Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

ESTRESSE OXIDATIVO E O DESENVOLVIMENTO DE SEPSE

Cristiane Ritter

Orientador: Dr. Felipe Dal Pizzol

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul como requisito para a obtenção do grau de Mestre em Bioquímica

Porto Alegre

2003

Agradecimentos

Ao professor Felipe Dal Pizzol pela orientação, apoio e dedicação.

Aos amigos do laboratório: Michael, Fernanda, Manoela, Marinho, Ramatis, Evandro, Ricardo e Fábio pela colaboração e pelo excelente ambiente de trabalho que me propiciaram.

Ao grande amigo Michael que se empenhou tanto na realização deste trabalho.

Ao professor José Cláudio pela orientação, aconselhamento e amizade.

Aos meus pais por tudo que fizeram e ainda fazem por mim.

Ao meu irmão Charles pela amizade e incentivo.

Às amigas Ticiana e Beatriz pelo incentivo.

À minha família por estarem sempre presentes nos momentos bons e ruins, oferecendo compreensão e amor. Ao meu filho Henrique que participou em tempo integral da realização desta dissertação e colaborou com a mamãe até no dia do nascimento. Vocês são a motivação para tudo que faço na vida.

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Abreviaturas

CLP: ligação cecal e punção

EAO: espécies ativas de oxigênio

•OH: radical hidroxil

DNA: ácido deoxirribonucleico

H_2O_2: peróxido de hidrogênio

Fe^{2+}: ferro II

Fe^{3+}: ferro III

O_2^-: superóxido

H^+: próton

O_2: oxigênio molecular

H_2O: água

ROOH: peróxido orgânico

GSH: glutationa reduzida

GSSG: glutationa oxidada

SOD: superóxido dismutase

MnSOD: manganês superóxido dismutase

CuZnSOD: cobre / zinco superóxido dismutase

GPx: glutationa peroxidase

CAT: catalase

LPS: lipopolissacarídeo

TNFα: fator de necrose tumoral α

IL-1β: interleucina 1β

NAC: N-acetilcisteína

DFX: deferoxamina

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Resumo

O estresse oxidativo tem um papel importante no desenvolvimento da falência orgânica múltipla e choque séptico. O presente estudo foi realizado para determinar diferentes parâmetros de dano oxidativo a biomoléculas, produção de superóxido mitocondrial, atividades da superóxido dismutase e catalase e suas relações com a mortalidade por sepse em um modelo animal. Além disso, nós avaliamos os efeitos da combinação de antioxidantes (N-acetilcisteína e deferoxamina) em um modelo murino de sepse polimicrobiana induzida por ligação cecal e punção (CLP). Ratos Wistar machos (250-300 g) foram randomicamente divididos em quatro grupos: sham-operated, CLP ressuscitado com solução salina (50 ml/Kg imediatamente e 12h após CLP), CLP ressuscitado com solução salina e antibioticoterapia (solução salina 50 ml/ Kg imediatamente e 12h após CLP + ceftriaxone 30 mg/Kg e clindamicina 25 mg/Kg de 6/6h), e CLP com N-acetilcisteína (20 mg/kg subcutâneo, 3h, 6h, 12h, 18h e 24h após CLP) + deferoxamina (20mg/kg, subcutâneo, 3h e 24h após CLP). A peroxidação lipídica, a carbonilação protéica e a atividade da superóxido dismutase se apresentaram significativamente aumentadas nos animais não-sobreviventes podendo predizer a mortalidade. Nós demonstramos que existe uma modulação diferente da superóxido dismutase e da catalase nos animais não-sobreviventes. Existe um grande aumento da atividade da superóxido dismutase sem um aumento proporcional da atividade da catalase nos não-sobreviventes. Os ratos tratados com antioxidantes apresentaram uma redução significante da atividade da mieloperoxidase e da peroxidação lipídica em todos os órgãos estudados. A produção de superóxido mitocondrial apresentou uma redução significante nos animais tratados com antioxidantes. Além disto, o tratamento com antioxidantes melhorou o equilíbrio entre a atividade da catalase e superóxido dismutase. A sobrevivências nos ratos sépticos não tratados foi de 10%. A sobrevivência aumentou para 40% com a ressuscitação volêmica e antibióticos. Ratos

tratados apenas con N-acetilcisteína e desferoxamina apresentaram uma sobrevivência de 47%, similar aos ratos que receberam suporte básico. Este estudo demonstrou pela primeira vez que a atividade da superóxido dismutase deve ser um marcador precoce de mortalidade em sepse. Nossos resultados auxiliam o entendimento de aspectos importantes da resposta oxidativa na sepse; um aumento na atividade da superóxido dismutase sem um aumento proporcional na atividade da catalase. Além disto, nossos resultados demonstraram que o tratamento combinado com N-acetilcisteína e desferoxamina reduzem as conseqüências da sepse, reduzindo o estresse oxidativo, limitando a ativação de neutrófilos e a disfunçào mitocondrial, levando a uma melhor sobrevida.

Abstract

Oxidative stress plays an important role in the development of multiple organ failure and septic shock. The present study was undertaken to assess different parameters of free radical damage to biomolecules, mitochondrial superoxide production, superoxide dismutase and catalase activities and its relation with sepsis mortality in an animal model. Besides this, 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 perforation (CLP). Male Wistar rats (250-300g) were randomly divided into four groups: sham-operated, CLP resuscitated with normal saline (saline at 50ml/kg immediately and 12 hours after CLP), CLP with normal saline plus antibiotics (saline at 50ml/kg immediately and 12 hours after CLP plus ceftriaxone at 30mg/kg and clindamycin 25mg/kg every 6h), and CLP with N-acetylcysteine (20 mg/kg, 3h, 6h, 12h, 18h and 24h after CLP, subcutaneous) plus deferoxamine (20mg/kg, 3h and 24h after CLP, subcutaneous). Lipid peroxidation, protein carbonyls and superoxide dismutase were significantly increased in non-survivor septic rats and could predict mortality. We demonstrated that there is a different modulation of superoxide dismutase and catalase in non-survivors during the course of septic response. There was a marked increase in superoxide dismutase activity without a proportional increase in catalase activity in non-survivors. Rats treated with antioxidants had significantly lower myeloperoxidase activity and lipid peroxidation 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%) that was similar to basic support. The present study is the first report showing that plasma superoxide dismutase might be an earlier marker of mortality.

Ours results might help to clarify an important aspect of oxidative response to sepsis, i.e., an increase in superoxide dismutase activity without a proportional increase in catalase activity. In addition, our data provide the first experimental demonstration that N-acetylcysteine plus deferoxamine reduces the consequences of septic shock induced by cecal ligation and perforation in the rat, by decreasing oxidative stress, limiting neutrophil activation and mitochondrial dysfunction, thereby leading to an improve in survival.

CAPÍTULO I

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 (1).

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) (1).

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 (1).

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 (2).

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 (1).

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 (2).

As EAO também constituem uma grande fonte de dano ao DNA (3). Aproximadamente 20 diferentes tipos de moléculas de DNA modificadas foram até hoje identificadas, muitas delas reconhecidamente indicativos de mutações (4tese).

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).

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 - conforme representado na figura 1.

Figura 1 - Formação de radical hidroxil via 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) (4).

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 (5).

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, conforme a figura 2 (6).

Figura 2 - Principais enzimas antioxidantes em eucariontes superiores:

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

$$2H_2O_2 \rightarrow O_2 + 2H_2O \text{ (catalase)}$$

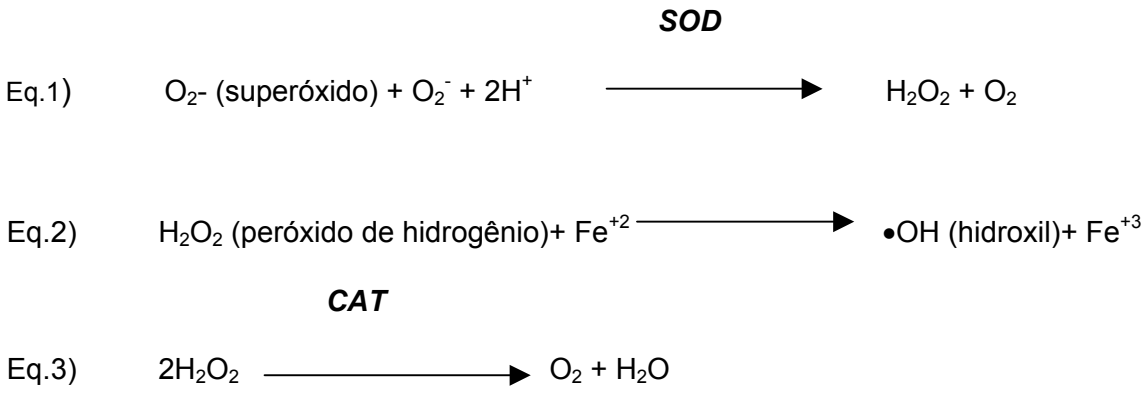
$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG \text{ (glutationa peroxidase)}$$

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 (7e), 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 aumenta o dano celular desencadeado pelas EAO e participa da gênese de várias doenças (8-10). 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”) (8). 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. 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 (11). 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 e mesmo nos pacientes em cujo sítio é altamente suspeito, uma proporção similar tem culturas estéreis ou de significado questionável. Pacientes com infecções presumidas ou condições inflamatórias graves não causada 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 precoce, 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 (12).

1.5 - 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 (13). 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 (13). Entretanto o modelo que parece simular mais adequadamente o quadro clínico de sepse, é o chamado ligação cecal e perfuração (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) (13). 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 (14).

1.6 - O papel das espécies ativas de oxigênio na fisiopatologia da 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 EAO (15,16).

As propriedades pró-inflamatórias dos EAO incluem dano às células endoteliais, formação de fatores quimiotáticos, recrutamento de neutrófilos, oxidação e peroxidação de lipídeos, dano ao DNA, liberação de TNF-α e IL-1β e formação de peroxinitrito (15,16). 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 O_2^- por estas células. Primariamente o 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 celeular irreverssível, como evidenciado no choque séptico.

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.

O 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 GPx e a CAT, mas em presença de metais de transição, ele é decomposto em radical hidroxil via reação de Fenton, um radical altamente tóxico e reativo. O dano 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 relacionado com mortalidade em modelos animais de sepse (SHOCK 2003).

1.7 - Antioxidantes e tratamento de sepse:

Intervenções que reduzem a produção das EAO exercem efeitos benéficos em diversos modelos de endotoxemia e choque séptico (18). Estas intervenções incluem a N-acetilcisteina (NAC) (19-21), α-tocoferol (22), alopurinol (21), deferoxamina (DFX) (23), catalase (24), superoxide dismutase (24), miméticos de superoxide dismutase (25), magnolol (26) e tempol (27). 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 fator nuclear kB (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. 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 (28-29). 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 (30), 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 (23).

