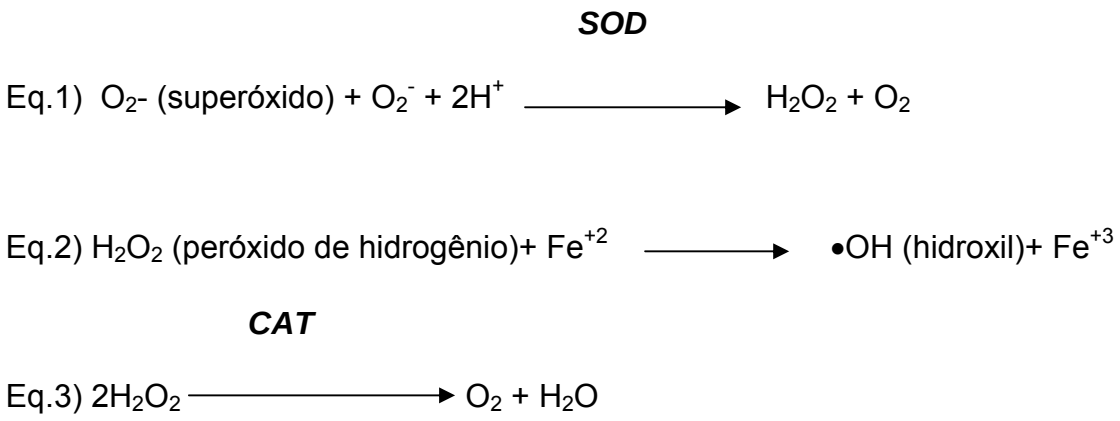
A superóxido dismutase (SOD) converte o ânion superóxido em peróxido de hidrogênio e oxigênio molecular. Todos os subtipos de superóxido dismutase apresentam pelo menos um metal de transição no seu sítio ativo. A MnSOD de células eucarióticas é estritamente localizada na membrana mitocondrial interna, sendo sua expressão regulada por EAO. A CuZnSOD, por outro lado, apresenta localização citosólica. Apesar de importante linha de defesa contra as EAO, uma grande atividade de SOD está envolvida com o aumento do estresse oxidativo em diversos modelos experimentais (Nelson et al 2006), provavelmente por produzir peróxido de hidrogênio além da capacidade de degradação da célula.

A glutationa peroxidase (GPx), uma enzima selênio-dependente, é importante para a proteção contra peróxidos orgânicos e peróxido de hidrogênio. Para sua atividade a GPx necessita da presença de glutationa reduzida (GSH) conforme reação acima.

A catalase (CAT), uma heme-proteína, tem o peróxido de hidrogênio como único substrato, sendo sua atividade intimamente relacionada com a concentração desta EAO. Ela atua, assim, complementarmente a GPx, não permitindo a produção de •OH a partir do peróxido de hidrogênio.

Uma ação orquestrada destas enzimas é necessária para a proteção celular contra o estresse oxidativo. Tem sido demonstrado que o desequilíbrio

entre SOD e CAT/GPx aumenta o dano celular desencadeado pelas EAO e participa da gênese de várias doenças (Park et al 2007, Nelson et al 2006). Uma superexpressão da SOD resulta na excessiva produção de peróxido de hidrogênio, que pode mediar dano às membranas através da peroxidação lipídica ou reagir com ferro para gerar radical hidroxil via reação de Fenton, conforme equação 1 e 2 , com diminuição da viabilidade celular ("toxicidade da SOD"). Um aumento proporcional da atividade da CAT poderia limpar este excesso de peróxido de hidrogênio (equação 3). Porém, já é descrito que o excesso de EAO poderia oxidar o sítio ativo da CAT, levando a inativação da enzima.



1.4 - Sepse- conceito e aspectos relevantes:

Sepse e suas conseqüências constituem as causas mais comuns de mortalidade em unidades de tratamento intensivo, chegando a taxas de 50-60% a despeito do tratamento (Silva et al 2004). É uma síndrome de resposta inflamatória sistêmica induzida por infecção e definida através da presença de

dois ou mais dos seguintes critérios: febre ou hipotermia, leucocitose ou leucopenia, taquicardia, taquipnéia ou hipocapnia. Quando existe uma falência orgânica devido a sepse, conceituamos de sepse grave, quando existe hipotensão refratária, conceituamos choque séptico e a disfunção em vários órgãos caracteriza falência orgânica múltipla.

A resposta do hospedeiro é tão importante quanto o sítio de infecção e o agente causador da sepse (Hotchkiss et al 2003). O pulmão é o sítio de infecção mais freqüente, seguido por abdômen e trato urinário, mas em 20-30% dos pacientes o sítio primário não é determinado. Pacientes com infecções presumidas ou condições inflamatórias graves não causadas por infecção (ex. pancreatite), apresentam alterações bioquímicas, fisiológicas, taxas de disfunção orgânica e mortalidade similares, o que vem suportar o argumento de que a resposta do hospedeiro é o maior determinante de sua evolução clínica.

Drogas antimicrobianas são necessárias, mas não suficientes para o tratamento da sepse, e paradoxalmente podem precipitar alterações sépticas pela liberação de produtos microbianos. Pacientes que não recebem prontamente antibioticoterapia apropriada têm uma mortalidade aumentada em 10-15 %.

A ocorrência de falência orgânica segue um padrão comum: a disfunção pulmonar ocorre quase sempre e precocemente, persiste durante o choque, que também ocorre precocemente, e se resolve precocemente ou é fatal; sérias anormalidades da função hepática, coagulação e neurológicas tendem a ocorrer horas a dias depois do início da sepse e persistem por tempo indeterminado. O número de falências orgânicas, além da gravidade destas, afeta o prognóstico do paciente, pois cada órgão adicional em falência

acrescenta 15-20% na taxa de mortalidade. Portanto, o tratamento da falência orgânica é essencial, mas, por enquanto se baseia em medidas de suporte, como ventilação mecânica, reposição volêmica generosa, drogas vasopressoras, suporte nutricional, sedação, diálise entre outras.

A taxa de mortalidade por sepse e suas complicações vem apresentando uma discreta redução nas últimas décadas, provavelmente devido a melhor definição da síndrome, a incrementos nas medidas de suporte a órgãos-alvo e prevenção de complicações, mesmo na ausência de uma terapêutica específica com impacto considerável na mortalidade.

1.5- Lesão pulmonar aguda(LPA) e síndrome da angústia respiratória aguda (SARA): conceitos e aspectos relevantes

A lesão pulmonar aguda e a síndrome da angústia respiratória aguda são síndromes comuns que complicam uma variedade de patologias graves clínicas ou cirúrgicas. Estas condições são quase sempre progressivas e caracterizadas por estágios com diferentes manifestações clínicas, histopatológicas e radiológicas (Ware e Matthay 2000, Goodman et al 2003).

A LPA/SARA é uma condição de injúria pulmonar, a expressão de uma patologia que produz inflamação sistêmica. As lesões pulmonares provavelmente se originam da ativação sistêmica dos neutrófilos circulantes, que se aderem ao endotélio vascular dos capilares pulmonares. Os neutrófilos liberam enzimas proteolíticas e metabólitos tóxicos do oxigênio, causando dano ao endotélio e rupturas nos capilares com exsudação para dentro do parênquima pulmonar, que então preenche os espaços alveolares. Outra

característica é a deposição de fibrina e com isso se desencadeia um remodelamento e fibrose pulmonar (Abraham 2003).

Clinicamente a LPA/SARA é caracterizada por aumento na permeabilidade da membrana alvéolo-capilar, levando a edema pulmonar e hipoxemia refratária. Nos estágios precoces da doença, se apresenta com taquipnéia e hipoxemia progressiva, em 24 horas já se observa comprometimento radiológico bilateral e em cerca de 48 horas o quadro progride para uma hipoxemia grave e refratária com necessidade de ventilação mecânica (Bernard et al 1994).

Para fins de prática clínica existem critérios para o diagnóstico de LPA e SARA: 1) início agudo; 2)presença de condição predisponente; 3)infiltrados bilaterais ao Rx de tórax; 4)relação PaO2/FiO2 menor que 200 mmHg para SARA e menor que 300 mmHg para LPA; 5) pressão de oclusão da artéria pulmonar ≤ 18 mmHg ou ausência de evidência clínica de hipertensão atrial esquerda (Bernard et al 1994, Wheeler e Bernard 2007).

Até o momento o tratamento da LPA e SARA tem sido de suporte, com entubação traqueal, ventilação mecânica com pressão positiva e altas concentrações de oxigênio inspirado. Embora essas medidas sejam salvadoras de vida, estudos mais recentes nos mostram que a própria ventilação mecânica contribua para a injúria pulmonar e possivelmente para a falência orgânica múltipla desses pacientes (Frank et al 2006).

A mortalidade dessas entidades clínicas chega a 40%, sendo que a maioria dessas mortes ocorre por falência orgânica múltipla e apenas uma pequena fração morre propriamente por insuficiência respiratória. (Wheeler e Bernard 2007).

1.6- Insuficiência hepática aguda: conceito e aspectos relevantes

A insuficiência hepática aguda pode ser fulminante ou subfulminante. A insuficiência hepática fulminante se caracteriza pelo desenvolvimento de encefalopatia hepática normalmente dentro das primeiras 8 horas do início da doença hepática, sendo que coagulopatia está invariavelmente presente. Já no quadro subfulminante, esses achados ocorrem em 8 semanas a 6 meses do início da doença hepática. Ambas as condições cursam igualmente com um pobre prognóstico.

Insuficiência hepática severa é uma complicação associada com uma miscelânea de patologias, entre elas, as hepatites virais agudas, intoxicações por acetaminofeno, choque, e várias outras, cursando sempre com uma alta mortalidade e sem um tratamento específico e eficaz até o momento.

Devido à evolução clínica imprevisível da patologia e a dificuldade no desenvolvimento de estudos clínicos em decorrência do pequeno número de casos e à heterogeneidade de etiologias, numerosos estudos experimentais se desenvolvem no intuito de elucidar os mecanismos de dano e regeneração hepática e de desvendar novas terapêuticas a fim de melhorar a morbimortalidade da patologia.

O dano celular hepático depende da natureza, duração e severidade do insulto e finalmente do grau de necrose, apoptose e necroapoptose. Clinicamente se apresenta com distúrbios de coagulação, desequilíbrio hidroeletrolítico e ácido-base, insuficiência renal, hipoglicemia e encefalopatia hepática. Em estados de choque cardiocirculatório, os pacientes podem desenvolver hepatite isquêmica, uma condição que cursa com necrose

centrilobular com intensa infiltração neutrofílica e aumento de transaminases e desidrogenase lática (DHL), além disto, estudos evidenciaram aumento nos "marcadores biológicos" de estresse oxidativo (produtos da peroxidação lipídica no plasma e em eritrócitos) (Biasi et al 1994).

1.7 - 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 gerar 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).

1.8- Modelo animal de lesão pulmonar aguda

A injúria pulmonar aguda em roedores pode ser induzida pela administração de LPS (lipopolissacarídeo) intratraqueal, que desencadeia uma intensa resposta inflamatória, caracterizada pelo influxo de polimorfonucleares, edema intersticial e alveolar e hemorragia intra-alveolar.

Produtos desta resposta inflamatória aguda pulmonar causando injúria tissular são liberados dos macrófagos ativados. Estes incluem produtos tóxicos do oxigênio e do nitrogênio, juntamente com proteases liberadas das células fagocitadas. A reação inflamatória aguda nos pulmões depende da ativação de NF-kB , que resulta na ativação de genes pró-inflamatórios, com liberação de citocinas . Como a reação inflamatória progride, produtos de genes reguladores (inibitórios) também aparecem, especialmente IL-10 e IL-13. Estas interleucinas são potentes mediadores anti-inflamatórios que suprimem a ativação de NF-kB, por prevenirem a hidrólise do IkB (Goodman et al 2003). O balanço entre essas duas classes de mediadores (pró-inflamatórios e anti-inflamatórios) define o quanto essa resposta inflamatória persiste ou se intensifica após a exposição a LPS e determina o desfecho desse processo. (Cavaillon e Annane 2006).

1.9 Modelo animal de insuficiência hepática aguda

O tetracloreto de carbono (CCl4) tem sido muito usado experimentalmente para induzir necrose hepatocelular aguda. A sua administração aumenta significativamente a liberação de enzimas hepáticas, a destruição do citocromo P450, os produtos da peroxidação lipídica e a resposta inflamatória.

O efeito hepatotóxico do CCl4 resulta da sua redução pelo sistema citocromo P450 a um radical livre altamente reativo, o radical triclorometil, e este reage com moléculas de oxigênio formando o radical triclorometilperoxil, que por sua vez, reagindo com ácidos graxos insaturados, retira destas átomos de hidrogênio formando os radicais centrados em carbono que rapidamente sofrem reação com o oxigênio, gerando o radical peroxil, iniciando assim o processo de peroxidação lipídica. O ferro pode catalizar as reações das EAOs que leva a maior peroxidação lipídica ou a inativação da atividade das enzimas antioxidantes, com conseqüente dano aos microssomos, mitocôndrias, núcleos e a um prejuízo nas funções fisiológicas dos hepatócitos (Weber et al 2003).

Esse modelo animal de indução de falência hepática aguda induzida pela administração de CCl4 intraperitonealmente em murinos simula o que desordens como a injúria hepática por intoxicação pelo álcool, porfiria, sobrecarga de ferro, hepatites virais fulminantes, demonstram histopatologicamente, com aumento da deposição de matriz extracelular, proliferação de fibroblastos e fibrose hepática (Weber et al 2003, Sakaguchi e Furusawa 2006).

1.10 - O papel das espécies ativas de oxigênio na fisiopatologia das doenças críticas do adulto

A sobrevida dos pacientes criticamente enfermos depende de uma resposta imune cuidadosamente orquestrada em todos os órgãos. Uma disfunção da resposta imune pode se apresentar sob duas formas: (1) ativação excessiva da resposta imune, manifestada clinicamente como a síndrome da resposta inflamatória sistêmica , e (2) excessiva “down regulation” da resposta celular imune, levando a um aumento na suscetibilidade a infecção. Uma produção excessiva de radicais livres contribui para uma resposta inflamatória intensa e o dano tissular (Crimi et al 2006).

O estresse oxidativo durante as doenças críticas pode ser relacionado com a ativação de fagócitos (neutrófilos, monócitos, macrófagos, eosinófilos), produção de óxido nítrico, liberação de íons ferro e metaloproteínas. Doenças críticas, assim como sepse, lesão pulmonar aguda e insuficiência hepática são caracterizadas pela produção de EAOs e outras espécies de radicais com conseqüente estresse oxidativo (Crimi et al 2006).

1.10.1. Sepse

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). 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-αe 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 a dano celular irreverssível, 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).

1.10.2. Lesão pulmonar aguda (LPA)/ Síndrome da angústia respiratória aguda (SARA)

A ruptura do balanço antioxidante-oxidante é provavelmente muito importante na fisiopatologia da LPA. Concentrações diminuídas de antioxidantes solúveis em água (urato, glutationa,ascorbato) estão presentes nas vias aéreas distais de pacientes com LPA (Bowler et al 2003). Concentrações elevadas de peróxido de hidrogênio assim como de produtos da peroxidação de fosfolipídeos de membrana são medidos no ar exalado de pacientes com SARA (Gessner et al 2003). Pacientes com SARA mostram diminuição nos níveis séricos de α-tocoferol, ascorbato, β-caroteno, selênio e níveis elevados de produtos da peroxidação lipídica (Richard et al 1990, Metnitz et al 1999). Kumar et al demonstraram que em pacientes com SARA, concentrações de peróxidos de lipídeos estão significativamente mais altas quando comparados a controles (pacientes com risco de desenvolver SARA), além disto, os pacientes com SARA mostram uma importante diminuição dos ácidos graxos saturados, sugerindo que ela possa ser uma doença com deficiência dos ácidos graxos essenciais (Kumar et al 2000). O mesmo estudo mostra uma diminuição dos níveis de óxido nítrico em pacientes com SARA e que contrasta com outros estudos que demonstram uma detecção de proteínas

nitratadas, aumento nos níveis de nitrato e nitratação do surfactante no lavado broncoalveolar de pacientes com SARA. (Kumar et al 2000, Lambe t al 1999, Sittipunt et al 2001). Na LPA/SARA o papel do óxido nítrico pode ter um efeito benéfico como uma espécie protetora ou ter um papel pró-oxidante como precursor do peroxinitrito (Anggard 1994).

O acúmulo de neutrófilos tem um papel no desenvolvimento da LPA/SARA, não somente na produção de radicais livres de oxigênio, mas também pela liberação de proteases, favorecendo a translocação bacteriana (Gullo et al 1996). A neutralização efetiva das proteases e das EAOs por antioxidantes previne a injúria pulmonar (Pacht et al 2003).

O dano celular e tissular resultante do estresse oxidativo pode ser conseqüência da ruptura do metabolismo normal do ferro e do aumento do ferro cataliticamente ativo. Concentrações elevadas deste metal são encontradas no trato respiratório inferior de pacientes com SARA (Ghio et al 2003) e outro estudo ainda sugere que a ferritina sérica do paciente teria uma relação com o desenvolvimento de SARA (Sharkey et al 1999).

1.10.3- Insuficiência hepática aguda

A produção de espécies ativas de oxigênio e a peroxidação lipídica no fígado em situações de insufiência hepática induzidas por sobrecarga de ferro, injúria colestática e intoxicações exógenas (etanol, tertracloreto de carbono) já foram descritas por alguns autores. (Bacon e Britton 1990, Parola et al 1996). Pacientes com insuficiência hepática aguda apresentam uma liberação significativamente elevada de enzimas hepáticas, aumento na destruição do

citocromo P450 e aumento nos produtos de peroxidação lipídica, o que justifica a intensa resposta inflamatória que ocorre nesta patologia (Castillo et al 1992, Luster et al 2001, Hua et al 2007). Todas essas desordens são caracterizadas por aumento na deposição de matriz extracelular e pelo desenvolvimento de fibrose. Um dos mecanismos responsáveis pela fibrose hepática poderia ser a indução de peroxidação lipídica (Chojkier et al 1989, Guimarães et al 2006). Outro estudo também demonstrou que uma depleção de glutationa, freqüentemente encontrada nas doenças hepáticas, poderia favorecer a produção de EAOs e a peroxidação lipídica (Han et al 2006).