1.8 - Objetivos:

1.8.1- Objetivo geral:

Avaliar o papel do estresse oxidativo na fisiopatologia da sepse, bem como o impacto terapêutico dos antioxidantes na mortalidade por sepse.

1.8.2- Objetivos específicos:

1. Avaliar medidas de dano oxidativo em modelo animal de sepse, bem como sua relação com a mortalidade.

2. Avaliar a atividade das enzimas antioxidantes (CAT e SOD) em modelo animal de sepse, bem como sua relação com a mortalidade.

3. Avaliar a produção mitocondrial de superóxido (como marcador de desacoplamento da cadeia de transporte de elétrons) em modelo animal de sepse, bom como sua relação com a mortalidade.

4. Definir marcadores plasmáticos de mortalidade em modelo animal de sepse.

4. Avaliar o efeito do tratamento com n-acetilcisteína (NAC) + deferoxamina (DFX) sobre as medidas de dano oxidativo em modelo animal de sepse.

5. Avaliar o efeito do tratamento com NAC + DFX sobre a atividade das enzimas SOD e CAT em modelo animal de sepse.

6. Avaliar o efeito do tratamento com NAC + DFX sobre a produção mitocondrial de superóxido e peroxidação lipídica mitocondrial em um modelo animal de sepse.

7. Avaliar o efeito do tratamento com NAC + DFX sobre a atividade da mieloperoxidase (enzima marcadora de ativação de neutrófilos) em modelo animal de sepse.

8. Definir o impacto do tratamento com NAC + DFX na mortalidade do modelo animal de sepse.

1.9- Organização dos trabalhos que compõe esta dissertação:

No capítulo II estão descritos os experimentos cujos resultados estão aceitos para publicação no periódico *Intensive Care Medicine*. Neste demonstramos que a peroxidação lipídica, dano a proteínas e a atividade da SOD estão significativamente aumentados nos ratos sépticos não sobreviventes e podem predizer mortalidade. Também demonstramos um desequilíbrio na atividade da SOD e CAT em não sobreviventes durante o curso da resposta séptica, sugerindo que a "toxicidade da SOD" pode ter um papel na gênese da sepse e choque séptico.

No capítulo III encontram-se os experimentos cujos resultados estão submetidos ao periódico *Critical Care Medicine.* Neste demonstramos que o tratamento com NAC + DFX atenuou as medidas de dano oxidativo tanto como o desequilíbrio entre SOD e CAT nos ratos sépticos. Também demonstramos que a associação terapêutica teve significativo impacto na mortalidade quando comparado com tratamento de suporte (reposição volêmica + antibiótico de amplo amplo espectro) ou cada droga isoladamente (NAC ou DFX).

CAPÍTULO II

Oxidative parameters and mortality in sepsis induced by cecal ligation and perforation

Intensive Care Medicine (2003) in press (publicado online em 29 maio de 2003 sob identificação DOI 10.1007/s00134-003-1789-9)

OXIDATIVE PARAMETERS AND MORTALITY IN SEPSIS INDUCED BY CECAL LIGATION AND PERFORATION

Cristiane Ritter.[1,2,3]; Michael Andrades [1,3];

Mário Luis C. Frota Jr.[1,3]; Fernanda Bonatto[1,3];

Ricardo A. Pinho [1,3]; Manuela Polydoro[1,3]; Fábio Klamt [1,3],

Cleovaldo T. S. Pinheiro [2]; Sérgio Menna-Barreto [1,4];

*José Cláudio F. Moreira [1,3]; Felipe Dal-Pizzol [1,3,5 *]*

[1]Laboratório de Estresse Oxidativo e Doenças, Centro de Pesquisa, HCPA;

[2]Serviço de Medicina Intensiva, HCPA;

[3]Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, UFRGS;

[4]Serviço de Pneumologia, HCPA;

[5]Departamento de Medicina, Universidade do Extremo Sul Catarinense

*To whom correspondence should be addressed:

Departamento de Bioquímica, UFRGS. Ramiro Barcelos, 2600 [anexo], Porto Alegre, Brazil. 90035-003. Fax: 55 51 33165535. e-mail: pizzol@ez-poa.com.br

Key Words: free radicals, oxidative stress, superoxide, antioxidants, mortality, sepsis

Word count: 2651

Word count (abstract): 242

Abstract

Objective: The present study was undertaken to assess different parameters of free radical damage to biomolecules, mitochondrial superoxide production, superoxide dismutase and catalase activities and its relation with sepsis mortality in an animal model. *Design*: prospective animal study. *Setting*: Laboratory for experimental studies at the University. *Subjects*: 140 male Wistar rats. *Interventions*: The animals were randomly divided into three groups: sham-operated (n=20), cecal ligation and perforation resuscitated with normal saline (n=40) and cecal ligation and perforation with normal saline plus antibiotics (n=40). *Measurements and results*: Blood samples were collected from all animals 3, 12 and 24 hours after CLP through a jugular catheter inserted before CLP. Rats were evaluated during 5 days after the intervention. Non-survivor animals were grouped according to time interval between sepsis induction and death and oxidative parameters were compared to survivors and sham-operated. Lipid peroxidation, protein carbonyls and superoxide dismutase were significantly increased in non-survivor septic rats and could predict mortality. We demonstrated that there is a different modulation of superoxide dismutase and catalase in non-survivors during the course of septic response. There was a marked increase in superoxide dismutase activity without a proportional increase in catalase activity in non-survivors. *Conclusions*: The present study is the first report showing that plasma superoxide dismutase might be an earlier marker of mortality. Ours results might help to clarify an important aspect of oxidative response to sepsis, i.e., an increase in superoxide dismutase activity without a proportional increase in catalase activity.

Introduction

Sepsis and the consequences of the response to sepsis are the most common causes of mortality in intensive care units [1]. Several molecular mechanisms of inflammation and cellular damage have been implicated in the pathogenesis of sepsis, septic shock, and multiple organ failure, including those related to overt generation of cytokines, eicosanoids and reactive oxygen species (ROS) [2,3]. ROS are believed to be important mediators of cellular injury contributing to the development of sepsis. The proinflammatory properties of ROS include endothelial cell damage, formation of chemotatic factors, recruitment of neutrophils, lipid peroxidation and oxidation, DNA damage, release of TNF-α, and IL-1β, and formation of peroxynitrite [4]. It is possible that antioxidants attenuates TNF-α secretion, oxidant mediated apoptosis, and cellular oxidative damage, thus reducing the mortality associated with sepsis mortality. The use of antioxidants in sepsis treatment is effective in reducing lipoperoxidation, diaphragmatic contractile dysfunction, NF-κB activation, and respiratory burst. Antioxidants also improve liver function [5-9], and increase neutrophil phagocytosis [8]. Regarding mortality the results are controversial. Some studies demonstrated an improved survival with antioxidant administration before [10-13] sepsis induction or in moderate sepsis [14] whereas other studies presented negative results [15-17]. Thus, the elucidation of the kinetics of sepsis-induced ROS production, its relation with antioxidants systems, and the identification of earlier markerers of sepsis mortality might be important to improve the use of antioxidants in sepsis treatment.

In the present study we aimed to assess different parameters of free radical damage to biomolecules, mitochondrial superoxide production, superoxide dismutase (SOD) and catalase (CAT) activities and their relation with sepsis mortality.

Material 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 month old, subjected to CLP as previously described [18,19]**,** 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 with a 14-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site. The cecum was then returned to the peritoneal cavity and the laparotomy was closed with 4.0 silk sutures. A sham operation (laparotomy and cecal exposure without any more manipulation) was performed as control. The sham-operated and CLP groups were allocated randomly during procedure. Animals were resuscitated with normal saline (50ml/kg b.w. subcutaneous, bolus injection) immediately after and every 6 hours after CLP with or without ceftriaxone at 30mg/kg b.w. and clindamycin 25mg/kg b.w. every 6h over a total of 3 days. All animals were then returned to their cages with free access to food and water. Rats were continuously monitored and followed up for 5 days after CLP. Non-survivor animals were grouped according to time interval between sepsis induction and death (12h-24h, 25h-36h, 37h-48h, 49h-60h after CLP) and organ systems (heart, lung, diaphragm, liver and kidney) were immediately isolated. After 5 days survival and sham-operated animals were sacrificed and organ systems were isolated for posterior analyses. In this cohort of animals were submitted to CLP 140 rats (20 sham-operated, 60 CLP plus fluid resuscitation, 60 CLP plus fluid resuscitation plus antibiotics).

To assess the relation of plasmatic oxidative parameters and mortality prediction animals exposed to CLP received fluid resuscitation with or without antibiotics as stated above. Blood samples were collected from all animals 3, 12 and 24 hours after CLP via a jugular catheter inserted before CLP for the determination of TBARS, protein carbonyls, superoxide dismutase and catalase activities. Each time we harvested approximately 150 μL of blood (total of 450μL per animal). This volume was replaced with normal saline to avoid hypovolemia. The mortality of the animals was recorded over a 5 day period.

Measurement of thiobarbituric acid reactive species (TBARS)

As an index of lipid peroxidation we used the formation of TBARS during an acid-heating reaction as previously described [20]. Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67%, then heated in a boiling water bath for 15 minutes. TBARS were determined by the absorbance at 535 nm.

Measurement of protein carbonyls

The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH) as previously described [21]. Briefly, proteins were precipitated by the addition of 20% trichloroacetic acid and redissolved in DNPH and the absorbance read at 370nm.

Measurement of catalase (CAT) and superoxide dismutase (SOD) activity

To determine CAT activity organ systems were sonicated in 50mM phosphate buffer and the resulting suspension was centrifuged at 3000 g for 10 minutes. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm [22]. SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described [23].

Measurement of mitochondrial superoxide generation

Submitochondrial particles (SMP) were isolated by differential centrifugation as previously described [24]. Superoxide was estimated by measuring adrenaline oxidation in a buffer containing SMP, succinate (as electron transfer chain initiator), and catalase [24]. To assure assay specificity a negative control was made in the presence of SOD.

Reagents

Thiobarbituric acid, catalase, superoxide dismutase, dinitrophenylhidrazine, adrenaline, hydrogen peroxide, luminol and succinate were purchased from Sigma, St. Louis, MO. 2,2'-azobis (2-

methylpropionamidine) dihydrochloride (AAPH) was purchased from Aldrich Chemical Co., Milwaukee, WI.

Statistical Analysis

All data are presented as means ± S.D. For repeated measurements (plasmatic oxidative parameters) a 2-factorial analysis of variance (ANOVA) was performed. Data without repeated measures was analyzed by 1-factorial ANOVA. Multiple comparisons were performed by a Newman-Keuls test. To determine if data was normally distributed we performed Bartlett's test for homogeneity of variance. p values below 0.05 were considered significant.

Results

Mortality description

Quantitative bacterial cultures demonstrated that all the animals subjected to CLP had positive cultures in blood (data not shown). Animals died between 18 and 60 hours after CLP. The overall mortality at the end of the four days was 90% with fluid resuscitation and 60% with fluid administration plus antibiotics (Figure 1).

Relation of plasmatic oxidative parameters and sepsis mortality

An increase in TBARS and protein carbonyls in plasma, as an index of oxidative injury, was observed in all groups after CLP plus fluid resuscitation in relation to sham-operated animals, but with a different magnitude in the survivors compared with the non-survivors (Table 1). The levels of TBARS and protein carbonyls in plasma increased up to 12h and remained at this level until 24 h. Plasmatic TBARS values higher than 2 nm/mg protein and 6 nm/mg protein in the first 12 and 24 hours respectively could predict mortality with 100% sensitivity and specificity in our model. This is also true to plasmatic carbonyl values higher that 0,3 nm/mg protein and 0,4 nm/mg protein in the first 12 and 24 hours respectively. Plasmatic SOD activity also increases in all groups after CLP in relation to sham-operated animals (Table 1), and seems to be an earlier marker of mortality in this model.

Three hours after sepsis induction SOD activity was higher in non-survivors when comparing to survivors (Table 1). After 3 hours of sepsis induction SOD activity can predict sepsis mortality in this model, with 100% of sensitivity and specificity to values higher than 5 U/mg protein. In contrast, CAT activity is similar between survivors and non-survivors, although we observed a trend toward higher CAT activity in survivors (Table 1). Plasmatic SOD/CAT relation illustrated the imbalance between these two antioxidant enzymes in non-survivors from CLP. SOD/CAT relation in sham-operated is 16.6, 16.1 and 21.9 respectively 3h, 12h and 24h after sham-operation. In survivors from CLP this relation is 5.3. 7.1 and 10.3 respectively 3h, 12h and 24h after CLP. This indicates that in survivors the increase in SOD activity is accompanied by an increase in CAT activity. In non-survivors, SOD/CAT relation is 27.1, 27 and 44.8 respectively 3h, 12h and 24h after CLP, demonstrating an imbalance between SOD and CAT activities. These results are similar in animals that receive fluid resuscitation plus antibiotics (data not shown).

Oxidative parameters in organ systems during sepsis development

An increase in TBARS and protein carbonyls was seen in all organs after CLP induction in the non-survivors group (Figure 2A and 2B). This is in accordance with reports that indicate an involvement of oxidative injury during the development of sepsis. There is a trend toward a higher organic oxidative injury 37-48h after CLP and tends to diminish at 49-60h, depending on the organ analyzed. The recovery phase is associated with cessation of oxidative injury, since TBARS and protein carbonyls returns to basal levels after 5 days of CLP (Figure 2A and 2B). These results are similar when analyzed animals that receive fluid resuscitation plus antibiotics (data not shown).

An increase in SOD activity was seen in all organ systems after CLP in non-survivors, and, as demonstrated for TBARS and protein carbonyls, tends to peak 37-48h after CLP (Figure 3A). This was not accompanied by an increase in CAT activity (Figure 3B). In contrast, CAT activity diminished in lung, liver and heart (Figure 3B). These are in accordance with plasmatic SOD and CAT activities demonstrated above. It seems that there is an imbalance between SOD and CAT activity during the course of septic response. As we demonstrated for TBARS and protein carbonyls the recovery phase from septic injury is associated with a return of SOD and CAT activities to basal levels (Figure 2B).

Since there was an increase in SOD activity we analyzed mitochondrial superoxide production after CLP. We demonstrated that in all organ systems there was an increase in mitochondrial superoxide production in the non-survivor group (Figure 4). The recovery phase is associated with a return of superoxide production to basal levels (Figure 4). These results are in animals that receive fluid resuscitation plus antibiotics (data not shown).

Discussion

The occurrence of lipid and protein oxidative damage during sepsis is well established. Free radicals have been detected by spin trapping techniques [25], and elevations in oxidative parameters have been measured in animal models as well as in humans [26-29]. Our results are in accordance with these reports. During septic response we demonstrated an increase in TBARS and protein carbonyls in all organs systems, and in the recovery phase (survivor animals) oxidative damage parameters returns to basal levels. Here we provide the first evidence that TBARS and protein carbonyl levels could predict sepsis mortality in CLP model. Probably, oxidative damage is one of several factors that lead to cell damage, organ dysfunction and death.

We demonstrated that there is a different modulation of SOD and CAT in non-survivors during the course of septic response. To the best of our knowledge there are no published data on the modulation of antioxidant enzyme activities during sepsis development. During septic response SOD activity seems to increase in both survivors and non-survivors (Table 1), probably as a response to oxidative stress induced by sepsis. The most striking finding was the marked increase in SOD activity observed in non-survivors without a proportional increase in CAT activity (Table 1 and Figure 3A and 3B). Cells that overexpress SOD [30] or mice transgenic for human SOD [30] showed some abnormalities related to oxidative stress. It seems that any concentrations of SOD other than the optimal leads to increased lipid peroxidation and therefore to decrease cell viability [30]. SOD activity results in the production of hydrogen peroxide (eq.1), which can mediate membrane damage by lipid peroxidation or react with iron to generate hydroxil radicals via Fenton chemistry, which is thought to

be the most toxic oxygen molecule *in vivo* (eq.2). CAT could clean an excess of peroxide, diminishing the oxidative effects of hydrogen peroxide (eq.3).

Eq. 1 O_2- (superoxide) + O_2^- + $2H^+$ $\xrightarrow{SOD}$ H_2O_2 + O_2

Eq.2 H_2O_2 (hydrogen peroxide)+ Fe^{+2} $\longrightarrow$ •OH (hydroxil)+ Fe^{+3}

Eq. 3 $2H_2O_2$ $\xrightarrow{CAT}$ O_2 + H_2O

Thus, an imbalance between SOD and CAT activity could lead to oxidative stress. We have previously demonstrated that an imbalance between SOD and CAT could participate in the genesis of several diseases [22,31-33]. This is in agreement with previous results that demonstrated higher plasma SOD levels in non-survival septic patients [33]. The therapeutic efficacy of SOD or its mimetic is controversial. Several references demonstrated an improvement in sepsis mortality with SOD or SOD mimetic administration [2, 6, 35,36]. On the other hand, several reports demonstrated an enhanced dysfunction cause by SOD mimetic in sepsis [37-40]. The best results are obtained when SOD mimetic presented catalase activity, but this is still an open field to research. In this way, we believe that our results presented new, and interesting data to this discussion. SOD activation could be a cellular response to the overproduction of mitochondrial superoxide demonstrated in Figure 4. Besides this, SOD activation, mainly manganese-SOD, could be a response to the increase in IL-1, TNF and lipopolysaccharide during sepsis development [41,42]. On the other hand, an excess of superoxide or hydrogen peroxide could oxidize CAT active site leading to enzymatic inactivation [43] and this could explain our results on CAT activity. To ascertain the exact role of this imbalance in sepsis development studies should be performed in transgenic animals lacking or overexpressing SOD and CAT.

To the best of our knowledge this is the first direct evidence of mitchondrial superoxide formation in CLP model. Boczkowski et al (1999) [44] demonstrated that diaphragm mitochondria from endotoxemic rats show a progressive increase in hydrogen peroxide production associated with uncoupling and decrease phosphorylating capacity. Probably, during sepsis development, the first step of mitochondrial impairment is an increase in nitric oxide that lead to a consecutive formation of

superoxide that reacts with nitric oxide to form peroxynitrite, which in turn impairs mitochondrial function [45-47]. The recovery from sepsis seems to be related to the attenuation of these effects since mitochondrial superoxide formation returns to basal levels 4 days after CLP. We believe that superoxide production could participate in several pathways of sepsis development [38]. Generally, superoxide is seen as the initiator of ROS-induced cell damage, and this could be associated with septic shock and failure of multiple organ systems [2]. Nowadays superoxide, and probably hydrogen peroxide, is though to participate in earlier steps of sepsis development. It seems that TNF-α secretion following lipopolysaccharide challenge in macrophages is dependent of Rac-1-induced ROS production [47]. Thus, their production seems to be involved in earlier [47] and in later steps of sepsis development [2]. and ROS generation may initiate a cascade of events that culminate with TNF-α secretion, contributing to further cellular ROS production that, as a final event, could lead to cell damage and death.

We demonstrated for the first time the importance of plasmatic SOD as an earlier marker of mortality. To date, antioxidants have not been shown to have beneficial effects on mortality in clinical trials [8,9]. In animal models, some studies demonstrated an improved survival with antioxidant administration before, but not after, sepsis induction [10-13]. The lack of an effective antioxidant treatment for sepsis could results from our incomplete knowledge about oxidative response during sepsis. Ours results could help to clarify an important aspect of oxidative response to sepsis, an increase in SOD activity without a proportional increase in CAT activity. This could lead to the production of hydrogen peroxide or hydroxil radicals that ultimately could induce cell damage. These findings suggest that a blockade in several steps of free radical formation is important to diminish the formation of the most reactive free radical, hydroxil (i.e. the association of a scavenger of superoxide and hydrogen peroxide together with iron chelator).

Acknowledgement: This work was supported by grants from FAPERGS, FIPE-HCPA and CNPq. The authors wish to express their thanks to Dr. R. Roesler for his critical revision of the manuscript.

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Figure legends

Figure 1 - ***Mortality description after cecal ligature and perforation with fluid resuscitation and antibiotics administration.*** Rats were submitted to cecal ligature and perforation (14-gauge puncture) or a sham operation (laparotomy and cecal exposure without any more manipulation). The sham-operated and CLP groups were allocated randomly during procedure. Animals were resuscitated with normal saline (50ml/kg b.w. subcutaneous, bolus injection) immediately after and every 6 hours after CLP with or without ceftriaxone at 30mg/kg b.w. and clindamycin 25mg/kg b.w. every 6h over a total of 3 days and followed up daily for 5 days after CLP.
(n=20 for sham-operated, n=40 fluid resuscitation, and n=40 for fluid resuscitation plus antibiotics).

Figure 2 - ***Thiobarbituric acid reactive species and protein carbonyls in organ systems during sepsis development***. Rats were submitted to cecal ligature and perforation (14-gauge puncture) or sham-operated and followed up daily for 5 days after CLP. Non-survivors animals were grouped by death time (12h-24h, 25h-36h, 37h-48h, 49h-60h after CLP), and the organ systems of non-survivors were immediately removed. After 5 days survival and sham-operated animals were sacrificed and organ systems were removed to the determination of *A,* thiobarbituric acid reactive species; *B,* protein carbonyls as described under “Material and Methods”. Values are expressed as means $\pm$ S.D. (n=20 for sham-operated, n=18 for survivor, n=7 for non-survivors 12h-24h, n=17 for non-survivors 25h-36h, n=8 for non-survivors 37h-48h, and n=10 for non-survivors 49h-60h).

* different from sham-operated and survivor ($p<0.05$).