1.11 - 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 2004), 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. 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. 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 a sua capacidade para reduzir o ferro para sua forma catalicamente ativa (Sprong et al 1998), favorecendo a reação de Fenton.

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).

1.12- Antioxidantes e o tratamento da LPA/SARA

O papel da NAC no tratamento da LPA/SARA tem sido estudado em vários estudos clínicos. A sua administração demonstrou efeitos protetores, restabelecendo níveis de glutationa dentro dos granulócitos pulmonares (Laurent et al 1996) e em fluido de lavado broncoalveolar (Ortolani et al 2000). No entanto, dois diferentes estudos falharam em demonstrar diferenças estatísticas nos end-points clínicos assim como mortalidade, tempo de ventilação mecânica, melhora da relação PaO2/FiO2 em pacientes com SARA. (Jepsen et al 1992, Domenighetti et al 1997).

Alguns estudos demonstram que monoterapia com NAC parece não mudar a história natural de doenças complexas como a SARA, entretanto usada mais precocemente poderia ser mais útil, diminuindo o dano induzido pelo estresse oxidativo (Spapen et al 1998).

A LPA/SARA é uma síndrome clínica que não é exclusivamente induzida por radicais livres, mas mostra uma fisiopatologia complexa envolvendo vias inflamatórias e não inflamatórias. Além disso, seria ingênuo pensar que existe um antioxidante ideal que bloquearia todas as vias, o que parece mais coerente seria usar uma combinação de antioxidantes, com a terapia de suporte já em uso.

1.13- Antioxidantes e o tratamento da insuficiência hepática aguda

A NAC é a droga de escolha no tratamento da insuficiência hepática induzida por paracetamol, tem uma variedade de propriedades farmacológicas

com benefícios potenciais em pacientes criticamente doentes (Atkinson 2002): restauração do poder antioxidante celular por restaurar os estoques depletados de glutationa, ser um “scavenger” de radicais livres tanto diretamente ou como precursor de GSH, vasodilatação e inibição da agregação plaquetária pelo aumento dos níveis de GMP-c e regenerar o óxido nítrico pela doação de sulfidril. A NAC pode diminuir a ativação de NF-kB com diminuição da produção de citocinas pró-inflamatórias, assim como IL-8 e TNF.

2 – OBJETIVO GERAL

Determinar o efeito da administração de NAC e DFX em diferentes modelos animais de doenças inflamatórias agudas.

2.1 – Objetivos específicos

Determinar o efeito da administração de NAC e DFX em parâmetros de dano oxidativo em modelos animais de doenças inflamatórias agudas;

Determinar o efeito da administração de NAC e DFX na relação SOD/CAT em modelos animais de doenças inflamatórias agudas;

Determinar o efeito da administração de NAC e DFX na produção de superóxido em partículas submitocondriais em modelos animais de doenças inflamatórias agudas;

Determinar o efeito da administração de NAC e DFX na resposta inflamatória em modelos animais de doenças inflamatórias agudas;

Determinar o efeito da administração de NAC e DFX na mortalidade em modelos animais de doenças inflamatórias agudas.

CAPÍTULO 2

Treatment with *N*-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis

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Crit Care Med 2004; 32:342–349

Treatment with *N*-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis*

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Objective: Oxidative stress plays an important role in the development of multiple organ failure and septic shock. Here we have evaluated the effects of a combination of antioxidants (*N*-acetylcysteine plus deferoxamine) in a murine model of polymicrobial sepsis induced by cecal ligation and puncture (CLP).

Design: Prospective, randomized, 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 (20 mg/kg, 3 hrs, 6 hrs, 12 hrs, 18 hrs, and 24 hrs after CLP, subcutaneously) plus deferoxamine (20 mg/kg, 3 hrs and 24 hrs after CLP, subcutaneously) 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: After 12 hrs, tissue myeloperoxidase (indicator of neutrophil infiltration), thiobarbituric acid reactive species (as a marker of oxidative stress), catalase and superoxide dismutase activities (antioxidant enzymes), and mitochondrial superoxide production (index of uncoupling of electron transfer chain) were measured in major organs involved in septic response. Rats treated with antioxidants had significantly lower myeloperoxidase activity and thiobarbituric acid reactive species formation in all organs studied. Mitochondrial superoxide production was significantly reduced by antioxidant treatment. Furthermore, antioxidants significantly improved the balance between catalase and superoxide dismutase activities. Survival in untreated septic rats was 10%. Survival increased to 40% with fluids and antibiotics. In rats treated only with *N*-acetylcysteine plus deferoxamine, survival was also significantly improved (47%) in a manner similar to basic support. Survival increased to 66% with basic support with *N*-acetylcysteine plus deferoxamine.

Conclusions: Our data provide the first experimental demonstration that *N*-acetylcysteine plus deferoxamine reduces the consequences of septic shock induced by CLP in the rat, by decreasing oxidative stress and limiting neutrophil infiltration and mitochondrial dysfunction, thereby improving survival. (Crit Care Med 2004; 32:342–349)

KEY WORDS: sepsis; antioxidants; oxidative stress; N-acetylcysteine; deferoxamine; free radicals

Septic shock has become one of the most frequent causes of morbidity and mortality in intensive care units (1). Treatment of sepsis consists of support of blood pressure, organ blood flow, and ventilation along with an emphasis on antibiotics and eradication of sources of infection. Despite significant advances in therapies available and understanding of pathogenesis, the mortality rate from septic shock has improved little over the last several decades (2).

Some investigators have demonstrated that reactive oxygen species (ROS) play an important role in the development of multiple organ failure and septic shock (3–5). One of the most important sources of ROS is the reduction of altered tetravalent oxygen as a consequence of endotoxic, hypoxic, and acidotic conditions. Muscle cell damage and acidosis increase the quantity of free iron released from myoglobin and hemoglobin and by the dangerous Fenton reaction (6). Leukocytes are activated, resulting in superoxide generation (7). In addition, elevated circulating nitric oxide has been reported in septic patients (8, 9). We previously reported that an imbalance between superoxide dismutase and catalase activities during sepsis response could predispose a subject to the accumulation of hydrogen peroxide (10). This environment leads to the formation of peroxynitrite and hydroxyl radicals, which are thought to be the most dangerous nitrogen and oxygen derivatives in biological systems.

Oxidant scavengers have been reported to inhibit lipopolysaccharide (LPS)-stimulated interleukin release (11), improve macrophage function (12), diminish the expression of adhesion molecules (13), and decrease tumor necrosis factor concentrations (5). Interventions that reduce the generation or the effects of ROS exert beneficial effects in a variety of models of endotoxic and septic shock (14). These therapeutic interventions include *N*-acetylcysteine (NAC) (5, 15–17), α-tocopherol (18), allopurinol (17), deferoxamine (19), catalase (20), superoxide dismutase (20), superoxide dismutase mimetics (21), magnolol (22), and tempol (23). To date, NAC is the most widely used antioxidant in the clinical and pre-

*See also p. 589.

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clinical setting. However, low-dose NAC protects against LPS toxicity whereas higher doses may have oxidant effects, probably by its interaction with iron, and these findings could restrict its use in the clinical setting (15). In this way, the use of deferoxamine (DFX), an iron chelator, seems to improve sepsis mortality rate when used before, but not after, endotoxin challenge (19).

We therefore hypothesized that the accumulation of hydrogen peroxide plus the presence of free iron during sepsis could lead to the formation of hydroxyl radicals that play a major role in sepsis mortality. In addition, the isolated use of NAC could have some limitations secondary to its pro-oxidant effects. Thus, the present study was designed to evaluate the effects of NAC plus DFX in a rat model of sepsis by monitoring oxidative stress, antioxidant enzyme activities, inflammatory variables and mortality rate.

MATERIALS AND METHODS

In vivo studies were performed in accordance with National Institutes of Health guidelines and with the approval of the local ethics committee.

Cecal Ligation Puncture (CLP) Model

Male Wistar rats 2–3 months old, subjected to CLP as previously described (24, 25), were used in this study. 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 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. The cecum then was returned to the peritoneal cavity and the laparotomy was closed with 4.0 silk sutures. Animals were resuscitated with normal saline (50 mL/kg subcutaneous) immediately after and 12 hrs after CLP. All animals were returned to their cages with free access to food and water. Septic rats in this model become bacteremic with Gram-negative enteric organisms (10, 25).

Experimental Protocols

For the purpose of biochemical measurements (described subsequently), 50 rats were made septic by CLP. The animals were randomly divided into five groups: group 1, sham operated; group 2, NAC (20 mg/kg) 3 hrs, 6 hrs, and 12 hrs after CLP plus DFX (20 mg/kg) 3 hrs after CLP with a subcutaneous injection; group 3, vehicle (isotonic saline) at same times after CLP; group 4, same as group 2 with "basic support" (saline at 50 mL/kg immediately after and 12 hrs after CLP plus ceftriaxone at 30 mg/kg and clindamycin 25 mg/kg every 6 hrs); group 5, same as group 3 with basic support. Twelve hours later the rats were killed by decapitation followed by the harvesting of samples from the lung, liver, kidney, heart, and diaphragm, which were immediately stored at −70°C until assayed for myeloperoxidase activity, thiobarbituric acid reactive species (TBARS) formation, superoxide dismutase and catalase activities, and superoxide production in submitochondrial particles as detailed subsequently.

Measurements

Thiobarbituric Acid Reactive Species. As an index of oxidative stress we used the formation of TBARS during an acid-heating reaction as previously described (26). 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 was determined by the absorbance at 535 nm using 1,1,3,3-tetramethoxypropane as an external standard. Results are expressed as malondialdehyde equivalents per milligram of protein (Lowry assay).

Myeloperoxidase Assay. As an index of neutrophil infiltration, we measured myeloperoxidase (MPO) activity in tissues homogenates. Tissues were homogenized (50 mg/mL) in 0.5% hexadecyltrimethylammonium bromide in 10 mM 3-*N*-morpholinopropanesulfonic acid and centrifuged at 15,000 × *g* for 40 mins. The suspension was then sonicated three times for 30 secs. An aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM hydrogen peroxide. Activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37°C, using a Spectramax microplate reader (27). Results are expressed as milliunits of MPO activity per milligram of protein, which were determined with the Bradford assay.

Measurement of Catalase and Superoxide Dismutase Activities. To determine catalase (CAT) activity, organ systems 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 (28). Superoxide dismutase (SOD) activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described (29).

Measurement of Mitochondrial Superoxide Generation. As an index of uncoupling of electron transfer chain, we measured the mitochondrial generation of superoxide as previously described (30). Briefly, submitochondrial particles were isolated by differential centrifugation. Superoxide was estimated by measuring adrenaline oxidation in a buffer containing submitochondrial particles, succinate (as electron transfer chain initiator), and catalase. To ensure assay specificity, a negative control was made in the presence of SOD.

Survival Experiments

Survival was tested in a separate cohort of animals. In a first protocol, animals exposed to CLP were randomly assigned to receive NAC (20 mg/kg) 3 hrs, 6 hrs, 12 hrs, 18 hrs, and 24 hrs after CLP plus DFX (20 mg/kg) 3 hrs and 24 hrs after CLP with subcutaneous injection or vehicle (isotonic saline at same times). In a second set of experiments, the effect of NAC plus DFX or vehicle with basic support was analyzed. The animals were challenged with CLP and administered saline at 50 mL/kg immediately after and 12 hrs after CLP plus ceftriaxone at 30 mg/kg and clindamycin 25 mg/kg every 6 hrs over a total of 3 days with either NAC (20 mg/kg) 3 hrs, 6 hrs, 12 hrs, 18 hrs, and 24 hrs after CLP plus DFX (20 mg/kg) 3 hrs and 24 hrs after CLP with subcutaneous injection or vehicle (isotonic saline at same times).

In this cohort, blood samples were collected from all animals 12 hrs after CLP via a jugular catheter inserted before CLP for the determination of TBARS and superoxide dismutase activity (approximately 150 μL of blood, this volume was replaced with normal saline to avoid hypovolemia). The mortality rate of the animals was recorded over a 5-day period.

Reagents

Thiobarbituric acid, CAT, SOD, dinitrophenylhidrazine, adrenaline, hydrogen peroxide, luminol, and succinate were purchased from Sigma Chemical (St. Louis, MO). 2,2'-azobis (2-methylpropionamidine) dihydrochloride was purchased from Aldrich Chemical (Milwaukee, WI). NAC was purchased from Zambon Labotatórios Farmacêuticos (Brazil). DFX was purchased from Novartis (Brazil).

Statistical Analyses

Data are expressed as mean ± SEM in all figures. For the biochemical measures, the means for the different treatment groups were compared by one-way analysis of variance followed by a Newman-Keuls test. In the survival experiments, the survival curves of the different treatment groups were compared using the log-rank test. Statistical significance was assigned to $p < .05$.

RESULTS

The measurement of TBARS and MPO activity in tissues revealed that NAC plus DFX treatment significantly attenuated diaphragm, heart, liver, lung, and kidney oxidative stress and inflammation during CLP when associated with basic support (Figs. 1 and 2). These results are similar when NAC plus DFX was administered without basic support (data not shown).

To determine the potential influence of NAC plus DFX on the balance between antioxidant enzyme activities during CLP, CAT and SOD activities were determined in homogenates from diaphragm, heart, liver, lung, and kidney. As illustrated in Figures 3 and 4, an imbalance between SOD and CAT activities occurred in rats challenged with CLP in several organs associated with septic response, as we demonstrated previously (10). This imbalance was secondary to SOD overactivation without proportional increase in CAT activity and was significantly suppressed by NAC plus DFX when associated with basic support (Fig. 3 and 4). These results are similar when NAC plus DFX was administered without basic support (data not shown).

Mitochondrial dysfunction was assessed by determining superoxide production in submitochondrial particles. CLP animals, but not the animals treated with NAC plus DFX with basic support, exhibited increased superoxide production compared with controls in all organs studied (Fig. 5).

These results are similar when NAC plus DFX was administered without basic support (data not shown). We also noted a significant increase in TBARS content in submitochondrial particles in CLP animals but not in the animals treated with NAC plus DFX with basic support (Fig. 6). These results are similar when NAC plus DFX was administered without basic support (data not shown).

The results of the survival experiments are shown in Figure 7. There were no deaths in sham-ligated control animals (n = 10). Survival in untreated septic rats (n = 20) was 10%. Survival increased to 40% with fluids and antibiotics (n = 30, $p < .05$). In rats treated only with NAC plus DFX, survival was also significantly improved (n = 30, 47%, $p < .05$) in a manner similar to basic support. Survival increased to 66% with basic support with NAC plus DFX (n = 30, $p < .05$). The administration of NAC (20 mg/kg 3 hrs, 6 hrs, 12 hrs, 18 hrs, and 24 hrs after CLP) or DFX (20 mg/kg 3 hrs and 24 hrs after CLP) with basic support did not significantly improve mortality rate or oxidative variables in comparison to the basic support group (data not shown).

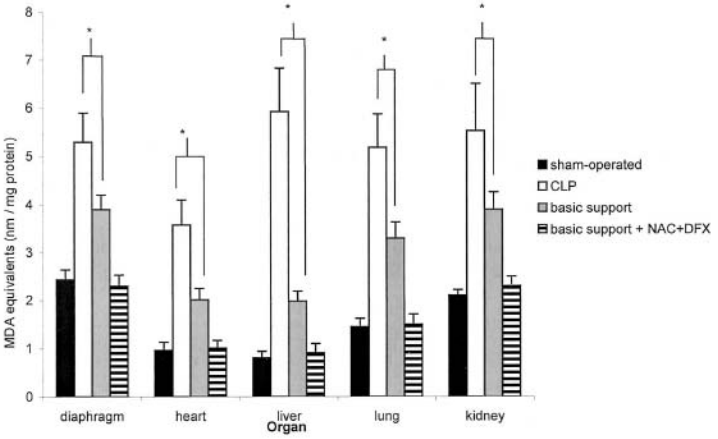

Figure 1. Thiobarbituric acid reactive species content in major organs associated with septic response. Rats were sham-operated or submitted to cecal ligation and puncture (*CLP*). CLP animals were randomly assigned to receive basic support, *N*-acetylcysteine (*NAC*) plus deferoxamine (*DFX*) with basic support, or only saline as described in Materials and Methods. Twelve hours after CLP, the heart, diaphragm, liver, lung, and kidney were removed to determine thiobarbituric acid reactive species content. Values are expressed as mean ± SD (n = 10 each group). *Different from sham-operated ($p < .05$). *MDA*, malondialdehyde.