** different from non-survivor ($p<0.05$)

Figure 3 - ***Superoxide dismutase and catalase activities in organ systems during sepsis development***. Rats were submitted to cecal ligature and perforation (14-gauge puncture) or sham-operated and followed up daily for 5 days after CLP. Non-survivors animals were grouped by death time (12h-24h, 25h-36h, 37h-48h, 49h-60h after CLP), and the organ systems of non-survivors were immediately removed. After 5 days survival and sham-operated animals were sacrificed and organ systems were removed to the determination of *A,* superoxide dismutase activity; *B,* catalase activity as described under “Material and Methods”. Values are expressed as means $\pm$ S.D. (n=20 for sham-

operated, n=18 for survivor, n=7 for non-survivors 12h-24h, n=17 for non-survivors 25h-36h, n=8 for non-survivors 37h-48h, and n=10 for non-survivors 49h-60h).

* different from sham-operated ($p<0.05$).

** different from survivor ($p<0.05$)

*** different from non-survivor 12-24h ($p<0.05$)

Figure 4 - ***Mitochondrial superoxide generation in organ systems during sepsis development***. Rats were submitted to cecal ligature and perforation (14-gauge puncture) or sham-operated and followed up daily for 5 days after CLP. Non-survivors animals were grouped by death time (12h-24h, 25h-36h, 37h-48h, 49h-60h after CLP), and the organ systems of non-survivors were immediately removed. After 5 days survival and sham-operated animals were sacrificed and organ systems were removed to the determination of mitochondrial superoxide generation as described under “Material and Methods”. Values are expressed as means $\pm$ S.D. (n=20 for sham-operated, n=18 for survivor, n=7 for non-survivors 12h-24h, n=17 for non-survivors 25h-36h, n=8 for non-survivors 37h-48h, and n=10 for non-survivors 49h-60h).

* different from sham-operated and survivor ($p<0.05$).

** different from non-survivor 12-24h ($p<0.05$)

Table 1 - Relation of plasmatic thiobarbituric acid reactive species, protein carbonyls and superoxide dismutase activity and prediction of sepsis mortality. Rats were submitted to cecal ligature and perforation (14-gauge puncture) or sham-operated and followed up daily for 5 days after CLP. After 3, 12 and 24 hours of sepsis induction blood were collected and thiobarbituric acid reactive species, superoxide dismutase activity, and protein carbonyl were measured as described under “Material and Methods”. Values are expressed as means ± S.D. (n=20 for sham-operated, n=18 for survivor and, n=42 for non-survivor).

Plasmatic oxidative markers	Sham-operated			Survivor			Non-survivor		
	Hours after CLP								
	3h	12h	24h	3h	12h	24h	3h	12h	24h
TBARS	0.9 ± 0.22	0.87 ± 0.34	0.84 ± 0.38	0.99 ± 0.49	1.2 ± 0.39*	3.73 ± 1.1*	1.37 ± 0.29	4.0 ± 0.97**	12.3 ± 2.1**
Protein Carbonyls	0.15 ± 0.03	0.18 ± 0.05	0.14 ± 0.05	0.13 ± 0.06	0.25 ± 0.03*	0.33 ± 0.03*	0.14 ± 0.05	0.41 ± 0.09**	0.56 ± 0.02**
Superoxide Dismutase	2.0 ± 0.9	2.1 ± 0.9	2.4 ± 1.2	2.4 ± 0.6	3.4 ± 0.3*	5.7 ± 0.4*	10 ± 1.0**	10.8 ± 4.0**	19.3 ± 6.5 **
Catalase	0,12 ± 0,03	0,13 ± 0,04	0,11 ± 0,02	0,45 ± 0,05*	0,48 ± 0,06*	0,55 ± 0,05*	0,37 ± 0,04*	0,40 ± 0,05*	0,43 ± 0,05*

* different from sham-operated, $p<0.05$

** different from survivor, $p<0.5$

Figure 1

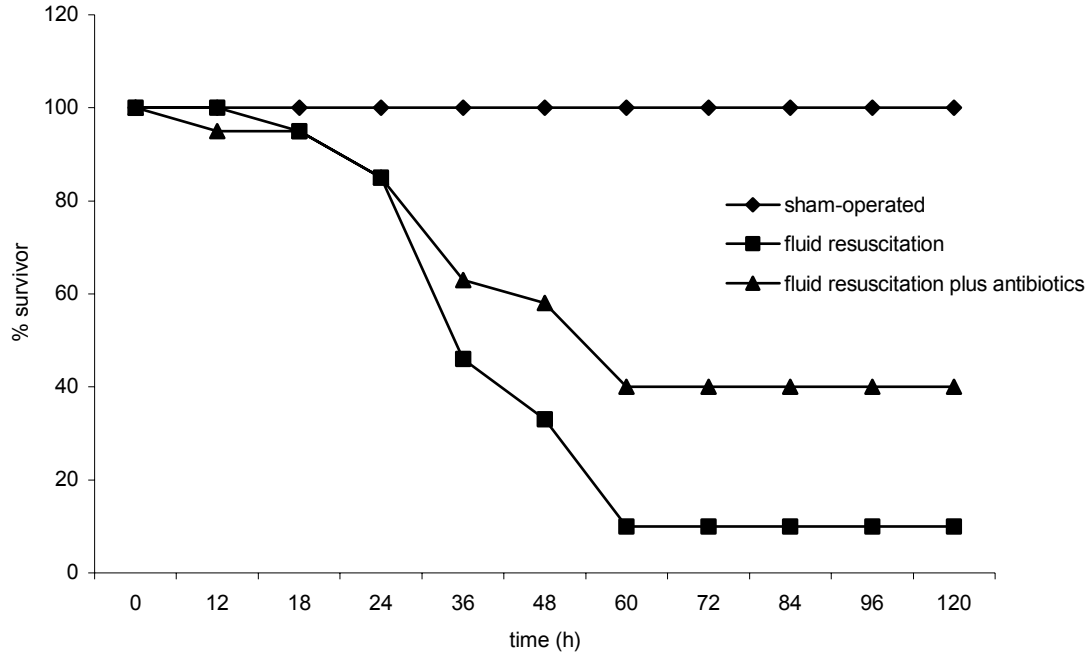
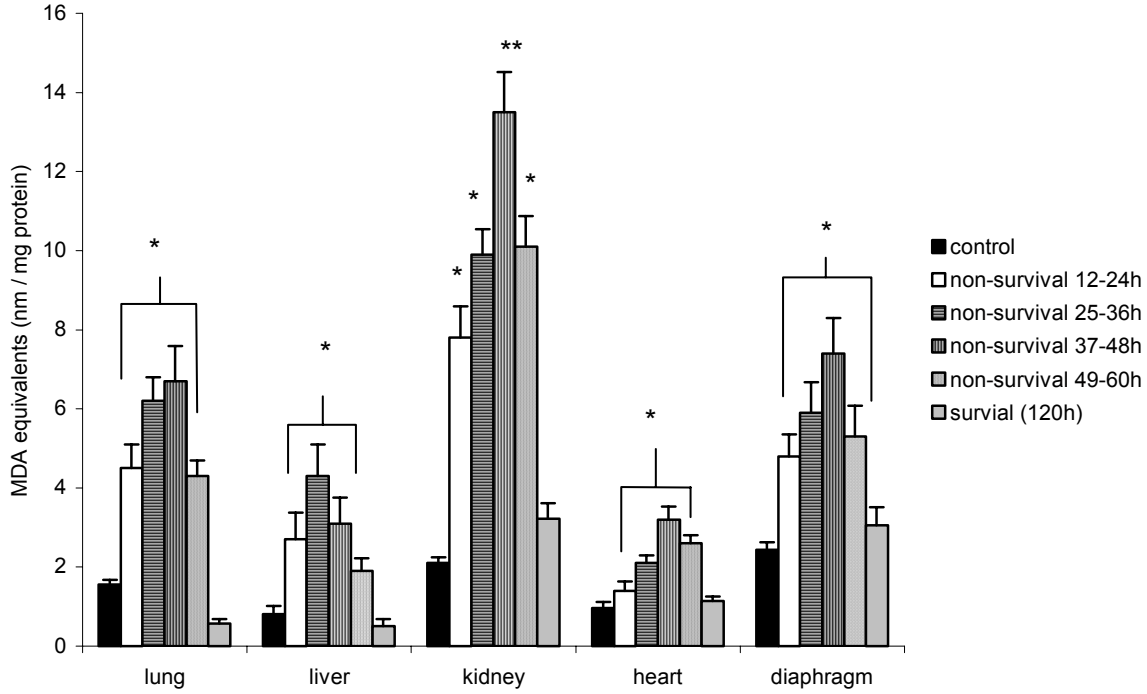

120
100
80
60
40
20
0
% survivor
0
12
18
24
36
48
60
72
84
96
120
time (h)
sham-operated
fluid resuscitation
fluid resuscitation plus antibiotics

Figure 2

A


MDA equivalents (nm / mg protein)
16
14
12
10
8
6
4
2
0
lung
liver
kidney
heart
diaphragm
control
non-survival 12-24h
non-survival 25-36h
non-survival 37-48h
non-survival 49-60h
survial (120h)

B

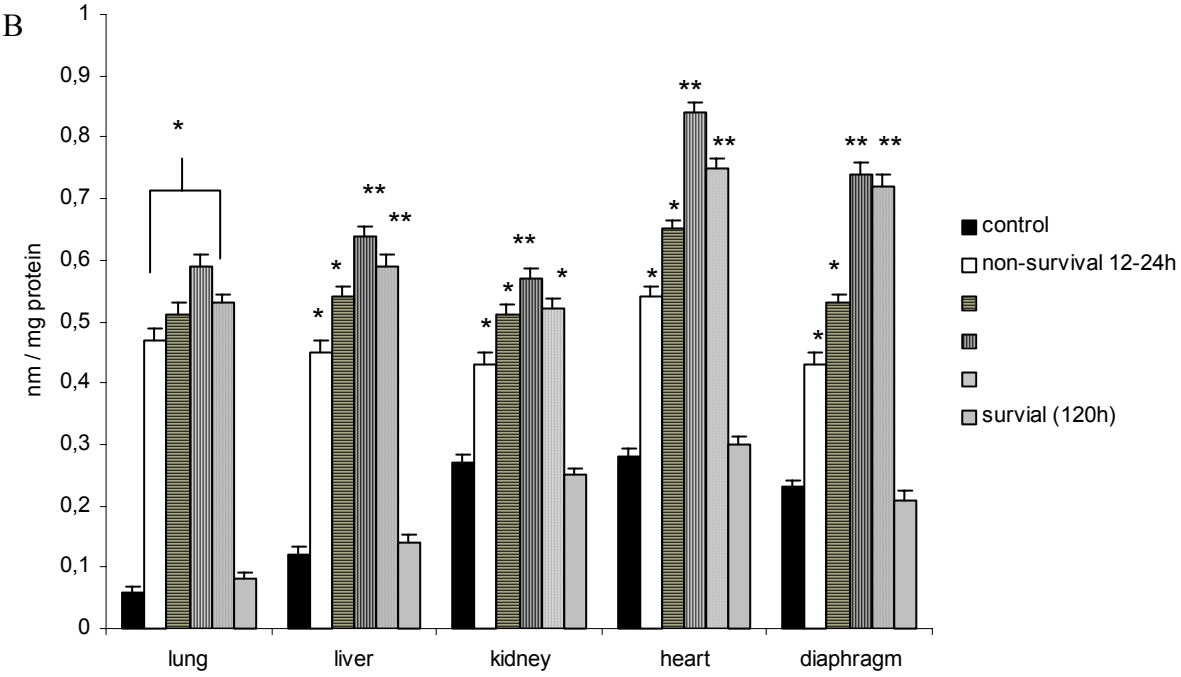

nm / mg protein
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
lung
liver
kidney
heart
diaphragm
control
non-survival 12-24h
survial (120h)

Figure 3

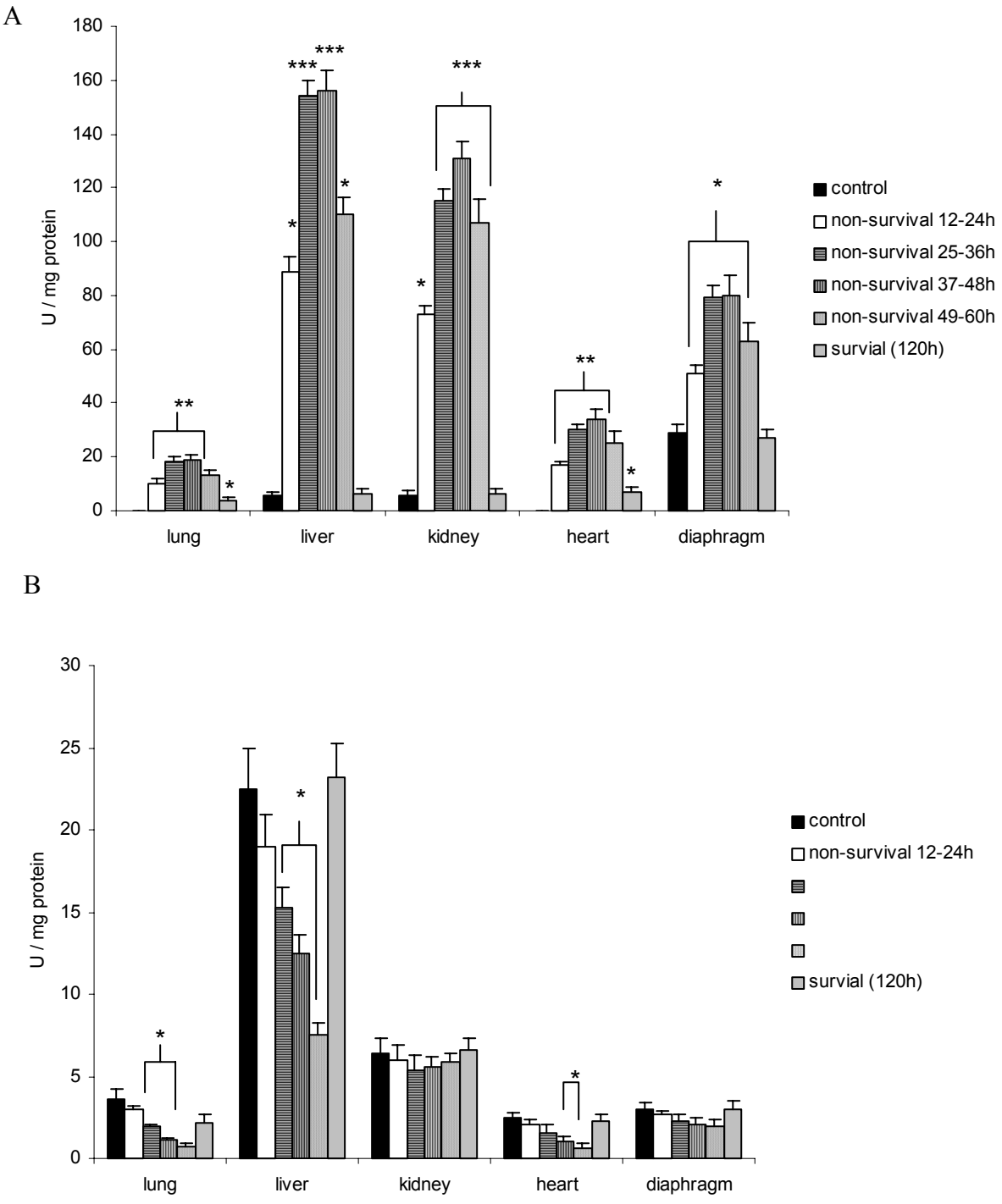
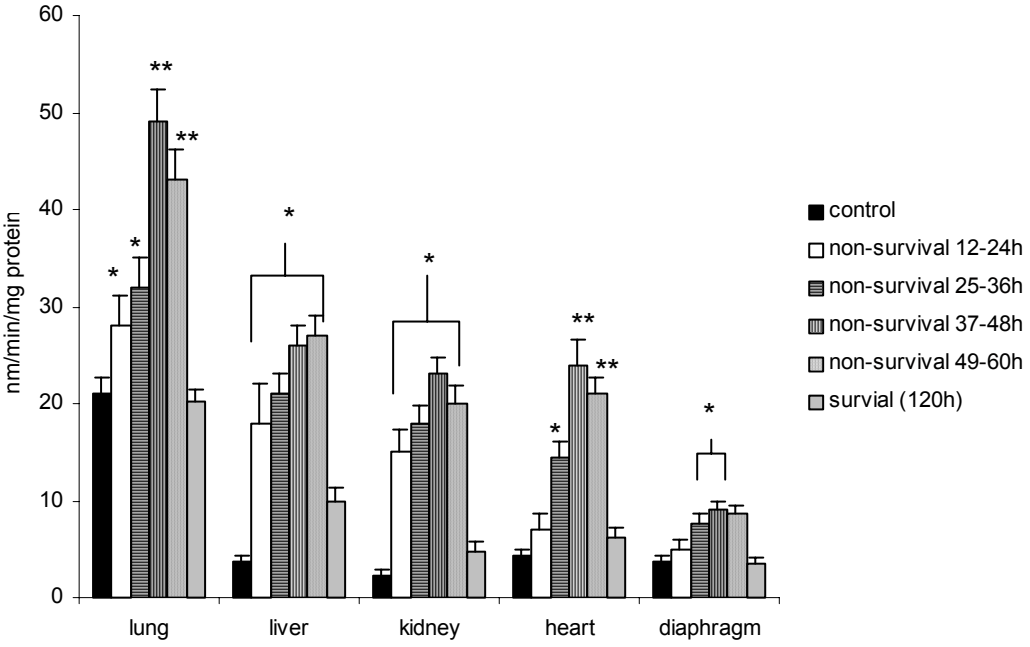

A
U / mg protein
180
160
140
120
100
80
60
40
20
0
lung
liver
kidney
heart
diaphragm
control
non-survival 12-24h
non-survival 25-36h
non-survival 37-48h
non-survival 49-60h
survial (120h)
B
U / mg protein
30
25
20
15
10
5
0
lung
liver
kidney
heart
diaphragm
control
non-survival 12-24h
survial (120h)

Figure 4


60
50
40
30
20
10
0
nm/min/mg protein
lung
liver
kidney
heart
diaphragm
control
non-survival 12-24h
non-survival 25-36h
non-survival 37-48h
non-survival 49-60h
survial (120h)

CAPÍTULO III

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

Submetido ao periódico Critical Care Medicine

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

Cristiane Ritter [1,2] MD, Michael E. Andrades [2,3] BSc,

Adalisa Reinke [1] BSc, Sérgio Menna-Barreto [3,4] MD, PhD,

José Cláudio F. Moreira [2,3] PhD, Felipe Dal-Pizzol [1,2,3] MD, PhD

[1] Laboratório de Fisiopatologia Experimental, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil. Avenida Universitária, 1105, 88006-000.

[2] Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[3] Centro de Pesquisa, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil.

[4] Serviço de Pneumologia, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil.

Address for reprints: Felipe Dal Pizzol. Laboratório de Fisiopatologia Experimental, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil. Avenida Universitária, 1105, 88006-000. phone: 55 48 4312641, fax: 55 48 431 2750, e-mail:piz@unesc.rct-sc.br

Acknowledgement: This work was supported by grants from FAPERGS, FIPE-HCPA and CNPq.

Key Words: sepsis, antioxidants, oxidative stress, N-acetylcysteine, deferoxamine, free radicals

Abstract

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 to 350 g.

Interventions: Rats subjected to CLP were treated with either N-acetylcysteine (20 mg/kg, 3h, 6h, 12h, 18h and 24h after CLP, subcutaneous) plus deferoxamine (20mg/kg, 3h and 24h after CLP, subcutaneous), vehicle or “basic support” (saline at 50ml/kg immediately and 12 hours after CLP plus ceftriaxone at 30mg/kg and clindamycin 25mg/kg every 6h).

Measurements and Main Results: After 12 h tissue myeloperoxidase (MPO, indicator of neutrophil activation), thiobarbituric acid reactive species (TBARS, indicator of lipid peroxidation), 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 MPO activity and TBARS 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 NAC plus DFX survival was also significantly improved (47%) that was similar to basic support.

Conclusions: Our data provide the first experimental demonstration that NAC plus DFX reduces the consequences of septic shock induced by cecal ligation and perforation in the rat, by decreasing oxidative stress, limiting neutrophil activation and mitochondrial dysfunction, thereby leading to an improve in survival.

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 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 from septic shock has improved little over the last several decades (2).

Some investigators have demonstrated that oxygen-derived free radicals (ROS) play an important role in the development of multiple organ failure and septic shock (3,4). ROS injure tissues through peroxidation of membrane lipids, breakage DNA strands, alteration of amino acids and disruption of cellular metabolism. Besides direct cell damage, there is increasing evidence that hydrogen peroxide, directly or indirectly via its reduction product hydroxyl, acts as a messenger molecule in the synthesis and activation of inflammatory cells and mediators (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). The monocyte and polymorphonuclear neutrophil pool undergoes alterations that are suggestive of leukocyte activation as a response to stimulation by tumor necrosis factor (TNF) and interleukins (IL) resulting in superoxide generation (7). In addition,

elevated circulating nitric oxide and nitric oxide synthase activation have been reported in septic patients (8,9). We had previously report that an imbalance between superoxide dismutase and catalase activities during sepsis response could predispose the accumulation of hydrogen peroxide (10). This environment predisposes the formation of peroxynitrite and hydroxyl radicals that are thought to be the most dangerous free radicals in biological systems.