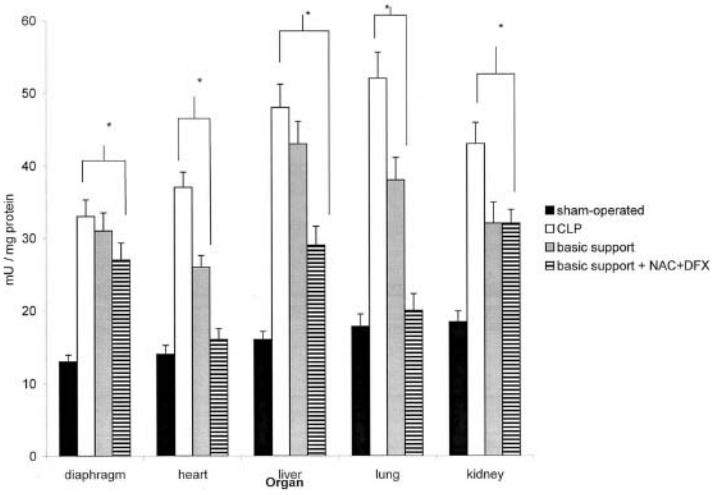

Figure 2. Myeloperoxidase activity in major organs associated with septic response. Rats were sham-operated or submitted to cecal ligation and puncture (*CLP*). CLP animals were randomly assigned to receive basic support, *N*-acetylcysteine (*NAC*) plus deferoxamine (*DFX*) with basic support, or only saline as described in Materials and Methods. Twelve hours after CLP, the heart, diaphragm, liver, lung, and kidney were removed to determine myeloperoxidase activity. Values are expressed as mean ± SD (n = 10 each group). *Different from sham-operated ($p < .05$).

Since mortality rate could be predicted 12 hrs after CLP determining plasmatic TBARS and SOD activity (10), in this cohort of animals we collected blood via a jugular catheter. The measurement of TBARS and SOD activity 12 hrs after CLP revealed that sepsis survivors had significantly reduced amounts of these

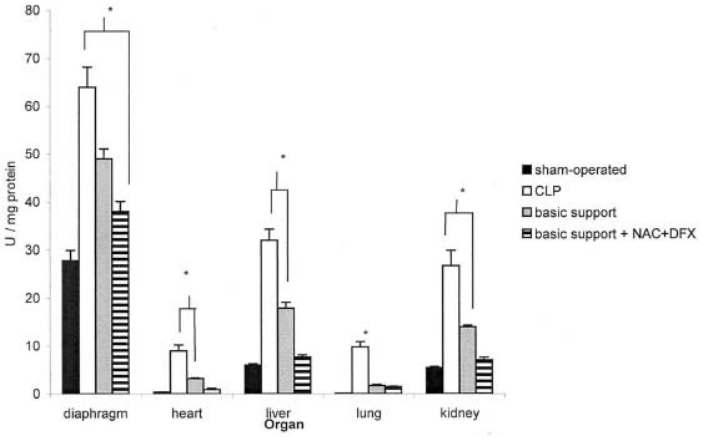

Figure 3. Superoxide dismutase activity in major organs associated with septic response. Rats were sham-operated or submitted to cecal ligation and puncture (*CLP*). CLP animals were randomly assigned to receive basic support, *N*-acetylcysteine (*NAC*) plus deferoxamine (*DFX*) with basic support, or only saline as described in Materials and Methods. Twelve hours after CLP, the heart, diaphragm, liver, lung, and kidney were removed to determine superoxide dismutase activity. Values are expressed as mean ± SD (n = 10 each group). *Different from sham-operated ($p < .05$).

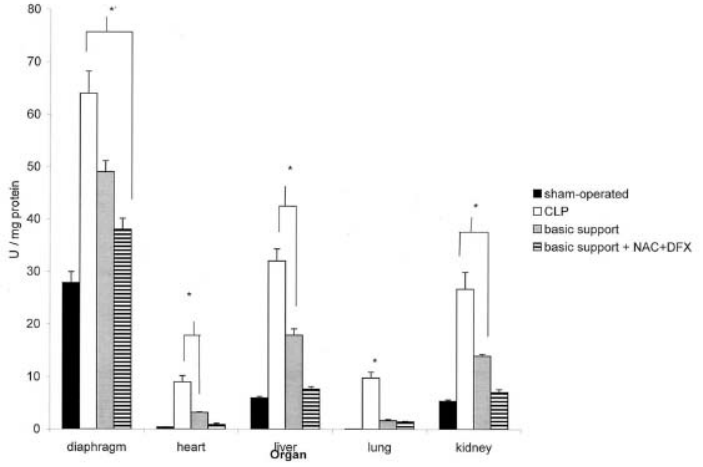

Figure 4. Catalase activity in major organs associated with septic response. Rats were sham-operated or submitted to cecal ligation and puncture (*CLP*). CLP animals were randomly assigned to receive basic support, *N*-acetylcysteine (*NAC*) plus deferoxamine (*DFX*) with basic support, or only saline as described in Materials and Methods. Twelve hours after CLP, the heart, diaphragm, liver, lung, and kidney were removed to determine catalase activity. Values are expressed as mean ± SD (n = 10 each group). *Different from sham-operated ($p < .05$).

plasmatic markers both in the basic support and in the NAC plus DFX groups when compared with the saline group (Figs. 8 and 9). Nonrespondents to both therapies maintained TBARS and SOD above the sham group. Only in the NAC plus DFX group did both markers return to sham group concentrations.

DISCUSSION

The main results of this study were that a combination of NAC plus DFX markedly reduced the systemic inflammation, organic oxidative stress, and mitochondrial dysfunction associated with sepsis induced by CLP, leading to a significant improvement in survival.

Background and Previous Work

ROS exhibits several proinflammatory properties pertinent to septic shock (31–35). Besides its proinflammatory effects, ROS possesses a number of cytotoxic mechanisms (36) and induces the activation of the nuclear enzyme poly(adenosine 5'-diphosphate-ribose) polymerase and depletion of nicotinamide adenine dinucleotide and adenosine triphosphate, which lead to irreversible cellular damage as evidenced in septic shock (37). Antioxidants inhibit the release of tumor necrosis factor, the activation of proinflammatory cytokines, cellular apoptosis, and necrosis (38). In this way many authors have proposed that the use of antioxidants decreases ROS damage in animal models (5, 15–23) and patients affected by sepsis (39, 40). The data presented in the current study confirm and extend these previous findings by showing that NAC plus DFX has major beneficial effects in a clinically relevant model of septic shock.

A number of studies have demonstrated the antioxidant role of NAC. Thus, NAC supplementation was found to reduce oxidative stress by improving the thiol redox status, to inhibit neutrophil and monocyte chemotaxis and oxidative metabolism, and to scavenge superoxide, hydrogen peroxide, and hydroxyl radicals (41–43). Other investigators have consistently shown that NAC, when administered before rather than after LPS challenge, protects animals against the hemodynamic (5, 43) and lethal effects of LPS (15, 16) or CLP (17). In contrast, Sprong et al. (15) demonstrated that high-dose NAC enhanced LPS-induced oxidative stress and mortality rate, possibly by its capacity to reduce iron to the catalytically active form. The administration of NAC in patients with sepsis decreased the oxidative stress and improved some hemodynamic variables and clinical scores, with little effect in mortality rate (39, 40). Some of these limitations of NAC therapy could be related to its adverse effects. It seems that high doses of NAC aggravate LPS toxicity (15). The oxidative metabolism of NAC can generate thiol free radicals that have been increasingly considered as intermediates in processes that may be involved in the development of biological damage resulting from oxidative stress (44). *In vitro*, NAC increased hydroxyl radical generation in a system with Fe(III)-citrate and H_2O_2 by reducing ferric iron to its catalytic, active

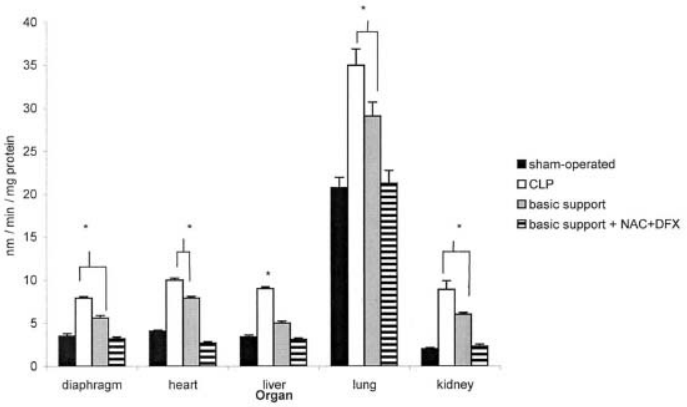

Figure 5. Superoxide production in submitochondrial particles in major organs associated with septic response. Rats were sham-operated or submitted to cecal ligation and puncture (*CLP*). CLP animals were randomly assigned to receive basic support, *N*-acetylcysteine (*NAC*) plus deferoxamine (*DFX*) with basic support, or only saline as described in Materials and Methods. Twelve hours after CLP, the heart, diaphragm, liver, lung, and kidney were removed to determine superoxide production in submitochondrial particles. Values are expressed as mean ± SD (n = 10 each group). *Different from sham-operated ($p < .05$).

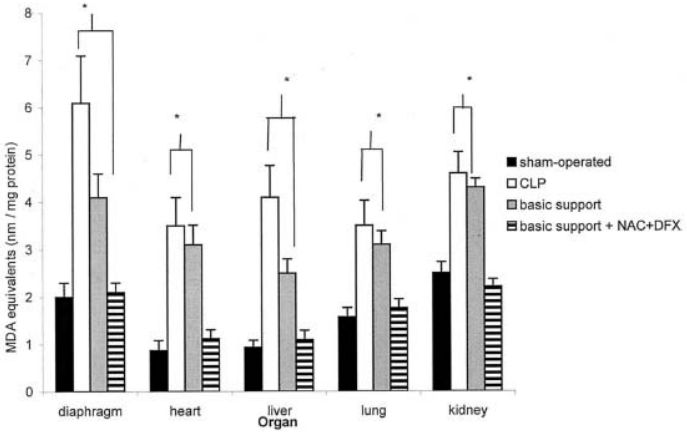

Figure 6. Thiobarbituric acid reactive species in submitochondrial particles in major organs associated with septic response. Rats were sham-operated or submitted to cecal ligation and puncture (*CLP*). CLP animals were randomly assigned to receive basic support, *N*-acetylcysteine (*NAC*) plus deferoxamine (*DFX*) with basic support, or only saline as described in Material and Methods. Twelve hours after CLP, the heart, diaphragm, liver, lung, and kidney were removed to determine thiobarbituric acid reactive species in submitochondrial particles. Values are expressed as mean ± SD (n = 10 each group). *Different from sham-operated ($p < .05$). *MDA*, malondialdehyde.

Fe^{+2} form (44). In this way, it seems reasonable to use an iron chelator in addition to NAC to improve its therapeutic effects in sepsis.

DFX is routinely employed in the treatment of several hematologic diseases with good safety profile. Despite its high stability constant for iron, DFX is poor at removing iron ions bound to transferrin and this could be associated with its safety profile (38). Because iron is essential for normal cell function, prolonged administration will probably cause side effects. The side effects of chronic DFX administration should not preclude its acute use; however, controlled clinical trials remain to be published in these areas. In this way, some caution is advisable after DFX use in diseases where major iron overload is not present. Treatment with the polymeric iron chelator, hydroxyethyl starch-DFX, significantly attenuates systemic oxidant injury (45) but not eicosanoid release or improves small bowel wall perfusion in a CLP model of sepsis (46). Other investigators have shown that DFX, when administered before rather than after LPS challenge, protects animals against lethal (19) effects of LPS.

Effects of NAC Plus DFX on Oxidative Variables During Sepsis

At 3 hrs after sepsis induction, rats uniformly were tachycardic and tachypneic and demonstrated lethargy and piloerection. We, for the first time, demonstrated that the association of NAC and DFX significantly improves mortality rate in a CLP model when administered after the onset of sepsis clinical signs. This approach protects some of the major organs involved in sepsis response against oxidative stress as demonstrated by the reduction of TBARS concentrations (Fig. 1). In addition, NAC plus DFX can reduce oxidative stress, restoring the balance between SOD and CAT activities (Fig. 3). It seems that any concentrations of SOD other than the optimal concentration lead to increased lipid peroxidation and therefore to decreased cell viability (47). SOD activity results in the production of hydrogen peroxide, which can mediate, in the presence of transition metals, membrane damage by lipid peroxidation or react with iron to generate hydroxyl radicals via Fenton chemistry, which is thought to be the most toxic oxygen molecule *in vivo* (47). CAT could clean an excess of peroxide, diminishing the oxidative effects of hydrogen peroxide. Thus, an imbalance between SOD and CAT activity could lead to oxidative stress, and we have previously demonstrated that this imbalance is associated with sepsis severity in the CLP model (10).

Effects of NAC Plus DFX on MPO Activity During Sepsis

It seems that NAC plus DFX reduces neutrophil infiltration in major organs involved in sepsis response as demonstrated by the reduction of MPO activity (Fig. 2). It is well known that NAC inhib-

its neutrophil activation and improves the function of macrophages (12, 38). Neutrophils have been regarded as double-edged swords in sepsis (7). Although neutrophils were thought to be essential for the eradication of pathogens, excessive release of oxidants and proteases by neutrophils also was believed to be responsible for injury to organs (7). Thus, in addition to its antioxidant effects, the effect of NAC plus DFX in CLP mortality could be related to the demonstrated reduction in MPO activity.

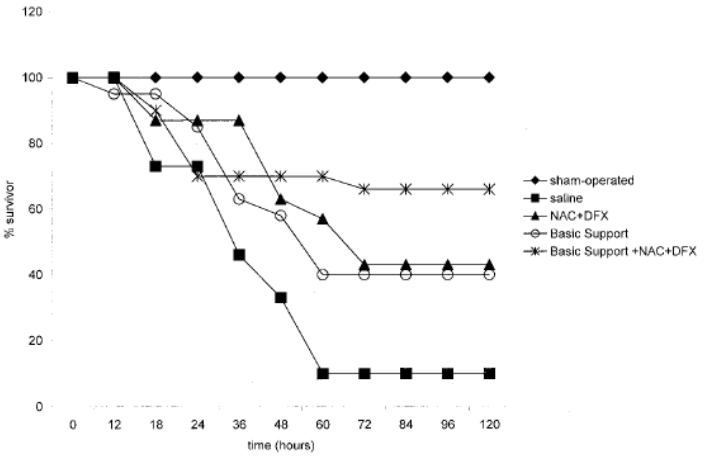

Figure 7. Percentage of rats surviving cecal ligation and puncture (CLP). Rats were sham-operated or submitted to CLP. CLP animals were randomly assigned to receive *N*-acetylcysteine (*NAC*) plus deferoxamine (*DFX*) with or without basic support, saline, or basic support as described in Materials and Methods. The mortality rate of the animals was recorded over a 5-day period.

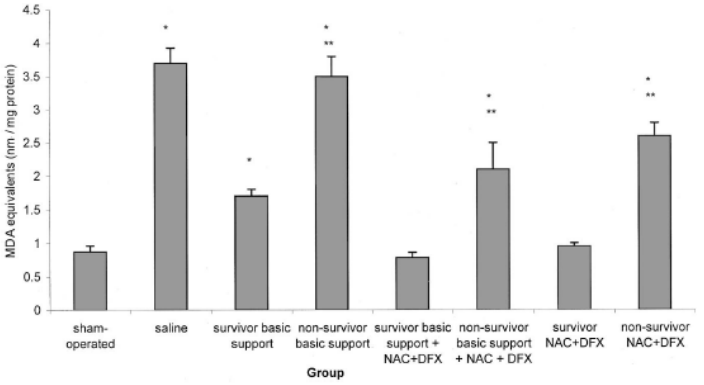

Figure 8. Plasmatic thiobarbituric acid reactive species 12 hrs after sepsis induction. Rats were sham-operated or submitted to cecal ligation and puncture (CLP). CLP animals were randomly assigned to receive *N*-acetylcysteine (*NAC*) plus deferoxamine (*DFX*) with or without basic support, saline, or basic support as described in Materials and Methods. Blood samples were collected from all animals 12 hrs after CLP via a jugular catheter inserted before CLP for the determination of thiobarbituric acid reactive species. Animals were followed over a 5-day period to record mortality rate. Values are expressed as mean ± SD. *Different from sham-operated ($p < .05$). **Different from survivors in same group ($p < .05$). *MDA*, malondialdehyde.

Effects of NAC Plus DFX on Mitochondrial Function During Sepsis

Sepsis causes a dysregulation of systemic oxygen metabolism that is characterized by increased oxygen delivery and impaired tissue oxygen extraction (48). In addition, cellular oxygen metabolism is disrupted, as indicated by the presence of lactic acidosis and other signs of accelerated anaerobic metabolism (49, 50). Recent investigations suggest that damage to mitochondria may contribute to the impaired oxygen metabolism that is associated with sepsis. Animal models demonstrate that ultrastructural injury to mitochondria commonly develops in various systemic organs during sepsis (51). In this regard, it has been reported that mediators of sepsis such as tumor necrosis factor (52) and LPS (53, 54) inhibit mitochondrial oxygen utilization and that tissue oxygen availability is maintained during the early stages of sepsis, at least in animal models (51, 55–58). We here demonstrated that NAC plus DFX improves mitochondrial electron transfer uncoupling as determined by superoxide production in submitochondrial particles (Fig. 4). One of the mechanisms of the uncoupling of mitochondrial electron transfer during sepsis is nitric oxide and superoxide-induced oxidative stress (30, 59), and we here demonstrated that NAC plus DFX treatment reduces mitochondrial TBARS in major organs involved in sepsis response (Fig. 5). In this way, the effect of NAC plus DFX on mortality rate also could be related to the reversion of mitochondrial impairment observed during sepsis development.