Oxidant scavengers have been reported to inhibit lipopolysaccharide (LPS) stimulated IL release (11), improves macrophage function (12), diminish the expression of adhesion molecules (13), and decrease TNF levels (5). Interventions, which 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 (5,15-17), α-tocopherol (18), allopurinol (17), deferoxamine (19), catalase (20), superoxide dismutase (20), superoxide dismutase mimetics (21), magnolol (22) and tempol (23). Generally these interventions are administered before, or shortly after sepsis induction, and these could restrict its use in the clinical setting. To date the most widely use antioxidant in the clinical and pre-clinical setting is N-acetylcysteine (NAC). NAC, which is a scavenger of superoxide, hydrogen peroxide and hydroxyl radical *in vitro*, has been successfully used in various models of adult respiratory distress syndrome and sepsis (5,15-17). However, low-dose NAC protects against LPS toxicity while 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 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 predispose the formation of hydroxyl

radicals that plays a major role in sepsis mortality. In addition, the isolate 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 parameters and mortality.

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 month 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 with a 14-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site. The cecum was then returned to the peritoneal cavity and the laparotomy was closed with 4.0 silk sutures. Animals were resuscitated with normal saline (50ml/kg subcutaneous) immediately after and 12 hours after CLP. All animals were then 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 (see below), 50 rats were made septic by CLP. The animals were randomly divided into four groups; *1* - sham operated, *2* - NAC (20mg/kg) 3h, 6h and 12h after CLP plus DFX (20mg/kg) 3h after CLP with a subcutaneous injection, or *3* - vehicle (isotonic saline) at same times after CLP, or *4* - "basic support" (saline at 50ml/kg immediately after and 12 hours after CLP plus ceftriaxone at 30mg/kg and clindamycin 25mg/kg every 6h). Twelve hours later the rats were killed by decapitation followed by the harvesting of samples from the lung, liver, kidney, heart, and diaphragm immediately stored at -70^0 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 below.

Measurements

Thiobarbituric acid reactive species (TBARS). As an index of lipid peroxidation we used the formation of TBARS during an acid-heating reaction as previously described (26). Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67%, then heated in a boiling water bath for 15 minutes. TBARS were 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 activation 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 (MOPS) and centrifuged at 15,000 *g* for 40 min. The suspension was then sonicated three times for 30 s. 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 ^{0}C, using a Spectramax microplate reader (27). Results are expressed as milliunits of myeloperoxidase (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 50mM phosphate buffer and the resulting suspension was centrifuged at 3000 g for 10 minutes. 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 (SMP) were isolated by differential centrifugation. Superoxide was estimated by measuring adrenaline oxidation in a buffer containing SMP, succinate (as electron transfer chain initiator), and catalase. To assure assay specificity a negative control was made in the presence of SOD.

Survival Experiments

Survival was tested in a separated cohort of animals. In a first protocol, animals exposed to CLP were randomly assigned to receive NAC (20mg/kg) 3h, 6h, 12h, 18h and 24h after CLP plus DFX (20mg/kg) 3h and 24h after CLP with subcutaneous injection, vehicle (isotonic saline at same times), or basic support (saline at 50ml/kg

immediately after and 12 hours after CLP plus ceftriaxone at 30mg/kg and clindamycin 25mg/kg every 6h over a total of 3 days). The mortality of the animals was recorded over a 5 day period.

Reagents

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

Statistical analyses

Data are expressed as means $\pm$ S.E.M. in all figures. For the biochemical measures the means for the different treatment groups were compared by ANOVA one-way 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 $p < 0.05$.

RESULTS

The measurement of TBARS and MPO activity on tissues revealed that NAC plus DFX treatment significantly attenuated diaphragm, heart, liver, lung and kidney oxidative stress and inflammation during CLP (Figures 1 and 2).

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 Figure 3 and 4 an imbalance between SOD and CAT activities occurs 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 (Figure 3 and 4).

Mitochondrial dysfunction was assessed by determining superoxide production in submitochondrial particles. CLP animals, but not the animals treated with NAC plus DFX, exhibited increased superoxide production when compared with controls in all organs studied (Figure 5). 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 (Figure 6).

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, $p<0.05$). In rats treated only with NAC plus DFX survival was also significantly improved (n=30, 47%, $p<0.05$) that was similar to basic support. The administration of NAC (20mg/kg 3h, 6h, 12h, 18h and 24h after CLP) or DFX (20mg/kg 3h and 24h after CLP) with basic support did not significantly improve mortality in comparison to basic support group (data not shown).

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 cecal ligation and perforation, leading to a significant improvement in survival.

Background and Previous Work

Previous data indicated that ROS have marked proinflammatory effects. Some of the proinflammatory properties of ROS pertinent to septic shock include recruitment of neutrophils at sites of inflammation (31), formation of chemotactic factors (32), DNA damage (33), initiation of lipid peroxidation (33), release of proinflammatory cytokines such as TNF and IL (34) via activation of nuclear factor (NF)-κB (35). Besides its proinflammatory effects, ROS possesses a number of cytotoxic mechanisms, including 1) the initiation of lipid peroxidation, 2) the inactivation of a variety of enzymes, and 3) the depletion of glutathione (36). Moreover, ROS can also cause DNA damage, resulting in the activation of the nuclear enzyme poly(adenosine 5'- diphosphate-ribose) polymerase, depletion of nicotinamide adenine dinucleotide (NAD) and adenosine triphosphate, which lead to irreversible cellular damage as evidenced in septic shock (37). Antioxidants inhibit the release of TNF, the activation of proinflammatory cytokines, cellular apoptosis and necrosis (38). In this way many authors have proposed the use of antioxidants to decrease 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 the oxidative stress by improving the thiol redox status, inhibit neutrophil and monocyte chemiotaxis and oxidative metabolism, to scavenge superoxide, hydrogen peroxide and hydroxyl radicals (42,43). Other

investigators have consistently shown that NAC, when administered before, not 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 demonstrated that high-dose NAC enhanced LPS-induced oxidative stress and mortality, possibly by its capacity to reduce iron to the catalytically active form (15). The administration of NAC in patients with sepsis decreased the oxidative stress, improved some hemodynamic variables and clinical scores with little effect in mortality (39,40). Some of these limitations of NAC therapy could be related to its adverse effects. Toxicity of NAC is low, although adverse effects, including anaphylactic responses, have been observed in accidental high-dose NAC infusion in humans. Nevertheless, it seems that high doses of NAC aggravate LPS toxicity (15). 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 (44). As in the case of ROS, the formation of thiyl radicals can occur in many different ways and is likely to depend on factors such the presence of metals (44). It seems that the ability of NAC to remove radicals do not include the hydroxyl radicals produced by the iron mediated Fenton reaction (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 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 hematological diseases with good safety profile. Based on histological and biochemical endpoints, 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, not after LPS challenge, protects animals against lethal (19) effects of LPS. As stated above, this could limit its isolate use in the clinical setting.

Effects of NAC plus DFX on oxidative parameters during sepsis

At 3 hours after sepsis induction, rats uniformly were tachycardic and tachypnic, demonstrated lethargy and piloerection. We, for the first time, demonstrated that the association of NAC and DFX significantly improves mortality in 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 levels (Figure 1). NAC plus DFX can increase the protection against free radical-mediated damage by increasing the intracellular reduced glutathione pool, scavenging superoxide and hydrogen peroxide, and reducing inflammatory response (38). Besides this, the addition of DFX can reduce the formation of hydroxyl radicals via Fenton reaction (38) and attenuates the pro-oxidative effects of NAC. In addition, NAC plus DFX can reduce oxidative stress restoring the balance between SOD and CAT activities (Figure 3). It seems that any concentrations of SOD other than the optimal leads to increased lipid peroxidation and therefore to decrease cell viability (47). SOD activity results in the production of hydrogen peroxide, which can mediate membrane damage by lipid peroxidation or react with iron to generate hydroxil 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 activation in major organs involved in sepsis response as demonstrated by the reduction of MPO activity (Figure 2). It is well known that NAC inhibit neutrophil activation and improve 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 was also believed to be responsible for injury to organs (7). Because of the intrapulmonary sequestration of neutrophils and the frequent complication of the acute respiratory distress syndrome in patients with sepsis, this link between overly exuberant neutrophil activation and organ injury was thought to affect the lungs in particular (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.

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). Based on these observations, circulatory disturbances have been considered to be the putative cause of altered oxygen metabolism during sepsis (50). On the other hand, 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. Moreover, the severity of mitochondrial injury correlates closely with the degree of impaired oxygen metabolism (51). In this regard, it has been reported that mediators of sepsis such as TNF (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). Together, these observations suggest that factors other than circulatory disturbances have been considered to be the putative cause of altered oxygen metabolism and the resultant tissue ischemia/ hypoxia could contribute significantly to the defective oxygen metabolism observed during sepsis. We here demonstrated that NAC plus DFX could improve mitochondrial electron transfer uncoupling as determined by superoxide production in SMP (Figure 4). One of the mechanisms of the uncoupling of mitochondrial electron transfer during sepsis is nitric oxide and superoxide induced oxidative stress (30,59). Thus, NAC plus DFX could improve mitochondrial function secondary to its antioxidant properties; and we here demonstrated that this treatment reduces mitochondrial TBARS in major organs involved in sepsis response (Figure 5). In this way, the effect of NAC plus DFX on mortality could also 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. The supportive therapy alone significantly improved mortality from 90% to nearly 60%, similar to antioxidant treatment alone. In addition, the administration of antioxidants after CLP challenge increases the relevance of the effect in mortality.

Several distinct strategies have been previously reported to improve survival in rodents challenged with CLP. The steroid hormone dehydroepiandrosterone reduced short-term mortality, an effect associated with a reduction of TNF release and an improvement of the activity of T cellular immunity (60). Recombinant heparin-binding protein, which enhances monocyte phagocytic activity, was also found to improve short-term survival when administered before CLP (61). In another study, a reduction in CLP mortality was reported after the administration for six consecutive days of recombinant granulocyte-macrophage colony-stimulating factor, which improved the killing of bacteria in various tissues (62). Neutralization of the macrophage migration inhibitory factor (MIF) by anti-MIF antibodies was shown to produce a marked increase in survival, even when treatment was delayed up to 8 h after CLP, defining a critical part for MIF in the pathogenesis of septic shock (63). Inhibition of adenosine deaminase improved both leukocyte-dependent and -independent mechanisms of endothelial injury in sepsis and mortality when administered immediately after CLP (64) or 2 hours after peritonitis induction (65). Liaudet et al demonstrated that inosine improved mortality even when administered 1 hour after CLP challenge (27).

Some authors reported different antioxidants approaches to the treatment of sepsis in animal models (15-23). Few of these employ the CLP model, and, for the best of our knowledge, here we describe the first study describing the effects of antioxidants in a clinically relevant model of rodents sepsis employing peritonitis. Not all animal models, particularly those without adequate fluid repletion, reproduce the typical hyperdynamic hemodynamics seen in resuscitated patients (25). 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 were administered 3 hours after the septic challenge. Also, our study provides evidence that NAC plus DFX, beyond its antioxidant effects, also affords protection from several important pathophysiologic alterations associated with sepsis: neutrophil activation and mitochondrial dysfunction.

CONCLUSIONS

In summary, our data provide the first experimental demonstration that NAC plus DFX reduces the consequences of septic shock induced by cecal ligation and perforation in the rat, by decreasing oxidative stress, limiting neutrophil activation and mitochondrial dysfunction, thereby leading to an improvement in survival. The current data, coupled with the safety profile of NAC and DFX may suggest that the concept of testing and developing NAC plus DFX as an antishock agent may be justified. 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 multidrug therapies. We believe that the approach described here is a more rational alternative to the use of antioxidants in sepsis treatment since can blockade free radical generation in several different steps.

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FIGURES LEGENDS

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 plus deferoxamine, or only saline as described under “Material and Methods”. 12 hours after CLP the heart, diaphragm, liver, lung and kidney were removed to the determination of thiobarbituric acid reactive species content as described under “Material and Methods”. Values are expressed as means $\pm$ S.D. (n=10 each group).

* different from sham-operated ($p<0.05$).

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 plus deferoxamine, or only saline as described under “Material and Methods”. 12 hours after CLP the heart, diaphragm, liver, lung and kidney were removed to the

determination of myeloperoxidase activity as described under “Material and Methods”. Values are expressed as means ± S.D. (n=10 each group).

* different from sham-operated ($p<0.05$).

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 plus deferoxamine, or only saline as described under “Material and Methods”. 12 hours after CLP the heart, diaphragm, liver, lung and kidney were removed to the determination of superoxide dismutase activity as described under “Material and Methods”. Values are expressed as means ± S.D. (n=10 each group).

* different from sham-operated ($p<0.05$).

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 plus deferoxamine, or only saline as described under “Material and Methods”. 12 hours after CLP the heart, diaphragm, liver, lung and kidney were removed to the determination of catalase activity as described under “Material and Methods”. Values are expressed as means ± S.D. (n=10 each group).

* different from sham-operated ($p<0.05$).

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 plus deferoxamine, or only saline as described under

“Material and Methods”. 12 hours after CLP the heart, diaphragm, liver, lung and kidney were removed to the determination of superoxide production in submitochondrial particles as described under “Material and Methods”. Values are expressed as means ± S.D. (n=10 each group).

* different from sham-operated ($p<0.05$).

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 plus deferoxamine, or only saline as described under “Material and Methods”. 12 hours after CLP the heart, diaphragm, liver, lung and kidney were removed to the determination of thiobarbituric acid reactive species in submitochondrial particles as described under “Material and Methods”. Values are expressed as means ± S.D. (n=10 each group).

* different from sham-operated ($p<0.05$).

Figure 7 - Percentage of rats surviving cecal ligation and puncture. Rats were sham-operated or submitted to cecal ligation and puncture (CLP). CLP animals were randomly assigned to receive N-acetylcysteine plus deferoxamine, “basic support”, or saline as described under “Material and Methods”. The mortality of the animals was recorded over a 5 day period.