Effects of NAC Plus DFX on Survival of CLP

We found that NAC plus DFX significantly improved the survival of CLP. After 12 hrs of sepsis induction, survivors and nonsurvivors presented significant differences in plasmatic SOD and TBARS, suggesting that these plasmatic markers could predict outcome after treatment (Fig. 8 and 9). We had previously described that these markers could predict mortality rate after CLP (10). Unlike other studies that used NAC or DFX (15–19), the present data are of greater clinical relevance because they were obtained in a model of polymicrobial sepsis with fluid resuscitation and antibi-

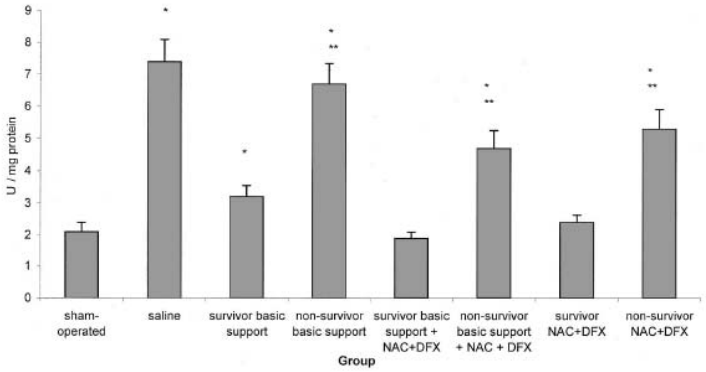

Figure 9. Plasmatic superoxide dismutase activity 12 hrs after sepsis induction. Rats were sham-operated or submitted to cecal ligation and puncture (CLP). CLP animals were randomly assigned to receive *N*-acetylcysteine (*NAC*) plus deferoxamine (*DFX*) with or without basic support, saline, or basic support as described in Materials and Methods. Blood samples were collected from all animals 12 hrs after CLP via a jugular catheter inserted before CLP to determine superoxide dismutase activity. Animals were followed over a 5-day period to record mortality rate. Values are expressed as mean ± SD. *Different from sham-operated ($p < .05$). **Different from survivor same group ($p < .05$)

otic administration that replicates the mortality rate seen in patients with septic shock (25). Our survival study design using fluids and antibiotics replicates more closely the supportive therapy performed in the clinical setting. This supportive therapy alone significantly improved mortality rate from 90% to nearly 60%. The additional significant improvement in survival achieved by adding NAC plus DFX to the treatment regimen suggests that this novel approach has significant therapeutic potential. In addition, the administration of antioxidants after CLP challenge increases the relevance of the effect on mortality rate.

Dehydroepiandrosterone (60), recombinant heparin-binding protein (61), recombinant granulocyte-macrophage colony-stimulating factor (62), anti-migration inhibitory factor antibodies (63), pentostatin (64, 65), and inosine (27) have been previously reported to improve survival in rodents challenged with CLP. Some authors reported different antioxidant approaches to the treatment of sepsis in animal models (15–23). Few of these used the CLP model, and, to the best of our knowledge, ours is the first study describing the effects of antioxidants in a clinically relevant model of rodent sepsis employing peritonitis with fluid resuscitation and antibiotics administration. Not all animal models, particularly those without adequate fluid repletion, reproduce the typical hyperdynamic hemodynamics seen in resuscitated patients (25). In addition, it is well recognized that supportive therapy can alter survival in an animal model considerably. Therapeutic interventions that are effective in untreated models may not work as well when combined with antibiotics and other supportive measures. Although it is difficult to compare the results of the aforementioned studies with our data, it is worth mentioning that NAC plus DFX was effective in a severe model of CLP and that its protective effects were present when NAC plus DFX was administered 3 hrs after the septic challenge.

CONCLUSIONS

Our data provide the first experimental demonstration that NAC plus DFX reduces mortality rate, decreases oxidative stress, and limits neutrophil infiltration and mitochondrial dysfunction induced by CLP in the rat. Ideally, the most effective form of antioxidant repletion is likely to include combinations of antioxidants with known synergistic actions. We do not expect that antioxidant therapy alone will greatly improve the survival of patients with sepsis, because sepsis cannot be simply reduced to a free-radical pathology; however, we consider antioxidants to be useful components of multiple-drug therapies. We believe that the approach described here is a more rational alternative to the use of antioxidants in sepsis treatment since they can blockade free radical generation in several different steps.

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CAPÍTULO 3

Protective effect of *N*-acetylcysteine and deferoxamine on carbon tetrachloride-induced acute hepatic failure in rats

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Objective: **Carbon tetrachloride (CCl_4) is a lipid-soluble potent hepatotoxic; thus, it widely is used as an animal model of severe hepatic failure. Treatment with antioxidants may modulate the toxic effects of CCl_4 on liver, generally with drug administration before CCl_4, which can restrict its use in the clinical setting. We here describe the effects of *N*-acetylcysteine, deferoxamine, or both in the treatment of CCl_4-induced hepatic failure.**

Design: **Prospective, randomized, controlled experiment.**

Setting: **Animal basic science laboratory.**

Subjects: **Male Wistar rats, weighing 200–250 g.**

Interventions: **Rats exposed to CCl_4 were treated with *N*-acetylcysteine and/or deferoxamine or vehicle.**

Measurements and Main Results: ***N*-acetylcysteine plus deferoxamine treatment significantly attenuated hepatic and central nervous system oxidative damage after acute hepatic failure induced by CCl_4. In addition, the serum levels of alanine aminotransferase, total bilirubin, and prothrombin time in the *N*-acetylcysteine plus deferoxamine group were significantly lower than those in the *N*-acetylcysteine or deferoxamine and saline groups. After *N*-acetylcysteine plus deferoxamine treatment, hepatocellular necrosis and inflammatory infiltration induced by carbon tetrachloride were greatly decreased. Survival in untreated rats was 5%. Survival increased to 25% and 35%, respectively, with *N*-acetylcysteine and deferoxamine treatment. In rats treated with *N*-acetylcysteine plus deferoxamine, survival was 80%.**

Conclusions: **Our data provide the first experimental demonstration that *N*-acetylcysteine plus deferoxamine reduces mortality rate, decreases oxidative stress, and limits inflammatory infiltration and hepatocyte necrosis induced by CCl_4 in the rat. (Crit Care Med 2004; 32:2079–2084)**

Key Words: severe hepatic failure; antioxidants; *N*-acetylcysteine; deferoxamine; oxidative stress

Severe hepatic failure is a common complication associated with acute viral hepatitis, acetaminophen toxicity, shock (septic or nonseptic), and several other diseases. The treatment of severe hepatic failure is directed toward correcting metabolic abnormalities associated with severe liver dysfunction. These include coagulation defects; disordered fluid, electrolyte, and acid-base balance; renal failure; hypoglycemia; and encephalopathy. Hepatic encephalopathy is an important cause of morbidity and mortality in patients with severe hepatic failure. Although the mechanisms responsible for hepatic encephalopathy remain elusive, the participation of ammonia and false neurotransmitters and the activation of benzodiazepine receptor have traditionally been considered as important in the pathogenesis of the disease (1). More recently, the participation of altered mitochondrial function and oxidative stress has been postulated (2, 3)

Carbon tetrachloride (CCl_4) is a lipid-soluble potent hepatotoxic that is widely used as an animal model of acute hepatocellular necrosis (4). The administration of CCl_4 significantly increases the release of hepatic enzymes, increases destruction of cytochrome P-450, increases lipid peroxidation products, and elicits an inflammatory response (5, 6). Some reports demonstrated that treatment with antioxidants modulated the toxic effects of CCl_4 on liver, generally with drug administration before CCl_4, which restricts its use in the clinical setting (7–12). Few studies have demonstrated the effects of different antioxidant combinations when administered after CCl_4 intoxication, the protective effects on central nervous system oxidative variables, or the effect of antioxidants on mortality rate after massive CCl_4 exposure (9).

We recently demonstrated that the combination of *N*-acetylcysteine (NAC) plus deferoxamine (DFX) is an effective treatment of severe sepsis in a rodent animal model (13). The combination of drugs is more efficient than the use of either alone, probably because of the decrease of NAC oxidation and Fenton chemistry. There are no reports in the literature that describe the effects of NAC plus DFX in animal or humans with massive hepatic failure.

Here we describe the effects of NAC, DFX, or both in the treatment of CCl_4-induced hepatic failure, measuring hepatic and central nervous system oxidative stress variables, plasmatic markers of hepatocellular death, degree of hepatic inflammatory response, histopathologic alterations, and mortality.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 200–250 g from our own breeding colony were

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housed in cages under conditions of controlled temperature and illumination. Animals were maintained on laboratory chow and water *ad libitum*. *In vivo* studies were performed in accordance with National Institutes of Health guidelines and with the approval of the local ethics committee.

Experimental Design. CCl_4 (mixed with an equal volume of soy oil) or vehicle was injected intraperitoneally in a volume of 4 mL/kg. We chose the dose by pilot experiments, because 4 mL/kg mixed solution of CCl_4 induced severe and reproducible acute hepatocellular necrosis in approximately 6 hrs and a high mortality rate (approximately 95%) under our experimental conditions. Rats in the control group were injected with soy oil at a volume of 4 mL/kg. For the purpose of biochemical measurements and histopathologic analyses (discussed subsequently), 50 rats were injected with CCl_4 or vehicle. The animals were randomly divided into five groups: group 1, control (soy oil); group 2, CCl_4 (4 mL/kg); group 3, CCl_4 (4 mL/kg) plus NAC (20 mg/kg subcutaneously 3 and 6 hrs after CCl_4); group 4, CCl_4 (4 mL/kg) plus DFX (20 mg/kg subcutaneously 3 hrs after CCl_4); and group 5, CCl_4 (4 mL/kg) plus NAC (20 mg/kg 3 and 6 hrs after CCl_4) and DFX (20 mg/kg 3 hrs after CCl_4). Groups 1 and 2 received saline volume corresponding to NAC and DFX administration in the same times. Six hours later the rats were killed by decapitation, and blood was collected for the determination of serum alanine aminotransferase (ALT), total bilirubin (TBil), and prothrombin time (PTA). Samples from the liver were isolated and immediately stored at −70°C until assayed for thiobarbituric acid reactive species (TBARS), protein carbonyls, and superoxide mitochondrial production or fixed in 4% formalin solution for histopathologic analyses. Samples from the central nervous system were isolated and immediately stored at −70°C until assayed for TBARS, protein carbonyls, or superoxide mitochondrial production.

Survival was tested in a separate cohort of animals. For this purpose, 90 rats were randomly divided into five groups: group 1, control (soy oil); group 2, CCl_4 (4 mL/kg); group 3, CCl_4 (4 mL/kg) plus NAC (20 mg/kg subcutaneously 3, 6, 12, and 24 hrs after CCl_4); group 4, CCl_4 (4 mL/kg) plus DFX (20 mg/kg subcutaneously 3 and 24 hrs after CCl_4); group 5, CCl_4 (4 mL/kg) plus NAC (20 mg/kg 3, 6, 12, and 24 hrs after CCl_4) and DFX (20 mg/kg 3 and 24 hrs after CCl_4). The mortality rate of the animals was recorded over a 2-day period.

Measurements. The formation of TBARS during an acid-heating reaction was measured as an index of oxidative stress as previously described (14). Briefly, the samples were mixed with 1 mL of trichloroacetic acid 10% and 1 mL of thiobarbituric acid 0.67% (Sigma Chemical, St. Louis, MO) and then heated in a boiling water bath for 15 mins. Malondialdehyde equivalents were determined by the absorbance at 535 nm using 1,1,3,3-tetramethoxypropane (Sigma Chemical) as an external standard. Results were expressed as malondialdehyde equivalents per milligram of protein (Lowry assay).

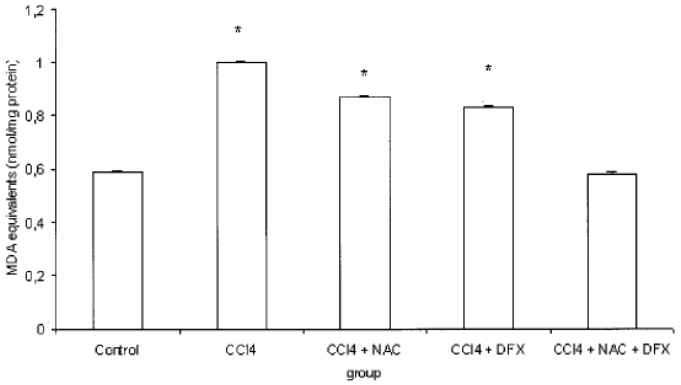

Figure 1. Malondialdehyde (*MDA*) content (nmol/mg protein) content in liver after acute hepatic necrosis induced by carbon tetrachloride (CCl_4). Rats were injected with CCl_4 and randomly assigned to receive *N*-acetylcysteine (*NAC*), deferoxamine (*DFX*), or both or only saline as described in Materials and Methods. Six hours after CCl_4 administration, the liver was removed to determine thiobarbituric acid reactive species content as described in Materials and Methods. Values are expressed as mean ± SD (n = 10 each group). *Different from control ($p < .05$).

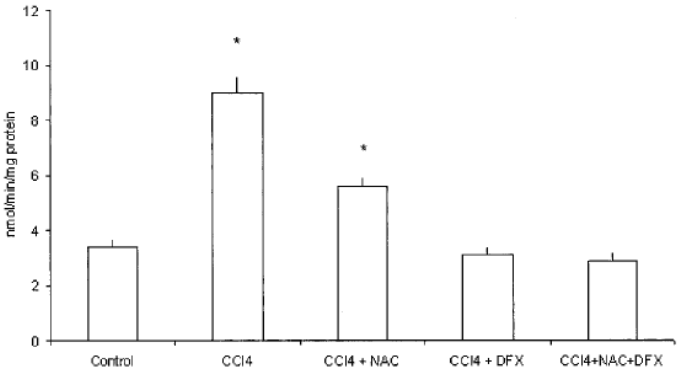

Figure 2. Mitochondrial superoxide production in liver after acute hepatic necrosis induced by carbon tetrachloride (CCl_4). Rats were injected with CCl_4 and randomly assigned to receive *N*-acetylcysteine (*NAC*), deferoxamine (*DFX*), or both or only saline as described in Materials and Methods. Six hours after CCl_4 administration, the liver was removed to determine superoxide production as described in Materials and Methods. Values are expressed as mean ± SD (n = 10 each group). *Different from control ($p < .05$).

The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhidrazine (Sigma Chemical) as previously described (15). Briefly, proteins were precipitated by the addition of 20% trichloroacetic acid and redissolved in dinitrophenylhidrazine, and the absorbance was read at 370 nm.

As an index of uncoupling of electron transfer chain, we measured the mitochondrial generation of superoxide as previously described (16). Briefly, submitochondrial particles were isolated in tissue homogenate by centrifugation (8,000 × *g*, 10 mins) followed by three successive freezing and melting procedures. Superoxide was estimated by measuring adrenaline oxidation in a buffer containing submitochondrial particles, succinate (as electron transfer chain initiator), and catalase. To ensure assay specificity, a negative control was made in the presence of commercial Cu-ZnSOD (Sigma Chemical).

Serum ALT, TBil, and PTA levels were determined routinely by commercially available kits (Labtest, Brazil).

For histopathologic analyses after fixation, excised liver tissues were embedded in paraffin and then routinely stained with hematoxylin and eosin. A blinded experienced pathologist performed histopathologic analyses. Inflammation and necrosis were graded as none, mild, moderate, or severe.

Statistical Analyses. Data are expressed as mean ± SEM in all figures. For the biochemical measures, the means for the different treatment groups were compared by one-way analysis of variance followed by a Newman-Keuls test. In the survival experiments, the survival curves of the different treatment groups were compared using the log-rank test. Statistical significance was assigned to $p < .05$.

Table 1. Effect of antioxidants on serum alanine aminotransferase (ALT), total bilirubin (TBil), and prothrombin time (PTA) levels after carbon tetrachloride (CCl_4) administration in rats

Group	ALT, units/L	TBil, mg/dL	PTA
Control	67 ± 4.3	0.6 ± 0.005	1.04 ± 0.08
CCl_4	532 ± 24.5[a]	4.6 ± 0.09[a]	2.3 ± 0.2[a]
NAC	577 ± 32.7[a]	5.4 ± 0.23[a]	2.1 ± 0.15[a]
DFX	302 ± 21.5[a,b]	3.2 ± 0.19[a]	1.96 ± 0.07[a]
NAC + DFX	113 ± 9.3[a,c]	0.9 ± 0.001	1.02 ± 0.04

[a]Different from control, $p < .05$; [b]different from CCl_4 group, $p < .05$; [c]different from DFX group, $p < .05$. Values are mean ± SD. Antioxidants were administered as described in Materials and Methods. Blood samples were collected 6 hrs after CCl_4 administration. n = 10 each group.

RESULTS

The measurement of TBARS and protein carbonyls on liver homogenates revealed that NAC plus DFX treatment significantly attenuated oxidative damage after severe hepatic failure induced by CCl_4 and NAC plus DFX, but neither NAC nor DFX alone decreased TBARS after CCl_4 administration (Fig. 1). The effects on protein carbonyls were similar between NAC or DFX alone compared with NAC plus DFX. CCl_4 administration increased protein carbonylation 20 times when compared with control (0.125 ± 0.05 vs. 2.81 ± 0.13 nm/mg protein, $p < .05$, n = 10 each group). NAC, DFX, or both attenuated this effect with similar

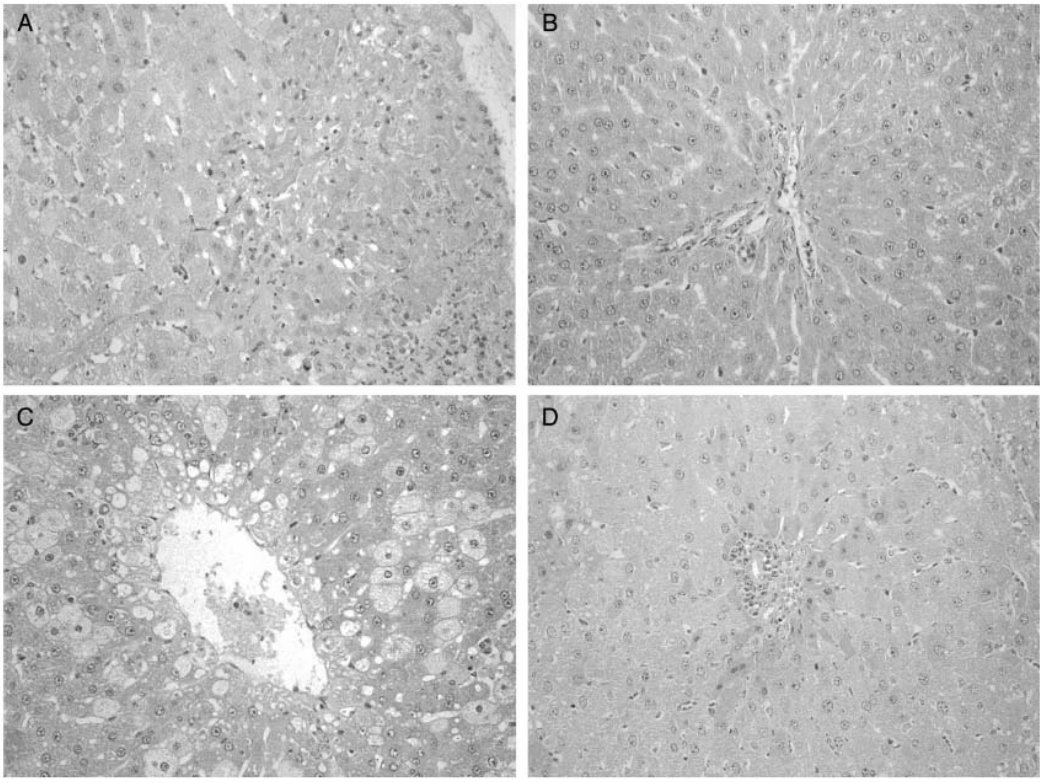

Figure 3. Liver histopathology after acute hepatic necrosis induced by carbon tetrachloride (CCl_4). Rats were injected with CCl_4 and randomly assigned to receive (*A*) saline, (*B*) *N*-acetylcysteine plus deferoxamine, (*C*) *N*-acetylcysteine, or (*D*) deferoxamine as described in Materials and Methods. Six hours after CCl_4 administration, the liver was removed for histopathologic analyses as described in Materials and Methods. Representative illustrations (n = 3). (Hematoxylin and eosin ×400.)

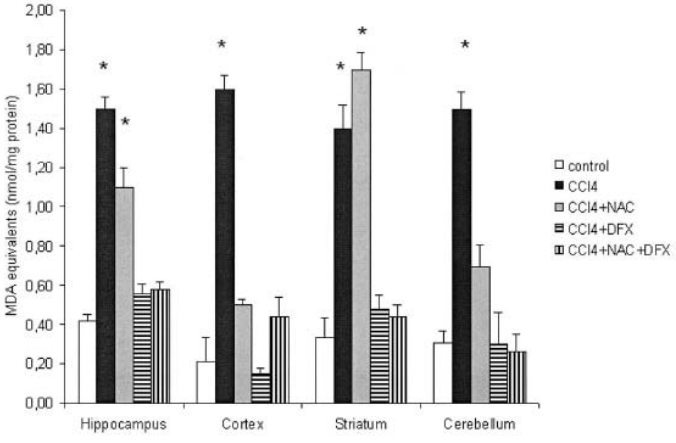

Figure 4. Malondialdehyde (*MDA*) content (nmol/mg protein) in the central nervous system after acute hepatic necrosis induced by carbon tetrachloride (CCl_4). Rats were injected with CCl_4 and randomly assigned to receive *N*-acetylcysteine (*NAC*), deferoxamine (*DFX*), or both or only saline as described in Materials and Methods. Six hours after CCl_4 administration, the cerebellum, hippocampus, striatum, and cortex were removed to determine thiobarbituric acid reactive species content as described in Materials and Methods. Values are expressed as mean ± SD (n = 10 each group). *Different from control ($p < .05$).

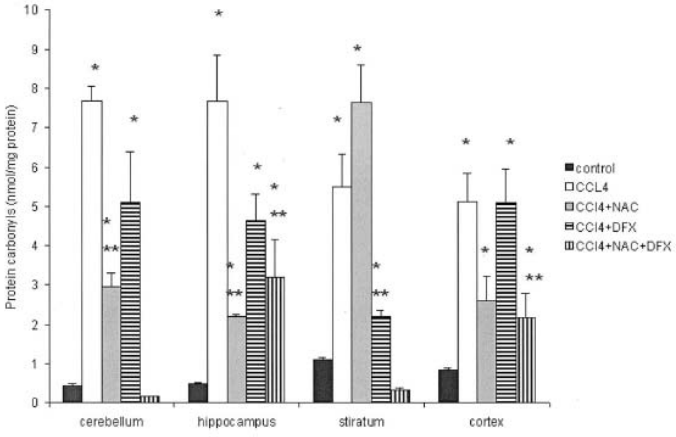

Figure 5. Protein carbonyls content (nmol/mg protein) in the central nervous system after acute hepatic necrosis induced by carbon tetrachloride (CCl_4). Rats were injected with CCl_4 and randomly assigned to receive acetylcysteine (*NAC*), deferoxamine (*DFX*), or both or only saline as described in Materials and Methods. Six hours after CCl_4 administration, the cerebellum, hippocampus, striatum, and cortex were removed to determine protein carbonyls content as described in Materials and Methods. Values are expressed as mean ± SD (n = 10 each group). *Different from control ($p < .05$; **different from CCl4 ($p < .05$)

magnitude (0.16 ± 0.023, 0.45 ± 0.037, 0.141 ± 0.012, nm/mg protein, $p < .05$ compared with CCl_4, n = 10 each group). Liver mitochondrial dysfunction was assessed by determination of superoxide production in submitochondrial particles. CCl_4 animals, but not the animals treated with NAC plus DFX, exhibited increased superoxide production compared with controls (Fig. 2).

The serum levels of ALT and TBil in NAC plus DFX group were significantly lower than those in NAC or DFX and CCl_4 groups (Table 1, $p < .05$). DFX alone decreased both ALT and TBil compared with CCl_4 group. In addition, the serum level of PTA was reduced to control levels only in the NAC plus DFX group but not in the NAC and DFX groups (Table 1).

Liver histopathology after CCl_4 administration revealed lobular disarray, ballooning degeneration, fatty degeneration, severe inflammatory cell infiltration, and severe necrosis of hepatocytes (Fig. 3*A*). All indicated that there was a severe liver failure in our model. After NAC plus DFX treatment, only mild hepatocellular necrosis was observed, and no inflammatory infiltration were observed (Fig. 3*B*). In contrast, there were no significant histopathologic differences between the CCl_4 and NAC groups (Fig. 3*C*). Moderate necrosis and inflammatory infiltration were observed in the DFX group (Fig. 3*D*).

Since hepatic encephalopathy is an important prognostic factor in severe hepatic failure, we determined oxidative variables and mitochondrial superoxide production in several central nervous system regions. Similar to the results demonstrated in liver, oxidative damage variables diminished in all analyzed regions only after NAC plus DFX treatment compared with CCl_4 animals (Figs. 4 and 5). This was also true regarding mitochondrial superoxide production in different central nervous system regions (Fig. 6).

The results of the survival experiments are shown in Figure 7. There were no deaths in control animals (n = 10). Survival in untreated CCl_4 rats (n = 20) was 5%. Survival increased to 25% with NAC (n = 20, $p > .05$ compared with CCl_4) and to 35% with DFX (n = 20, $p < .05$ compared with CCl_4). In rats treated with NAC plus DFX, survival was also significantly improved (n = 20, 80%, $p < .05$ compared with CCl_4, NAC and DFX groups).

DISCUSSION

The main results of our study were that a combination of NAC plus DFX markedly reduced the hepatic necrosis and inflammatory infiltration, organic oxidative stress, and plasmatic markers of hepatic dysfunction associated with severe hepatic failure induced by CCl_4, leading to a significant improvement in survival.

The lipid solubility of CCL_4 allows it to cross cell membranes, and any CCl_4 administered is distributed to all organs. However, the main toxic effects are shown on the liver; thus, it widely is used as an animal model of acute hepatocellu-

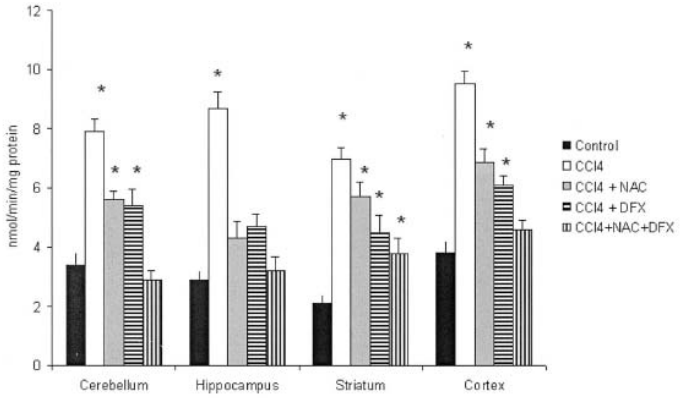

Figure 6. Superoxide mitochondrial production in the central nervous system after acute hepatic necrosis induced by carbon tetrachloride (CCl_4). Rats were injected with CCl_4 and randomly assigned to receive *N*-acetylcysteine (*NAC*), deferoxamine (*DFX*), or both or only saline as described in Materials and Methods. Six hours after CCl_4 administration, the cerebellum, hippocampus, striatum, and cortex were removed to determine superoxide mitochondrial production as described in Materials and Methods. Values are expressed as mean ± SD (n = 10 each group). *Different from control ($p < .05$).

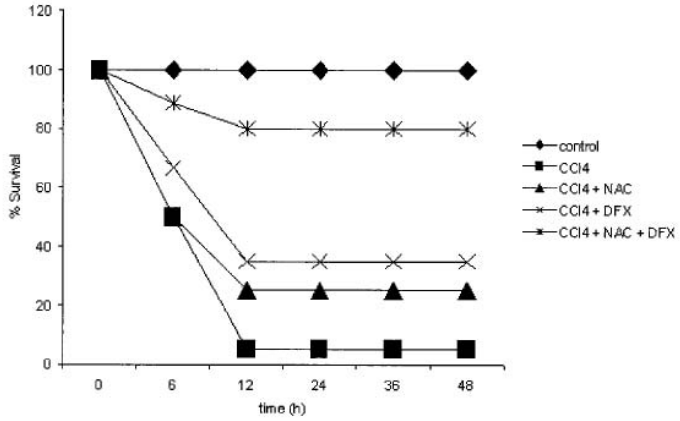

Figure 7. Percentage of rats surviving carbon tetrachloride administration (CCl_4). Rats were injected with CCl and randomly assigned to receive *N*-acetylcysteine (*NAC*), deferoxamine (*DFX*), or both or only saline as described in Materials and Methods. The mortality rate of the animals was recorded over a 2 day period. n = 10 (control) and n = 20 (all other groups).

lar necrosis (17, 18). CCl_4 toxicity is secondary to its metabolism by the P450 system to give the trichloromethyl radical, which, in part, explains its main toxic effects on the liver (18). Oxidative stress and inflammatory response seem to be important in the pathogenesis of severe hepatic failure independent of the initial insult (4, 19–23). In this way, the use of antioxidants could be an alternative adjuvant therapy in severe hepatic failure (9–12). Reactive oxygen species exhibit several proinflammatory properties and a number of cytotoxic mechanisms (24–26). Antioxidants inhibit the release of proinflammatory cytokines, cellular apoptosis, and necrosis. Thus, many authors have proposed the role of oxidative stress and the use of antioxidants to attenuate reactive oxygen species damage in animal models and humans with acute inflammatory diseases (10, 27–31), including

Our data provide the first experimental demonstration that N-*acetylcysteine plus deferoxamine reduces mortality rate, decreases oxidative stress, and limits inflammatory infiltration and hepatocyte necrosis induced by carbon tetrachloride in the rat.*

severe hepatic failure (9–12, 32). Valles et al. (9) demonstrated that the administration of NAC after CCl_4 administration prevented liver necrosis, probably by its conversion to cysteine and glutathione. Siegers et al. (33) demonstrated that DFX administration before, not after CCl_4, prevented lipid peroxidation and liver necrosis.

We supposed that, as demonstrated previously for sepsis, the addition of DFX could increase NAC therapeutic effects in a severe hepatic failure model. This is of particular importance since liver is the major organ responsible for iron storage (34). We supposed that hepatocyte necrosis could liberate free iron ions that contribute to the pathogenesis of severe hepatic failure. The significant survival advantage of the NAC plus DFX treatment, even when rendered 3 hrs after CCl_4 administration, provides compelling support for the notion that oxidative stress and free iron play a role in the development of severe hepatic failure. In addition, the oxidative metabolism of NAC can generate thiyl free radicals, which have been increasingly considered as intermediates in processes involved in the development of biological damage resulting from oxidative stress (35). *In vitro*, NAC increased hydroxyl radical generation in a system with Fe(III)-citrate and H_2O_2 by reducing ferric iron to its catalytic, active Fe (II) form (35). In this way, it seems reasonable to use an iron chelator in addition to NAC to improve its therapeutic effects in acute liver failure.

Since hepatic encephalopathy seems to be important in the prognosis of severe hepatic failure, prevention of central nervous system damage is important when designing new therapeutic targets. Ytrebo et al. (32) demonstrated that NAC administration improved cerebral blood flow and increased survival in an animal model of acute hepatic failure. We demonstrated that NAC plus DFX attenuates oxidative damage and mitochondrial dysfunction associated with CCl_4 intoxication. Mitochondrial dysfunction and oxidative stress seem to be related to the development of hepatic encephalopathy (2, 3), and our results support these findings. We cannot ascertain if central nervous system alterations are a result of direct CCl_4 toxicity or hepatic failure itself, once CCl_4 can cross blood-brain barrier. We suppose that the major component of central nervous system damage in this model is related to hepatic failure itself, since hepatic metabolism is necessary to CCl_4 toxicity, which makes liver the primary target of drug toxicity (18).

We previously demonstrated that the association of NAC plus an iron chelator (DFX) is superior to each alone in the treatment of severe sepsis in an animal model (13). Here we demonstrated that the effect of NAC plus DFX is superior to that of each drug used alone in the treatment of severe hepatic failure induced by CCl_4 in rats. The effect observed on mortality rate is, at least, secondary to a decrease in liver and central nervous system oxidative stress and superoxide mitochondrial production. In addition, NAC plus DFX reduced plasmatic markers of hepatic damage, hepatic inflammatory infiltration, and hepatocyte necrosis induced by CCl_4.

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CAPÍTULO 4

Effects of N-acetylcysteine plus deferoxamine in lipopolysaccharide-induced acute lung injury in the rat

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Effects of N-acetylcysteine plus deferoxamine in lipopolysaccharide-induced acute lung injury in the rat*

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Objectives: Interventions that reduce the generation or the effects of reactive oxygen species exert controversial effects in animal models of lung injury, and these could be secondary to the pro-oxidant effects of antioxidants generally by their interaction with iron. We here describe the effects of N-acetylcysteine, deferoxamine, or both in the treatment of acute lung injury induced by intratracheal lipopolysaccharide injection.

Design: Prospective, randomized, controlled experiment.

Setting: Animal basic science laboratory.

Subjects: Male Wistar rats, weighing 200–250 g.

Interventions: Rats exposed intratracheally to lipopolysaccharide were treated with N-acetylcysteine (20 mg/kg subcutaneously 3, 6, and 12 hrs after lipopolysaccharide instillation), deferoxamine (20 mg/kg subcutaneously 3 hrs after lipopolysaccharide instillation), N-acetylcysteine (20 mg/kg, 3, 6, and 12 hrs after lipopolysaccharide instillation) plus deferoxamine (20 mg/kg 3 hrs after lipopolysaccharide instillation), or vehicle.

Measurements and Main Results: Acute lung injury was induced by intratracheal instillation of lipopolysaccharide in Wistar rats. The animals were randomly divided into five groups: group 1, control with instillation of isotonic saline; group 2, lipopolysaccharide treated with saline; group 3, lipopolysaccharide treated with N-acetylcysteine; group 4, lipopolysaccharide treated with deferoxamine; and group 5, lipopolysaccharide treated with N-acetylcysteine plus deferoxamine. Several times after lipopolysaccharide instillation, the rats were killed and a bronchoalveolar lavage was performed to determine thiobarbituric acid reactive species, protein carbonyls, superoxide dismutase and catalase activities, mitochondrial superoxide production (oxidative stress variables), the degree of the alveolar-capillary membrane compromise, and inflammatory infiltration. Samples from the lung were isolated and assayed for oxidative stress variables or histopathologic analyses. N-acetylcysteine plus deferoxamine decreased bronchoalveolar lavage fluid protein, inflammatory cells, oxidative damage variables, and proinflammatory cytokines. N-acetylcysteine plus deferoxamine treatment significantly attenuated lung oxidative damage, mitochondrial superoxide production, and histopathologic alterations after lipopolysaccharide instillation.

Conclusions: Our data provide the first experimental demonstration that N-acetylcysteine plus deferoxamine decreases oxidative stress and mitochondrial dysfunction and limits inflammatory response and alveolar pathology induced by lipopolysaccharide in the rat. (Crit Care Med 2006; 34:471–477)

KEY WORDS: acute respiratory distress syndrome; antioxidants; iron; oxidative stress; acute lung injury; mitochondria

Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are common clinical syndromes that affect both medical and surgical patients. These conditions are often progressive, characterized by distinct stages with different clinical, histopathologic, and radiographic manifestations (1, 2). The acute, or exudative, phase is manifested by the rapid onset of respiratory failure in a patient with a risk factor for the condition. Pathologic findings include diffuse alveolar damage, with neutrophils, macrophages, erythrocytes, hyaline membranes, and protein-rich edema fluid in the alveolar spaces (1, 2).

Clinical and experimental studies have provided circumstantial evidence of the occurrence of neutrophil-mediated injury in ALI (1, 2). A complex network of cytokines and other compounds initiates and amplifies the inflammatory response in the ALI (2–4). In this way, the production of reactive oxygen species (ROS) could be, in part, responsible for the pathologic abnormalities seen in ALI (5–7). Several studies demonstrate depressed antioxidant levels in animal models and patients with ALI (8–10). In addition, it seems that during ALI development, there is aberrant regulation of iron metabolism, with increased concentrations of available iron that may participate in catalyzing ROS production (11–13). Interventions that reduce the generation or the effects of ROS exert controversial effects in animal models of lung injury (14–21), and these could be secondary to the pro-

*See also p. 569.

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oxidant effects of antioxidants generally by their interaction with iron (17). For example, the oxidative metabolism of N-acetylcysteine (NAC) can generate thiyl free radicals, and NAC can reduce Fe^{+3} ions to participate in the generation of hydroxyl radical via the Fenton reaction (17). We recently demonstrated that the combination of N-acetylcysteine (NAC) plus deferoxamine (DFX), but not their isolated use, is an effective treatment of severe sepsis and acute hepatic failure rats (22, 23). We cannot find reports on the use of iron chelators in lipopolysaccharide (LPS)-induced acute lung injury.

We here describe the effects of NAC, DFX, or both in the treatment of ALI induced by intratracheal LPS injection, with or without iron overload, measuring lung and alveolar fluid variables of oxidative stress, variables of disruption of the alveolar-capillary barrier, degree of lung inflammatory response, lung histopathologic alterations, and alveolar macrophage cytokine release.

MATERIALS AND METHODS

In vivo studies were performed in accordance with National Institutes of Health guidelines and with the approval of the Universidade do Extremo Sul Catarinense ethics committee.

ALI (Acute Lung Injury) Model. Adult male Wistar weighing approximately 250–300 g were used in this study. Rats were anesthetized by an intraperitoneal injection of ketamine (80 mg/kg), and ALI was induced by intratracheal instillation of LPS (*Escherichia coli* 055:B5; Sigma Chemical, St. Louis, MO) at dose of 100 μg/100 g of body weight.

Several times (3, 6, 12, 24, and 48 hrs) after LPS instillation, separate sets of animals were killed and a bronchoalveolar lavage (BAL) was performed. BAL fluid (BALF) was collected three times after instillation and withdrawn with 6 mL of phosphate-buffered saline. We always retrieved approximately 15 mL of BAL administered (n = 3 rats each time, per treatment group). The BALF was centrifuged (1000 × *g* for 10 mins), and the resultant cell-free supernatant was analyzed for the different biochemical and for oxidative stress variables. The cell pellet was used to determine the total cell count and differential. BALF cells were evaluated using a Neubauer chamber stained with Giemsa or trypan blue exclusion dye. BALF total protein content was determined by the Lowry assay. BALF tumor necrosis factor (TNF)-α and interleukin (IL)-1β content was quantified 3 and 6 hrs after LPS instillation by enzyme-linked immunosorbent assay with commercially available kits (R&D Systems, Minneapolis, MN; n = 4 rats each group). The times for cytokine determination were chosen since in longer times both cytokines tended to decrease to undetectable levels.

In a separated cohort of animals, ALI was induced as described previously to isolate lung tissue (n = 6 each treatment group). Twelve hours after LPS instillation, the rats were killed, lungs were perfused from the right ventricle with saline, and samples from the lungs were isolated and immediately stored at −70°C until assayed for oxidative stress and mitochondrial function variables or fixed in 4% formalin solution for histopathologic analyses (described subsequently). These animals were divided into five groups in a blinded manner: group 1, control with intratracheal instillation of isotonic saline; group 2, ALI treated with saline; group 3, ALI treated with NAC (20 mg/kg subcutaneously 3, 6, and 12 hrs after LPS instillation); group 4, ALI treated with DFX (20 mg/kg subcutaneously 3 hrs after LPS instillation); and group 5, ALI treated with NAC (20 mg/kg, 3, 6, and 12 hrs after LPS instillation) plus DFX (20 mg/kg 3 hrs after LPS instillation).

To determine the effects of iron overload on LPS-induced lung injury in a separate cohort of animals, ALI was induced as described previously (n = 6 each treatment group) and iron overload was induced as previously described (24) by a bolus of 0.75 mL of 1 mM ferric chloride immediately before LPS instillation. Twelve hours after LPS instillation, BALF was performed, lungs were perfused from the right ventricle with saline, and samples from the lungs were isolated and immediately stored at −70°C until assayed for oxidative stress variables (described subsequently). Animals were divided into the same five groups as stated previously.

Oxidative Stress Variables. As an index of oxidative stress in the lung, we used the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction as previously described (25). In addition, we determined in lung tissue the oxidative damage to proteins as previously described (26). We also determined mitochondrial generation of superoxide as previously described (22).

To determine antioxidant defenses against free radicals, we measured the activity of the major enzymatic defenses in lung tissues (catalase and superoxide dismutase). Catalase (CAT) activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm (27). Superoxide dismutase (SOD) activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described (28).

Total Nonheme Iron Levels. To determine nonheme iron level, lung homogenates were analyzed spectrophotometrically with commercially available kits (Labtest, Brazil).

Histopathologic Analyses. For histopathologic analyses after fixation, excised lung tissues were fixed intratracheally and then embedded in paraffin and routinely stained with hematoxylin and eosin. A blinded experienced pathologist performed histopathologic analyses. Inflammation and alveolar edema were graded as none, mild, moderate, or severe.

Effects of NAC and/or DFX on Cytokine Production From Alveolar Macrophages. To elucidate the direct effects of NAC plus DFX on cytokine production from alveolar macrophages, the cells were purified from BALF and cultured with NAC and/or DFX. Briefly, rats were anesthetized as described previously, and lungs were lavaged via the tracheal tube with phosphate-buffered saline (5 × 1 mL 37°C). Cells were washed and 1×10^5 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. After 24 hrs of incubation, the media were replaced by fetal bovine serum-free medium with or without NAC (10 mM), DFX (0.1 mM), or both 1 hr before LPS treatment (100 ng/mL for 6 hrs). After this period, the medium was recovered to determine TNF-α and IL-1β. To obtain sufficient macrophages for these experiments, BALF was pooled from two to three rats (n = 3 each group).

Statistical Analyses. Data are expressed as mean ± SEM in all figures. For the biochemical measures, the means for the different treatment groups were compared by two-way analysis of variance followed by a Newman-Keuls' test. Statistical significance was assigned to $p < .05$.

RESULTS

The kinetics of changes in the levels of inflammation in BALF were studied in rats 3, 6, 12, 24, and 48 hrs after LPS challenge. The total cell influx (with predominance of neutrophils) in BALF showed an almost immediate response after LPS challenge, increasing already after 3 hrs to reach maximal values at 12 hrs. This inflammatory response progressively resolved (Table 1). The concentration of total protein in BALF increased rapidly after LPS challenge like inflammatory cells (Table 1). Treatment with NAC plus DFX reduced the inflammatory response to LPS as evidenced by cell infiltration and protein leakage in BALF as early as 6 hrs (Table 1), returning to control levels 24 hrs after LPS instillation. Although NAC or DFX alone decreased BALF protein and inflammatory cells, the time and magnitude of this response were significantly different from NAC plus DFX treatment. BALF TBARS and protein carbonyls increased after LPS challenge accompanied by the inflammatory response described previously (Table 2). As we demonstrated for inflammatory variables, treatment with NAC plus DFX reduced BALF TBARS content as early as 6 hrs (Table 2), returning to control levels 24 hrs after LPS. There were no differences in the cytokine levels between

Table 1. Time course of cellular and biochemical variables in the bronchoalveolar lavage fluid of rats after intratracheal lipopolysaccharide (LPS)

Time After Instillation, Hrs	Total Cell Count, $\times 10^5$					Total Protein, mg/mL				
	Control	LPS	NAC	DFX	NAC + DFX	Control	LPS	NAC	DFX	NAC + DFX
3	13 ± 2.1	190 ± 12.3[a]	210 ± 15.4[a]	180 ± 21.2[a]	197 ± 16.3[a]	33 ± 2.1	65 ± 2.7[a]	71 ± 3.1[a]	73 ± 2.2[a]	72 ± 3.4[a]
6	11 ± 1.2	339 ± 18.9[a]	298 ± 21.5[a]	270 ± 18.7[a]	150 ± 14.8[b]	38 ± 1.6	98 ± 3.3[a]	85 ± 2.7[a]	88 ± 3.6[a]	42 ± 1.2[b]
12	55 ± 5.2	535 ± 24.5[a]	210 ± 17.6[b]	325 ± 28.7[a]	127 ± 10.3[b]	42 ± 2.7	72 ± 2.8[a]	55 ± 2.1	52 ± 1.4	44 ± 1.7[b]
24	30 ± 4.6	225 ± 13.1[a]	195 ± 15.9[a]	210 ± 12.6[a]	85 ± 9.3[b]	40 ± 2.4	64 ± 2.5[a]	50 ± 1.9	47 ± 1.7	32 ± 2.1
48	33 ± 4.7	195 ± 15.7[a]	100 ± 10.1[b]	110 ± 10.2[b]	50 ± 5.8[b]	35 ± 1.9	40 ± 2.2	47 ± 2.4	44 ± 1.4	30 ± 1.8

NAC, N-acetylcysteine; DFX, deferoxamine.

[a]Different from control $p < .05$; [b] different from LPS $p < .05$. Rats were treated intratracheally with LPS 100 μg/100 g of body weight. Several times after LPS instillation, the rats were killed and a bronchoalveolar lavage was performed to determine total and differential cell count and total protein content (n = 3 each time). Values are expressed as mean ± SEM.

LPS and treated groups 3 hrs after LPS instillation (data not shown). In contrast, BALF cytokine content was changed by NAC plus DFX treatment 6 hrs after LPS instillation. NAC treatment did not modify the levels of BALF TNF-α and IL-1β at 6 hrs (Table 3). DFX treatment increased IL-1β but did not modify TNF-α content (Table 3). NAC plus DFX treatment decreased both TNF-α and IL-1β (Table 3). Since these results could be secondary to the influence of antioxidants on macrophage function, we determined cytokine levels in cultured alveolar macrophage. In contrast to the *in vivo* data, NAC diminished the release of TNF-α and IL-1β from cultured alveolar macrophages activated by LPS (Table 4). The effect of DFX seemed to be similar to the *in vivo* effect and increased both IL-1β and TNF-α (Table 4). NAC plus DFX decreased both TNF-α and IL-1β, but only the release of TNF-α was significantly different from NAC alone (Table 4).

The measurement of TBARS and protein carbonyls on lung homogenates revealed that NAC plus DFX treatment significantly attenuated oxidative damage after ALI. NAC plus DFX, but neither NAC nor DFX alone, decreased TBARS and protein carbonyls after LPS administration (Fig. 1). Interestingly, NAC administration increased TBARS in ALI (Fig. 1). Lung mitochondrial dysfunction was assessed by determination of superoxide production in submitochondrial particles. ALI animals, but not the animals treated with NAC plus DFX, exhibited increased superoxide production compared with controls (Fig. 2). These oxidative alterations induced by LPS instillation were accompanied by an increase the lung content of nonheme iron (Table 5), and this was reversed by DFX or NAC plus DFX treatment. Although not statistically significant, NAC treatment alone increased total nonheme iron content compared with the LPS group (Table 5). TBARS and protein carbonyl content, including the increase in TBARS content after NAC treatment, were potentiated by iron overload, reinforcing the role of iron ions in LPS-induced ALI (data not shown).

To determine the potential influence of NAC plus DFX on the balance between antioxidant enzyme activities during ALI, CAT and SOD activities were determined in lung homogenates. As illustrated in Figures 3 and 4, an imbalance between SOD and CAT activities occurred in rats challenged with LPS. This imbalance was secondary to SOD inhibition and an increase in CAT activity, and this was significantly suppressed by antioxidant treatment, especially with NAC plus DFX (Figs. 3 and 4). This pattern of lung SOD activity was similar to the BALF SOD activity (data not shown).

Lung histopathology after LPS administration revealed alveolar disarray, severe inflammatory cell infiltration, and abundant alveolar exudates (Fig. 5*A*). All indicated that there was ALI in this model. After NAC plus DFX treatment, only mild inflammatory infiltration and no alveolar exudate were observed (Fig. 5*B*). The administration of DFX alone diminished alveolar inflammation (to a mild to moderate infiltrate), but there was moderate to severe peribronchial inflammation (Fig. 5*C*). In contrast, with NAC there were minimal histopathologic differences between the LPS and NAC groups (Fig. 5*D*).

DISCUSSION

The combination of NAC plus DFX could interfere with several steps of ALI induced by LPS. We demonstrated that this treatment interferes with oxidative stress, lung inflammatory response, antioxidant enzyme imbalance, and proinflammatory cytokine release from alveolar macrophage.

Oxidative stress and inflammatory response seem to be important in the pathogenesis ALI and ARDS; in this way, the use of antioxidants could be an alternative adjuvant therapy in these processes since they inhibit the release of proinflammatory cytokines, cellular apoptosis, and necrosis (29–31). Besides these mechanisms, it seems that oxidative stress could induce airway hyperreactivity in LPS-treated lungs (32). Studies in ARDS patients have demonstrated both a decrease in total glutathione and a relative increase in oxidized glutathione in BALF (33–35).

We here demonstrated that *in vitro* NAC diminished alveolar macrophage release of TNF-α and IL-1β as previously described (29), and this effect was similar to NAC plus DFX. The *in vivo* results suggested that the complex network of alterations occurring in the whole lung during LPS exposure modifies the observed *in vitro* response. NAC did not interfere with BALF cytokines, but the addition of DFX restored the expected NAC anti-inflammatory effect. We cannot ascertain the exact reason for these differences, but the persistence of oxidative stress in the NAC group, but not the NAC plus DFX group, could explain this in part. It was previously described that NAC pretreatment reduced TNF-α release from peritoneal macrophages in a model of endotoxic shock (36). In contrast, our *in vivo* design demonstrated that NAC alone did not interfere with BALF TNF-α and IL-1β. Probably these differences were secondary to the postinjury administration of NAC. We supposed that in a postinjury protocol, the alterations elicited by the initial immune response in-

Table 2. Time course of oxidative stress variables in the bronchoalveolar lavage of rats after intratracheal lipopolysaccharide (LPS)

Time After Instillation, Hrs	TBARS Content, nmol/mL					Protein Carbonyls, nmol/mL				
	Control	LPS	NAC	DFX	NAC + DFX	Control	LPS	NAC	DFX	NAC + DFX
3	0.05 ± 0.001	0.14 ± 0.02[a]	0.21 ± 0.03[a]	0.19 ± 0.02[a]	0.15 ± 0.02[a]	0.012 ± 0.001	0.09 ± 0.01[a]	0.11 ± 0.04[a]	0.08 ± 0.01[a]	0.12 ± 0.05[a]
6	0.043 ± 0.001	0.31 ± 0.04[a]	0.27 ± 0.07[a]	0.23 ± 0.03[a]	0.10 ± 0.02[a]	0.015 ± 0.0012	0.14 ± 0.06[a]	0.17 ± 0.07[a]	0.12 ± 0.03[a]	0.07 ± 0.01[a]
12	0.034 ± 0.002	0.25 ± 0.03[a]	0.30 ± 0.04[a]	0.22 ± 0.05[a]	0.06 ± 0.01	0.014 ± 0.0018	0.12 ± 0.05[a]	0.23 ± 0.05[b]	0.13 ± 0.03[a]	0.023 ± 0.006
24	0.041 ± 0.0012	0.12 ± 0.01[a]	0.25 ± 0.04[b]	0.10 ± 0.01[a]	0.03 ± 0.006	0.019 ± 0.0021	0.11 ± 0.02[a]	0.14 ± 0.02[a]	0.09 ± 0.01[a]	0.031 ± 0.004
48	0.039 ± 0.0023	0.09 ± 0.006	0.13 ± 0.05[b]	0.03 ± 0.003	0.04 ± 0.003	0.016 ± 0.0012	0.08 ± 0.01[a]	0.13 ± 0.05[a]	0.021 ± 0.005	0.011 ± 0.003

TBARS, thiobarbituric acid reactive species; NAC, N-acetylcysteine; DFX, deferoxamine.

[a]Different from control $p < .05$; [b] different from LPS $p < .05$. Rats were treated intratracheally with LPS 100 μg/100 g of body weight. Several times after LPS instillation, the rats were killed and a bronchoalveolar lavage was performed to determine TBARS and protein carbonyls content (n = 3 each time). Values are expressed as mean ± SEM.

Table 3. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β in the bronchoalveolar lavage fluid 6 hrs after intratracheal lipopolysaccharide (LPS)

	TNF-α, pg/mL	IL-1β, pg/mL
Control	UD	UD
LPS	130 ± 15	112 ± 11
LPS + NAC	100 ± 12[a]	94 ± 13[a]
LPS + DFX	145 ± 12[a]	234 ± 23[a,b]
LPS + NAC + DFX	31 ± 3[b]	49 ± 6[b]

UD, undetectable; NAC, N-acetylcysteine; DFX, deferoxamine.

[a]Different from LPS + NAC + DFX $p < .05$; [b] different from LPS $p < .05$. Rats were treated intratracheally with LPS 100 μg/100 g of body weight. Six hours after LPS instillation, the rats were killed and a bronchoalveolar lavage was performed to determine TNF-α and IL-1β content (n = 4 each time). Values are expressed as mean ± SEM.

Table 4. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β released by alveolar macrophages exposed to lipopolysaccharide (LPS)

	TNF-α, pg/mL	IL-1β, pg/mL
Control	UD	UD
LPS	45 ± 5	34 ± 3
LPS + NAC	20 ± 2[a,b]	18 ± 2[a]
LPS + DFX	78 ± 8[a,b]	150 ± 19[a,b]
LPS + NAC + DFX	8 ± .9[a]	15 ± 2[a]

UD, undetectable; NAC, N-acetylcysteine; DFX, deferoxamine.

[a]Different from LPS; [b] different from LPS + NAC + DFX. Macrophages were isolated from bronchoalveolar lavage and cultured for 24 hrs in medium supplemented with 10% fetal bovine serum. After 24 hrs of incubation, the media were replaced by fetal bovine serum-free medium with or without NAC (10 mM), DFX (0.1 mM), or both 1 hr prior to LPS treatment (100 ng/mL for 6 hrs). After this period, the medium was recovered to determine TNF-α and IL-1β (n = 3 each group).

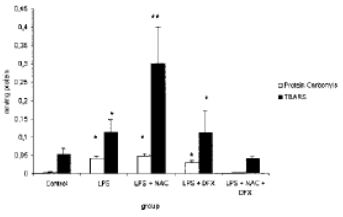

Figure 1. Thiobarbituric acid reactive species (*TBARS*) and protein carbonyls content in the lung after lipopolysaccharide (*LPS*) instillation. Rats were submitted to intratracheal administration of LPS or saline. LPS-administered animals were assigned to receive N-acetylcysteine (*NAC*), deferoxamine (*DFX*), or both as described in the text. Twelve hours after LPS administration, the lung was removed to determine TBARS and protein carbonyls content. Values are expressed as mean ± SEM (n = 6 each group). *Different from control, $p < .05$; **different from LPS, $p < .05$.

terfere with NAC antioxidant and proinflammatory effects. In addition, iron seemed to increase TNF-α secretion induced by LPS (37), and this is supported by our *in vivo* results. The addition of DFX to NAC restores its expected effect on oxidative variables and lung cytokines. In cultured alveolar macrophages, a system with low iron content, the addition of DFX to NAC had a modest but significant effect on TNF-α, but not IL-1β, release from alveolar macrophages. These results, together with our previously published results (22, 23), support an interference of iron on the therapeutic effects of NAC in different models of acute severe illness.

Previous studies demonstrated a preventive action of NAC in animal models of lung injury (16–18). These effects are less expressive when NAC is administered up to 2 hrs after endotoxin challenge (1, 16, 17). Some of these limitations of NAC therapy could be related to its adverse effects. It seems that high doses of NAC aggravate LPS toxicity (17). The oxidative metabolism of NAC can generate thiyl free radicals that have been increasingly considered as intermediates in processes that may be involved in the development of biological damage resulting from oxidative stress. *In vitro*, NAC increased hydroxyl radical generation in a system with Fe(III)-citrate and H_2O_2 by reducing ferric iron to its catalytic, active Fe^{+2} form (38). We believe that this oxidative property of NAC could, in part, explain the lung TBARS and BALF cytokine levels. We supposed that the addition of DFX to the NAC regimen prevents its oxidation, and the occurrence of the Fenton chemistry maintained by the iron recycling mediated by NAC. We demonstrated that LPS increased nonheme iron content in the lung, as previously described (39). Upton et al. (39) demonstrated that after LPS, lung iron increased and this was accompanied by a decrease in ferritin levels. NAC treatment seemed to increase total nonheme iron content (Table 5),

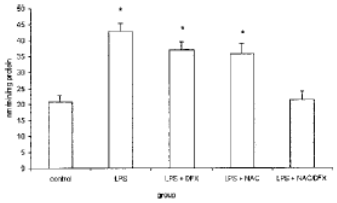

Figure 2. Mitochondrial superoxide production in the lung after lipopolysaccharide (*LPS*) instillation. Rats were submitted to intratracheal administration of LPS or saline. LPS-administered animals were assigned to receive N-acetylcysteine (*NAC*), deferoxamine (*DFX*), or both as described in the text. Twelve hours after LPS administration, the lung was removed to determine the mitochondrial superoxide production. Values are expressed as mean ± SEM (n = 6 each group). *Different from control, $p < .05$.

Table 5. Total non-heme iron (nmol/mg protein) in lung homogenates 12 hrs after intratracheal lipopolysaccharide (LPS)

	Total Non-Heme Iron, nmol/mg of Protein
Control	2.02 ± 0.2
LPS	5.5 ± 0.5[a]
LPS + NAC	7.1 ± 1.2[a]
LPS + DFX	3.1 ± 0.4
LPS + NAC + DFX	3.2 ± 0.5

NAC, N-acetylcysteine; DFX, deferoxamine.

[a]Different from control $p < .05$. Rats were treated intratracheally with LPS 100 μg/100 g of body weight. Twelve hours after LPS instillation, the rats were killed and the lung was isolated to determine total non-heme iron content (n = 6 each group). Values are expressed as mean ± SEM.

and this could be associated with the effects of NAC on iron regulatory protein and ferritin synthesis (40, 41). Thus, the alterations of iron metabolism after LPS could be associated, in part, with the observed pro-oxidant NAC effects.

It seems reasonable to use an iron chelator in addition to NAC to improve its therapeutic effects in ALI, and our *in vivo* results support this. In addition, we loaded DFX with excess of iron and this did not modify the effects of DFX on oxidative variables and BALF protein and cellular content (data not shown), supporting the importance of its chelator potential on the observed effects. We previously demonstrated that the association of NAC plus an iron chelator (DFX) is superior to each alone in the treatment of severe sepsis and acute hepatic failure in an animal model (22, 23). These effects seem to be of great importance in ALI and

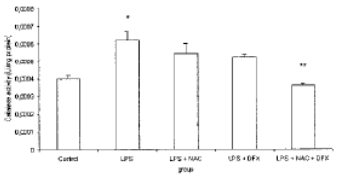

Figure 3. Catalase activity in the lung after lipopolysaccharide (*LPS*) instillation. Rats were submitted to intratracheal administration of LPS or saline. LPS-administered animals were assigned to receive N-acetylcysteine (*NAC*), deferoxamine (*DFX*), or both as described in the text. Twelve hours after LPS administration, the lung was removed to determine the catalase activity. Values are expressed as mean ± SEM (n = 6 each group). *Different from control, $p < .05$; **different from LPS, $p < .05$.

ARDS. Aberrant regulation of iron metabolism and deficient antioxidant protection are associated with ARDS (11), but we cannot find in the literature the adjuvant use of DFX in the treatment of LPS-induced ALI. Increased concentrations of available iron in ARDS may participate in catalyzing oxidant generation destructive to the tissues of the lower respiratory tract (12). The concentrations of total and nonheme iron seem to be increased in the BALF of ARDS patients (12). This increased metal availability elicits an increased expression of transferrin receptor, lactoferrin, ferritin, and heme oxygenase in the lower respiratory tract, which will function to diminish this oxidative stress (12, 13, 42). The significant advantage of the NAC plus DFX treatment, even when rendered 3 hrs after LPS administration, provides compelling support for the notion that oxidative stress and free iron play a role in the development of LPS-induced ALI.

DFX is an indiscriminate and very powerful iron chelator. Although patients with ARDS/ALI have altered body iron chemistry, they are not iron-overloaded. In contrast, in the setting of sepsis and several other critical illnesses, patients can be anemic and the use of DFX in such situations can outweigh any clinical benefit. Thus, the use of iron chelators for conditions in which there are changes in iron mobilization and storage of a transient nature, such as occur in ARDS/ALI or sepsis, must be viewed with caution. Iron has many important biological functions related to biosynthesis, proliferation, gene regulation, and cell signaling processes. The indiscriminate interference with such iron pools is likely to

Our data provide the first experimental demonstration that N-acetylcysteine plus deferoxamine decreases oxidative stress and mitochondrial dysfunction and limits inflammatory response and alveolar pathology induced by lipopolysaccharide in the rat.

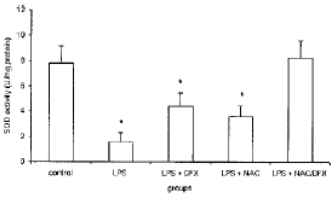

Figure 4. Superoxide dismutase activity in the lung after lipopolysaccharide (*LPS*) instillation. Rats were submitted to intratracheal administration of LPS or saline. LPS-administered animals were assigned to receive N-acetylcysteine (*NAC*), deferoxamine (*DFX*), or both as described in the text. Twelve hours after LPS administration, the lung was removed to determine the superoxide dismutase activity. Values are expressed as mean ± SEM (n = 6 each group). *Different from control, $p < .05$.

cause major disruption to a range of cellular functions. Thus, the use of such an antioxidant cocktail in the clinical setting deserves several preclinical evaluations of its efficacy and security. Another potential limitation of our study was the occurrence of *ex vivo* oxidation of the samples. Since one set of samples contained antioxidants, this could limit *ex vivo* oxidative damage; thus, there was a potential for artifacts in our analyses. We tried to minimize such artifacts by preserving samples at −80°C until their utilization and freezing samples separately for each technique to avoid refreezing.

In addition, the demonstrated effect of NAC plus DFX on SOD activity seems to be of importance. Several studies demon-

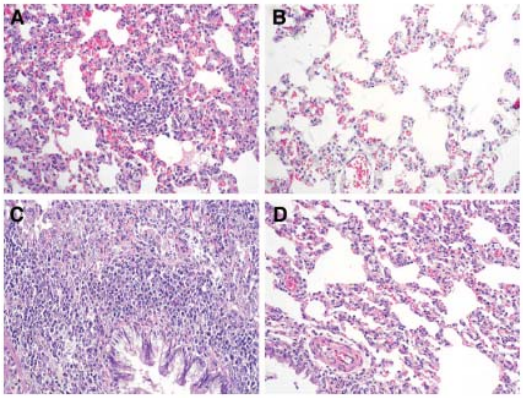

Figure 5. Histopathologic findings after lipopolysaccharide (LPS) instillation. Rats were submitted to intratracheal administration of LPS or saline. LPS-administered animals were assigned to receive (*A*) saline, (*B*) N-acetylcysteine plus deferoxamine, (*C*) deferoxamine, or (*D*) N-acetylcysteine as described in the text. Twelve hours after LPS administration, the lung was removed for histopathologic analyses. Representative illustrations from three animals each group (hematoxylin and eosin ×400).

strated the relation of extracellular SOD with decreased recruitment of neutrophils (43, 44). Extracellular SOD reduced the expression of adhesion molecules. One mechanism by which extracellular SOD might modulate neutrophil inflammation is by reducing cytokine release from macrophages (43). This suggests that extracellular SOD should be considered as an anti-inflammatory enzyme as well as a bulk antioxidant. In this way, some of the protective effects of NAC plus DFX could be secondary to its effects on SOD activity demonstrated here.

CONCLUSIONS

Our data provide the first experimental demonstration that NAC plus DFX decreases oxidative stress and mitochondrial dysfunction and limits inflammatory response and alveolar disarray induced by LPS-induced ALI in the rat.

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5- DISCUSSÃO

Neste trabalho determinamos que o uso da combinação de NAC e DFX é mais eficiente ao uso isolado dos compostos em diferentes modelos animais de doenças inflamatórias. Tentamos explorar diferentes mecanismos responsáveis por esta efetividade, e determinamos que estes antioxidantes atuam em pelo menos três diferentes aspectos da fisiopatologia das doenças estudadas: 1) dano oxidativo em órgãos alvo, 2) disfunção mitocondrial associada a sepse, 3) atenuação da resposta inflamatória.

5.1- Atenuação do dano oxidativo em órgãos alvo

De todos estes mecanismos talvez o mais óbvio seja a atenuação do dano oxidativo em diferentes órgãos associados ao desenvolvimento destas doenças. No capitulo II demonstramos que o uso de NAC + DFX atenua o dano oxidativo em diversos órgãos associados à resposta séptica. A disfunção de múltiplos órgãos é central na mortalidade associada à sepse em humanos. Diversas evidências sugerem que o estresse oxidativo tem um papel na falência de órgãos durante o desenvolvimento de sepse (Crimi et al, 2006a, 2006b; Jao et al 2005; Singer et al 2004; Zhang et al 2000). Este estresse pode ser secundário a ativação de xantina oxidase durante a isquemia/reperfusão (Conlon et al 2005, Galley et al 1996), ativação do sistema imune (Cavaillon e Adib-Conquy 2005, Brown et al 2006), disfunção mitocondrial (Protti e Singer 2006, Crouser 2004), depleção de antioxidantes (Mishra et al 2005, Voigt et al 2002, Crimi et al 2006, Cowley et al 1996; Goode et al 1995, Pascual et al

(1998. Chuang et al 2006). Apesar destas evidências, estudo realizado por Linares e cols utilizando técnicas elegantes para a detecção de radicais livres demonstrou que a presença de estresse oxidativo durante a endotoxemia não parece ser tão importante quanto previamente descrito (Linares et al 2003). De qualquer forma 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. 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. NAC é efetiva em diferentes modelos de sepse, incluindo endotoxemia (Hsu et al 2006) e CLP (Ozdulger et al 2003). Cabe ressaltar que se deve levar em conta a limitação do uso de LPS como modelo de sepse (Rittirsch et al 2007) e que o uso de NAC em modelos de CLP normalmente é feito antes, ou logo após a CLP. Além disto estudo de Sprong e cols demonstrou que doses altas de NAC são associadas a aumento de mortalidade, provavelmente pelo seu efeito pró-oxidante (Sprong et al 1998). NAC também foi estudada em modelos de sepse utilizando animais de maior porte, encontrando-se efeitos positivos (Zhang et al 1995). Poucos estudos avaliam o uso isolado de DFX em modelos animais de sepse. Vulcano et al demonstraram que DFX diminui estresse oxidativo e reduz mortalidade em modelo animal de endotoxemia (Vulcano et al 2000). Em

modelo de CLP, Messaris e cols demonstraram que o uso profilático de DFX reduz apoptose e mortalidade (Messaris et al 2004). Este último estudo utiliza a mesma dose de DFX que nossos estudos, porém o tempo do inicio da administração e o tempo de seguimento dos animais pós-CLP limitam a interpretação dos dados. Nossos resultados, portanto, somam ao conhecimento prévio do tema, determinando que a associação de antioxidantes parece ser mais efetiva em modelo de sepse, insuficiência hepática aguda e lesão pulmonar aguda, mesmo quando administrados após o desenvolvimento das doenças. A redução de estresse oxidativo nestes modelos pode ser efeito direto dos antioxidantes utilizados, ou secundários aos seus efeitos sobre a disfunção mitocondrial e resposta inflamatória que serão abordados posteriormente. Outro aspecto importante do papel antioxidante de NAC mais DFX parece ser a restauração do equilíbrio entre atividade de SOD e CAT que nosso grupo sugere ser importante na geração de estresse oxidativo durante o desenvolvimento de sepse (Andrades et al 2005).

Mesmo sem uma definição apropriada em modelos animais diversos, ensaios clínicos foram desenvolvidos para avaliar o efeito de antioxidantes no tratamento da sepse (para excelente revisão veja Crimi et al 2006b). A maioria deles utiliza NAC como antioxidante e avalia desfechos clínicos secundários (como p.ex. variáveis hemodinâmicas e respiratórias, escores de gravidade e perfil plasmático de citocinas). Mesmo assim, os resultados são conflitantes, com estudos positivos, estudos negativos e estudos neutros (Crimi et al 2006). O ensaio clínico melhor desenhado para avaliar o uso de antioxidantes em pacientes sépticos, recentemente publicado, utiliza selênio como antioxidante e avalia desfechos clínicos primários (Angstwurm et al 2007) Diferentemente dos

ensaios que utilizam NAC este estudo administra selênio por tempo prolongado e demonstrou que a suplementação com selênio reduz significativamente a mortalidade em 28 dias em pacientes com SIRS e sepse.

Outro aspecto relevante para se ressaltar dos nossos resultados foi a demonstração de que estes antioxidantes conseguem diminuir o estresse oxidativo no sistema nervoso central (SNC) de animais submetidos a insuficiência hepática aguda. Isoladamente parece que estes resultados representam o efeito dos antioxidantes em mais um órgão envolvido em doenças inflamatórias agudas. Entretanto este achado iniciou uma importante linha de pesquisa em nosso laboratório onde avaliamos o estresse oxidativo em SNC durante a sepse e suas possíveis conseqüências a longo prazo (Barichello et al 2005a e 2005b). Estudos conduzidos por Barichello e colaboradores demonstraram pela primeira vez que existe estresse oxidativo em diferentes regiões do SNC de ratos sépticos, e que este estresse diferentemente de outros órgãos é precoce e transitório (Barichello et al 2006). Esta mesma autora posteriormente demonstrou que animais sobreviventes de sepse apresentam alterações de memória e que estas alterações dependem em parte da ocorrência de estresse oxidativo no SNC (Barichello et al 2007). O entendimento do papel do SNC no desenvolvimento de alterações agudas e crônicas em doenças inflamatórias agudas ainda é limitado, e nossos resultados demonstrados no capitulo III auxiliam neste entendimento e, principalmente, serviram de incentivo para ampliação dos leques de oportunidade para serem explorados no futuro por nosso laboratório.

5.2- Disfunção mitocondrial associada à sepse

Além dos efeitos diretos sobre o estresse oxidativo discutido anteriormente, no capítulo II demonstramos que o tratamento com antioxidantes diminui a produção de superóxido em partículas sub-mitocondriais o que sugere um possível efeito do tratamento sobre disfunção mitocondrial tão importante para o desenvolvimento de sepse. Este aspecto é ressaltado no editorial publicado pelo Dr Crouser (Crouser 2004), provavelmente um dos autores com maior número de publicações sobre disfunção mitocondrial e sepse nos últimos anos, acerca de nosso artigo (anexo 1).

A relação entre oferta inadequada de oxigênio e mortalidade de doentes criticamente enfermos foi proposta 40 anos atrás (Broder et al 1964). Ao assumir que os níveis altos de lactato era conseqüência do metabolismo anaeróbio, a correlação entre o nivel de lactato e mortalidade levou a conclusão de que a falta de oferta de oxigênio deve ser fator primário no desenvolvimento de choque séptico. Este e outros achados apoiavam esta conclusão e por muito tempo se assumiu estas observações como verdade. Nos anos 90, dois ensaios clínicos randomizados desenhados para otimizar a oxigenação tecidual em pacientes com sepse falharam em comprovar esta teoria (Hayes et al 1994, Gattinoni et al 1995). Análises retrospectivas destes dados sugeriam que não sobreviventes de sepse apresentavam uma redução na capacidade de aumentar o consumo de oxigênio tissular em resposta a aumento da oferta de oxigênio (Hayes et al 1997). Recentemente VanderMeer e cols demostraram que a acidose tissular durante a sepse se desenvolvia

independente da oxigenação tissular sugerindo que o mecanismo da acidose poderia ser independente do metabolismo anaeróbico (VanderMeer et al 1995). A partir deste estudo uma série de evidências apontou para a teoria de que a desregulação do metabolismo do oxigênio durante a sepse é manifestação, não de redução de oferta de oxigênio, mas de diminuição da utilização celular de oxigênio, atualmente denominada hipóxia citopática (Fink 2001), sendo a disfunção mitocondrial central neste contexto.

No contexto da hipóxia citopática diversas teorias são aventadas para explicar este fenômeno. Dentre as propostas de mecanismos talvez a mais antiga sugira que a produção de NO seria o responsável primário pela disfunção mitocondrial. 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. Entretanto em um modelo felino de sepse com adequada perfusão tecidual o dano mitocondrial antecede o aumento da iNOS e da detecção de nitrotirosina (Crouser et al 2000). Outra possibilidade é a de que os diferentes mediadores da resposta inflamatória da sepse possam levar a disfunção mitocondrial. Entre estes, o TNF-α tem capacidade de levar a 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). Neste sentido, já foi descrito que as concentrações de ceramida estão aumentadas durante sepse em humanos (Delogu et al 1999). Estes achados corroboram com dados não publicados de nosso grupo que demonstraram que em um modelo de CLP com ressuscitação volêmica a produção de superóxido em partículas submitocondriais não é alterada pela inibição farmacológica de NOS, induz estresse oxidativo e *swelling* mitocondrial. Todos estes efeitos são parcialmente inibidos por antioxidantes.

Além disto, o genoma mitocondrial é particularmente suscetível a estresse oxidativo (pela ausência de histonas e enzimas de reparo) e dano ao DNA mitocondrial parece acontecer durante a sepse (Sulliman et al 2003). Com isto há redução na quantidade de proteínas da cadeia de transporte de elétrons o que contribui para a disfunção mitocondrial da sepse. Diferentes subunidades dos complexos da cadeia de transporte de elétrons têm sua transcrição e traduções diminuídas durante o desenvolvimento de sepse (Callahan et al 2005a 2005b).

Independente da possível causa da disfunção mitocondrial na sepse ela pode levar a redução da extração tecidual de oxigênio (Fink 2002), depleção de reservas energéticas celulares (Fredriksson et al 2006), produção de radicais livres por desestruturação da cadeia de transporte de elétrons (Kozlov et al 2006). Estas alterações fisiológicas e celulares podem contribuir para a morte celular e disfunção de múltiplos órgãos. Estudos em animais demonstram que a disfunção mitocondrial associada à sepse se correlaciona com a gravidade

da disfunção orgânica (Fredriksson et al 2006, Brealey et al 2004, Crouser et al 2002, Crouser et al 1999). Os resultados em estudos experimentais são reforçados por alguns estudos em humanos. Em apoio à teoria da hipóxia citopática, o aumento nos níveis de lactato em pacientes sépticos não parece ser secundário a hipoxia, mas a um aumento na atividade da Na/K ATPase (Levy et al 2005). Estudo elegante realizado por Svistunenko e cols demonstrou a ocorrência de semiquinonas em músculo de pacientes sépticos e isto se correlaciona com critérios de gravidade da doença (Svistunenko et al 2006). Em músculo esquelético de pacientes sépticos os níveis energéticos se correlacionam com a gravidade da doença e com desfechos clínicos relevantes (Brealey et al 2002). Neste mesmo sentido, estudo de Vanhorebeek e cols demonstraram que o controle glicêmico estrito em pacientes criticamente enfermos restaura a função mitocondrial e a energética celular em paralelo a melhora de sobrevida observada (Vanhorebeek et al 2005).

Neste sentido nossos resultados sugerem um papel protetor da associação de NAC e DFX sobre a função mitocondrial associada a sepse e insuficiência hepática aguda. Entretanto, é difícil com nossos dados determinar o exato mecanismo do tratamento com antioxidantes. O tratamento com antioxidantes pode interferir com diferentes mecanismos responsáveis pela disfunção mitocondrial. Demonstramos no modelo de lesão pulmonar aguda que o tratamento com NAC e DFX tem efeito sobre a secreção de citocinas inflamatórias, sendo que, como descrito acima o TNF pode ter efeito direto sobre a função mitocondrial. Certamente não temos, em nosso estudo, determinação destas citocinas no modelo de CLP, mas este efeito já foi previamente descrito para o uso de NAC (Victor et al 1999). 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.

5.3- Resposta inflamatória e antioxidantes

A ativação do sistema imune e inflamatório é essencial no decorrer da resposta séptica. A infecção leva, em um primeiro momento, a ativação da resposta imune inata. A ativação desta serve como primeira linha de defesa contra a infecção e sinaliza para a ativação do sistema imune adquirido. A eficácia da resposta inflamatória / imune vai depender da comunicação entre estes dois sistemas e isto parece ter papel central na fisiopatologia da sepse (Hotchkiss et al 2003). Os patógenos, ou produtos do seu metabolismo, disparam a resposta inflamatória por ativar a transcrição de diversos genes inflamatórios, levando liberação de citocinas, quimiocinas, ROS. Simultaneamente vias antiinflamatórias são ativadas como mecanismos contra-regulatórios para conter a resposta inflamatória. O desequilíbrio destas vias pode levar a SIRS ou CARS e estas têm papel fundamental na mortalidade da sepse (Hotchkiss et al 2003).

Pelo menos em dois grandes processos relevantes a função do sistema imune existe participação central dos radicais livres: 1) erradicação do patógeno; 2) controle da expressão gênica em células imunes. Certamente

entre os dois o papel mais comumente discutido é a erradicação do patógeno. Diferentes estratégias relativas às EAO são utilizadas pelo sistema imune para erradicação dos patógenos. De qualquer forma como este tipo de mecanismo de defesa é inespecífico pode ser um dos geradores do dano orgânico descrito anteriormente. Diferentes espécies derivadas de oxigênio e nitrogênio podem ter papel na erradicação do patógeno. Estas espécies são geralmente associadas à resposta inflamatória disparada predominantemente por macrófagos e neutrófilos (Segal 2006, Forman e Torres 2001). O superóxido produzido pela NADPH oxidase pode diretamente exercer atividade bactericida (Roos et al 2003). Entretanto é fundamental na produção da espécie reativa de oxigênio central no papel de erradicar patógenos, o peróxido de hidrogênio (Kettle et al 2007). A partir deste podemos formar o radical hidroxil pela reação de Fenton, ou originar ácido hipocloroso, via mieloperoxidase. Nosso grupo sugere que a formação de peróxido de hidrogênio é favorecida, durante a sepse, pelo aumento desproporcional entre a atividade da SOD e da CAT e GPx e nossos resultados demonstram que o tratamento com antioxidantes atenua o desequilíbrio entre SOD e CAT. Além destes, a ativação do sistema imune pode aumentar a produção de NO que ao reagir com superóxido forma peroxinitrito que também tem poder bactericida (Fang 2004).

A estes mecanismos de erradicação de patógenos se soma o papel dos radicais livres na ativação da resposta inflamatória (Fialkow et al 2007, Fernando de Souza et al 2007). O processo de indução de genes inflamatórios é mediado pela ativação de fatores de transcrição como o AP-1 e o NF-κB (Roebuck et al 1999) e recentemente o papel do Nrf-2 na sepse foi descrito (Thimmulappa et al 2006). Destes o NF-κB parece ser fundamental na

fisiopatologia da sepse e ele pode ser regulado por radicais livres (Liu et al 2006). Durante a evolução de sepse o NF-κB pode ser ativado por patógenos ou por citocinas levando a amplificação da resposta inflamatória (Liu et al 2006). Além da especulação teórica, diversas evidências demonstram a participação do NF-κB na fisiopatologia da sepse, por exemplo, o NF-κB é altamente ativo em células mononucleares de pacientes com sepse e seu nível de ativação se correlaciona com o APACHE II e mortalidade nestes pacientes (Arnalich et al 2000, Bohrer et al 1997). Além disto a inativação deste fator parece ter efeito favorável no desfecho de sepse em modelos animais (Mitaka et al 2005, Ikezoe et al 2003).

As vias de transdução de sinal que levam a ativação do NF-κB são múltiplas e complexas. Estímulos particulares podem ativar algumas vias de sinalização especifica, enquanto algumas vias são compartilhadas por diferentes estímulos, por outro lado o mesmo estimulo pode ativar o NF-κB por diferentes vias. Neste último caso encontra-se, por exemplo, a ativação por LPS, que pode ativar NF-κB por ativação de mitogen-activated protein (MAP) quinase ou de proteína quinase C (Gloire et al 2006, Doyle et al 2006, Zhang e Ghosh 2000). A regulação redox do NF-κB parece ocorrer através da oxidação/redução de resíduos de cisteínas altamente conservados no sítio de ligação ao DNA do NF-κB (Gloire et al 2006). Em geral, antioxidantes e oxidantes tem papel opostos na regulação deste fator, antioxidantes inibem e oxidantes ativam o mesmo (Meyer et al 1993). Porém, existem diferenças conforme o tipo celular estudado e os mecanismos associados a esta regulação ainda não são completamente esclarecidos, fugindo dos objetivos de nosso trabalho. Não sabemos se o esquema antioxidante utilizado em nossos

estudos interfere com NF-κB, mas já existe demonstração que NAC exerce este papel em células do sistema imune (Victor et al 2003). Além disto, não sabemos sobre qual regulador do estado redox intracelular o esquema atua. Provavelmente atue sobre os níveis de glutationa reduzida (GSH), por ser a NAC doadora de cisteína para a produção de GSH via gama-glutamilcisteina sintase, porém não podemos excluir o papel sobre outros fatores que mantém o estado redox como, por exemplo, a tioredoxina (Deneke 2000).

Por todas estas considerações o uso de antioxidantes pode apresentar uma dupla face na sepse (como bem citado no editorial a respeito do artigo do capítulo 4 assinado pelo Dr. Barry Halliwell – anexo 2). 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, ou Compensatory Antiinflamatory Response Syndrome (CARS) 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.

5.4- Outras possibilidades de mecanismos dos antioxidantes

Além das possibilidades de mecanismos para a efetividade dos antioxidantes abordados nesta tese, alguns outros mecanismos possíveis não foram abordados diretamente, mas são tópicos de discussão na literatura. Um dos grandes desafios clínicos no tratamento da sepse é o choque refratário a vasopressores. Neste sentido existem evidências de que baixas doses de corticóides podem melhorar a sobrevida de pacientes em choque séptico refratário, que apresentem insuficiência adrenal relativa (Annane et al 2006, Annane et al 2002), entretanto esta medida parece não ser suficiente. Catecolaminas podem ser desativadas por um processo dependente de superóxido, neste cenário sua atividade vasocontritora é perdida (Macarthur et al 2000, Salvemini et al 2002). Além disto, estes derivados oxidados podem ser citotóxicos (Yates et al 1981, Singal et al 1982). Neste sentido o uso de miméticos de SOD parece ter efeito benéfico na sepse experimental, predominantemente por evitar a oxidação das catecolaminas, além de prevenir os efeitos deletérios do superóxido, como a geração de peroxinitrito (Macarthur et al 2000). Curiosamente nosso grupo demonstrou que a atividade plasmática de SOD pode prever mortalidade em modelo animal de sepse (Ritter et al 2003). Isto pode refletir uma menor quantidade de SOD extracelular ancorada a membrana do endotélio vascular, com maior inativação de catecolaminas, alteração do controle do tônus vascular e maior produção de peroxinitrito. Neste sentido, Wang e cols demonstraram que animais submetidos a endotoxemia apresentam níveis reduzidos de SOD extracelular (Wang et al 2003). O dano endotelial também pode participar na redução da resposta às catecolaminas, além de aumentar a permeabilidade capilar (Szabo et al 1996) e o estresse oxidativo parece participar do dano endotelial durante a sepse

(Huet et al 2007). Talvez o mais importante mecanismo de dano endotelial seja a ativação da poli-ADP ribose polimerase (PARS) pelo peroxinitrito (Boulos et al 2003). A ativação excessiva desta enzima leva a depleção de energia celular, secundária ao consumo de NAD^+, e morte celular (Kirkland et al 1991). A ativação da PARS por peroxinitrito tem sido descrita em diversas alterações induzidas pela sepse (Soriano et al 2006), incluindo a disfunção endotelial. Células endoteliais humanas tratadas com soro de pacientes sépticos apresentam uma diminuição da respiração mitocondrial e dos níveis de ATP, sendo que estas alterações são revertidas por inibidores de PARS e de iNOS, sugerindo o papel central do peroxinitrito e da PARS na lesão endotelial associada a sepse (Boulos et al 2003). Além da ativação da PARS a apoptose de céluas endotelias pode ser secundária a exossomos derivados de plaquetas com atividade de NAD(P)H oxidase e conseqüente produção de radicais livres (Janiszewski et al 2004). Neste sentido o uso de antioxidantes poderia reduzir a lesão endotelial ao diminuir os níveis de peroxinitrito e conseqüente ativação da PARS.

5.5- Limitações do estudo

1 - 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. O modelo de insuficiência hepática aguda, apesar de ser amplamente utilizado em estudos de toxicologia, tem uma série de limitações intrínsecas. Talvez a principal delas seja o fato de que o dano hepático é mediado por estresse oxidativo, o que em nosso caso pode ser uma grande limitação. A proteção mediada por antioxidantes pode não ser válida para outros tipos de lesão hepática, não primariamente oxidativas. Apesar disto, apesar de o dano inicial ser mediado diretamente por oxidação, a perpetuação do dano é feita por resposta inflamatória e expressão de diversas citocinas comuns a outros modelos de lesão hepática aguda (Weber et al 2003, Luster et al 2000). O modelo de lesão pulmonar aguda empregado não apresenta mortalidade, apenas mimetiza as alterações inflamatórias encontradas em pacientes com SARA. Além disto, as alterações mecânicas observadas em pacientes com SARA também não se aplicam ao modelo de instilação intratraqueal de LPS (Kobayashi et al 2001), e este modelo não permite caracterizar as diferenças entre SARA pulmonar e extra-pulmonar. Normalmente os melhores modelos animais de SARA utilizam animais de grande porte, sob ventilação mecânica, para mimetizar as alterações clínicas e o suporte terapêutico de situações reais, mas este tipo de modelo extrapola os objetivos do presente estudo;

2 - As medidas de estresse oxidativo determinadas certamente não são padrão ouro para a detecção de radicais livres. A medida destas espécies pode ser feita diretamente, normalmente por técnicas que utilizam detecção por

ressonância eletrônica. Outra maneira de detectar sua presença é indireta, não se mede o radical livre, mas seu efeito em biomoléculas, como as técnicas empregadas em nossos estudos. Mesmo dentre as técnicas de detecção indiretas, as empregadas neste estudo são sujeitas a criticas, principalmente a medida de TBARS. Entretanto, para os objetivos deste estudo as técnicas utilizadas para determinação do estresse oxidativo não parecem influenciar nos resultados, uma vez que foram utilizadas apenas como indicativo de estresse oxidativo e não como marcador específico de peroxidação lipídica ou oxidação de proteínas.

3 – Com os presentes dados não podemos determinar os exatos mecanismos envolvidos na efetividade do uso de antioxidantes em doenças inflamatórias agudas. Determinamos que o uso de antioxidantes atenua a disfunção mitocondrial, o dano oxidativo e a reposta inflamatória, mas não podemos afirmar se um destes mecanismos é o principal, se eles são complementares ou se são os únicos. A resposta para estas questões é complexa, provavelmente difícil de ser atingida. Além disto, não determinamos se as doses empregadas e o tempo de administração são os ideais para as doenças estudadas.

5.6- Perspectivas futuras

Diversas possibilidades de continuidade deste trabalho podem ser propostas, desde a bancada até a beira do leito. Um melhor entendimento dos mecanismos de ação dos antioxidantes sobre macrófagos e outras células do sistema imune, as vias de sinalização intracelulares envolvidas, os genes modulados entre outros. Determinar os efeitos celulares dos antoxidantes, predominantemente sobre a função mitocondrial, determinando se há

mecanismo principal, entre os diversos mecanismos postulados, para a citoproteção demonstrada neste estudo. Determinar os efeitos dos antioxidantes sobre variáveis fisiológicas, como cardiovasculares, pulmonares e de sistema nervoso central, este último abrindo diversas possibilidades tanto de efeitos agudos, quanto de seqüelas tardias de doenças inflamatórias agudas. Transportar os dados dos modelos animais para pacientes em unidades de terapia intensiva parece ser algo factível. Os antioxidantes utilizados em nosso estudo já são empregados na prática clínica com outras indicações. A NAC parece ter razoável segurança inclusive em pacientes com sepse. A DFX ainda não foi utilizada nesta condição, já foram descritas reações graves com o seu uso, predominantemente lesão pulmonar aguda e predisposição a infecções. Por outro lado, cada vez mais a sobrecarga de ferro parece ser deletéria para o paciente criticamente enfermo, por isso parece ser razoável propor o uso de NAC e DFX em ensaios clínicos em pacientes com sepse.

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Anexo 1

Anexo 2