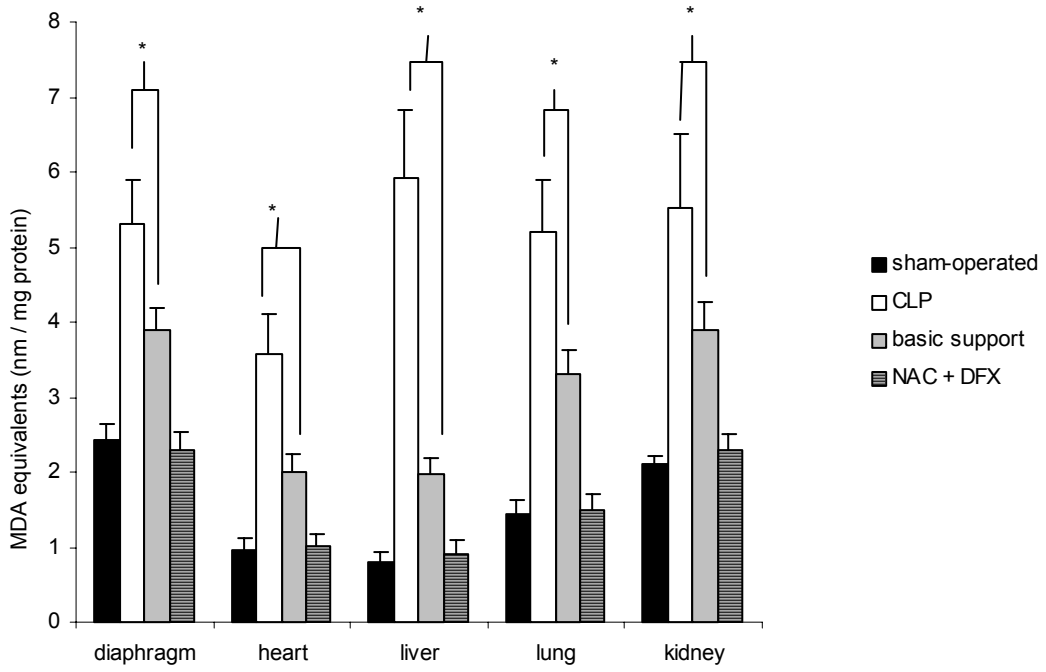

Figure 1

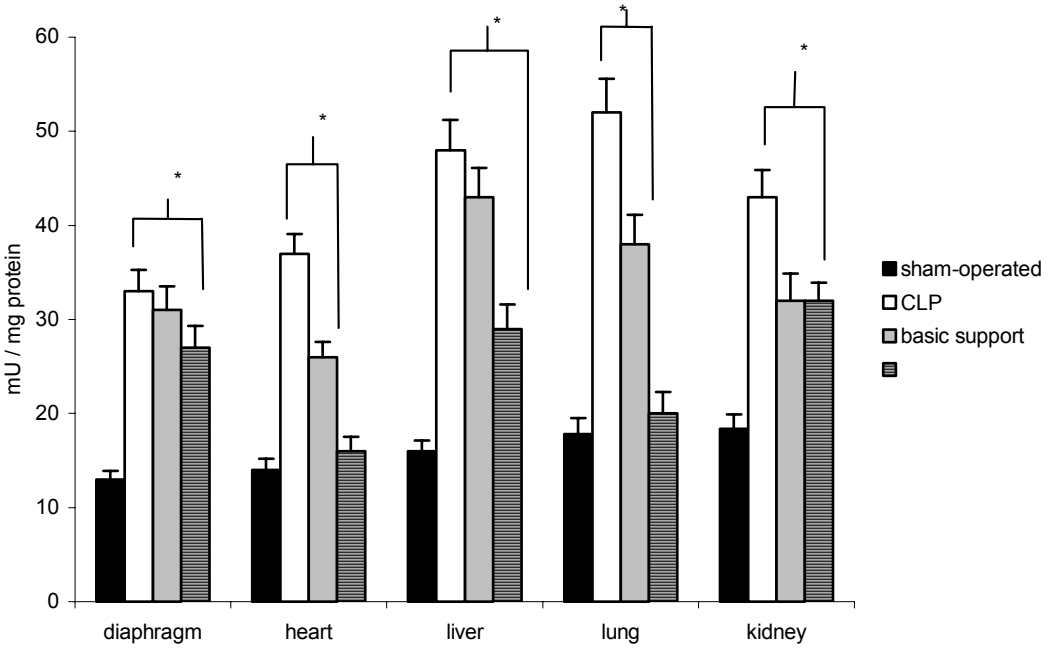

Figure 2

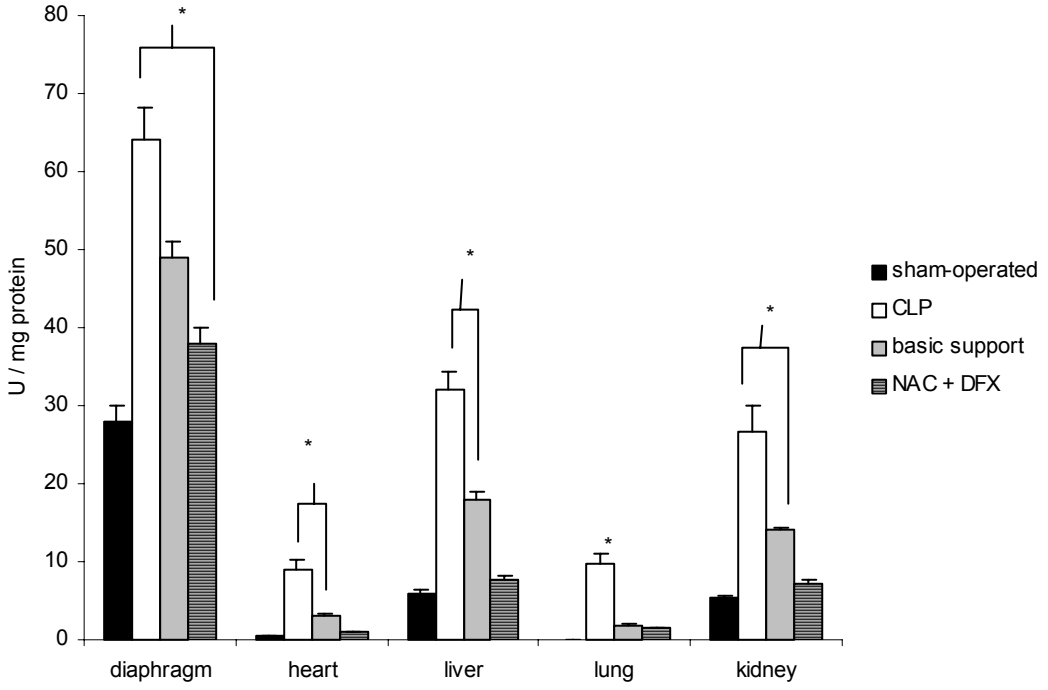

Figure 3

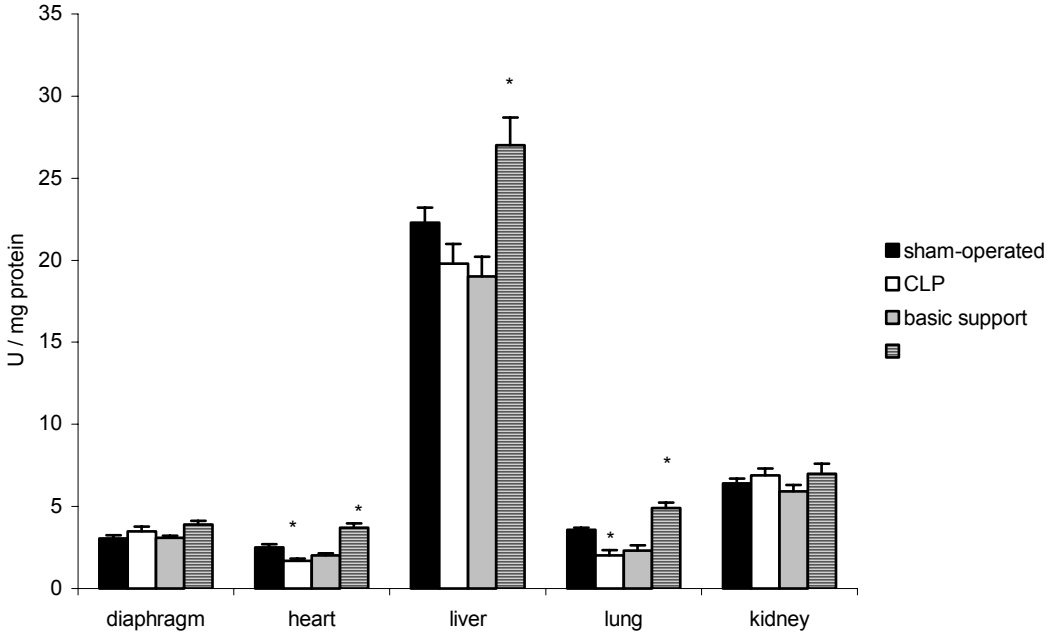

Figure 4

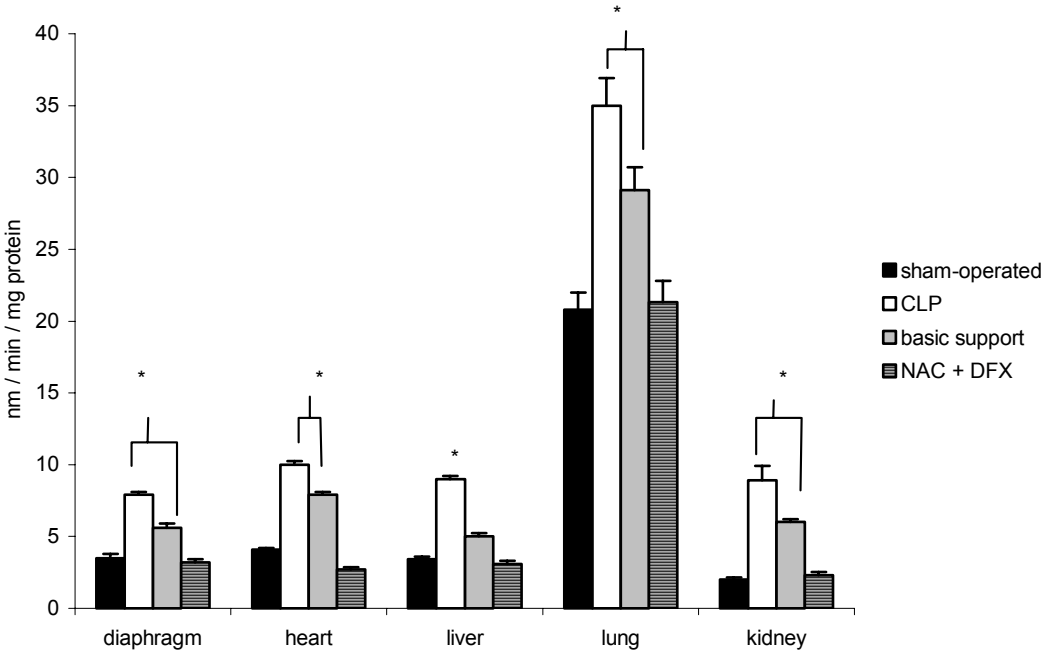

Figure 5

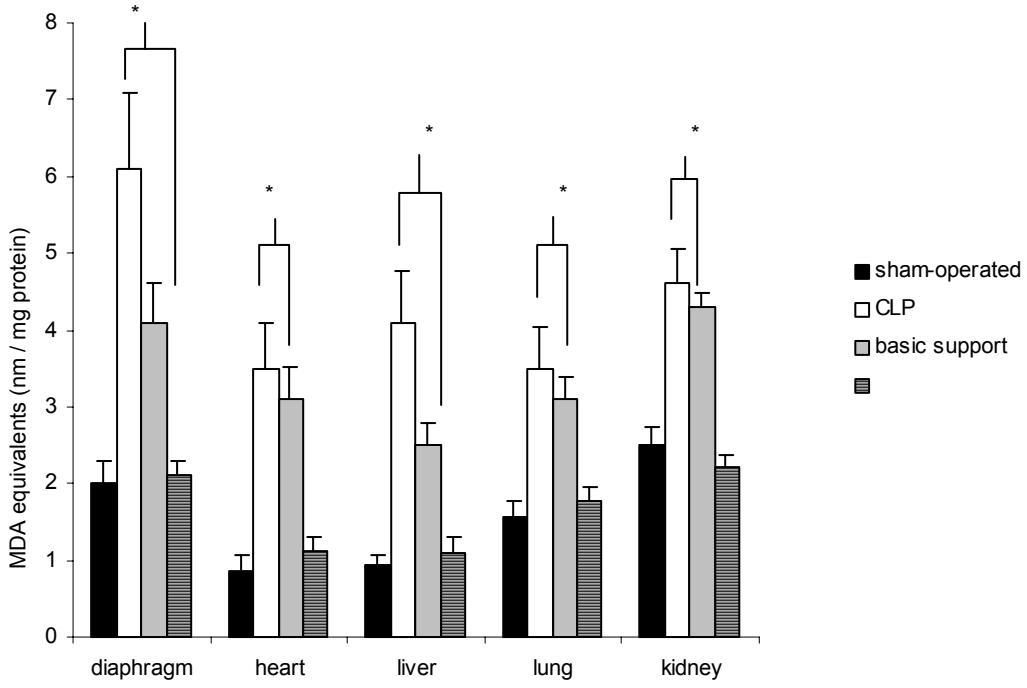

Figure 6

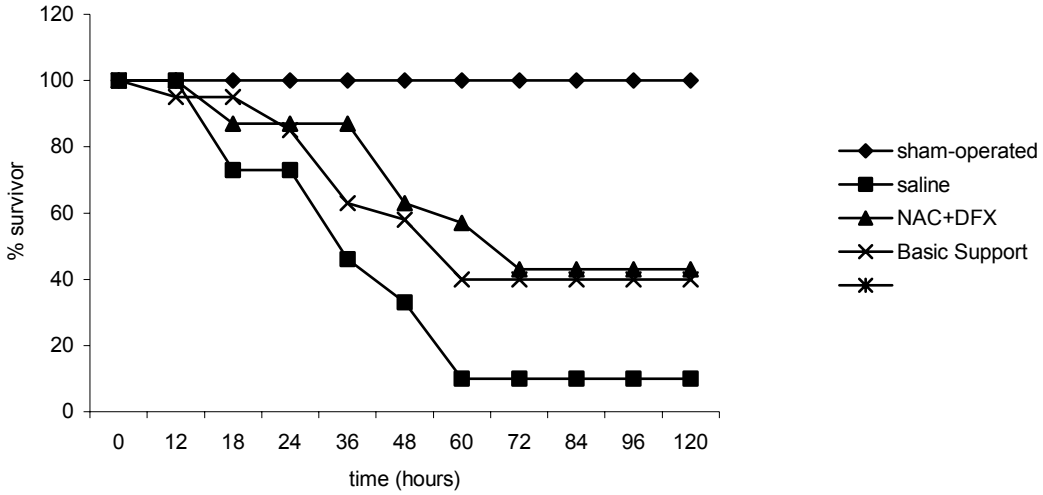
120
100
80
60
40
20
0
% survivor
0
12
18
24
36
48
60
72
84
96
120
time (hours)
sham-operated
saline
NAC+DFX
Basic Support

Figure 7

CAPÍTULO IV

DISCUSSÃO

Os resultados apresentados nesta dissertação podem ser resumidos:

No primeiro estudo (capítulo II) verificou-se um aumento de TBARS, grupamentos carbonil em todos os órgãos de animais sépticos e estes valores retornaram a níveis basais no grupo sobrevivente. Demonstrou-se também um desequilíbrio entre SOD e CAT no grupo não sobrevivente, sendo que o aumento excessivo da SOD pode predizer mortalidade muito precocemente (3h após sepse). Os valores de TBARS e carbonil podem predizer mortalidade com 12h após indução da sepse.

Muitos estudos com modelos animais e em humanos já haviam mostrado elevações nos parâmetros oxidativos durante o curso da sepse e que o estresse oxidativo é um dos fatores que levam a dano celular, disfunção orgânica e morte (15-16). Nosso estudo mostra a primeira evidência de que elevações de TBARS e grupamentos carbonil podem predizer mortalidade por sepse no modelo CLP, com 12h e 24 h após indução da sepse. Também demonstramos que há uma modulação diferente entre SOD e CAT em sobreviventes e não sobreviventes durante a sepse. Nós observamos um marcado aumento da SOD sem elevação proporcional da atividade da CAT em não sobreviventes e essa ativação da SOD foi um marcador precoce de mortalidade, já com 3h de evolução da sepse. A elevada atividade da SOD pode estar relacionada à produção aumentada de superóxido. Além disso, a ativação da SOD, principalmente a SOD-Mn, pode ser uma resposta ao aumento de IL-1, TNF e LPS durante a sepse (31,32). Uma grande atividade da SOD geralmente não protege a célula do estresse oxidativo, ao contrário, sua ativação resulta na produção de peróxido de hidrogênio que pode mediar dano às membranas pela peroxidação lipídica ou reagir com ferro via reação de Fenton. A CAT poderia limpar esse excesso de peróxido de hidrogênio se tivesse sua atividade proporcionalmente

aumentada, mas o excesso de superóxido e de peróxido de hidrogênio pode oxidar o sítio ativo da CAT, levando a inativação enzimática (33).

No segundo estudo demonstramos que o tratamento com NAC+ DFX diminui todos os parâmetros de estresse oxidativo, a ativação de leucócitos e atenuou o desequilíbrio de SOD e CAT. Houve significativa diminuição da mortalidade no grupo da NAC+ DFX em comparação com os grupos que receberam apenas uma das drogas; efeito semelhante ao tratamento de suporte.

Antioxidantes inibem a liberação de TNF, a ativação de citocinas pró-inflamatórias, apoptose e necrose celular (1). Baseando-se nisso, muitos autores têm proposto o uso de antioxidantes para diminuir o dano por radicais livres em modelos animais e pacientes com sepse. Nosso estudo confirmou esses dados e ampliou os achados previamente descritos, demonstrando que o tratamento com NAC + DFX tem um maior efeito benéfico em um modelo clinicamente relevante de sepse.

Alguns estudos mostraram que NAC, quando administrada antes, não depois da indução da sepse, protegeu animais contra os efeitos hemodinâmicos e letais da sepse. Já a administração de NAC em humanos, diminuiu o estresse oxidativo, melhorou algumas variáveis hemodinâmicas e escores clínicos, mas teve um impacto muito pequeno na mortalidade (28,29). Algumas das limitações da terapia com NAC pode ser associada ao fato de que o metabolismo oxidativo desta droga pode gerar radicais thyil, considerados intermediários nos processos de dano resultante de radicais livres (34). Como é sugerido que a formação de radicais thyil depende da presença de metais (34), nosso estudo utilizou a associação de NAC com um quelante de ferro (deferoxamina), no intuito de melhorar os efeitos terapêuticos da NAC. Além disso, a habilidade da NAC em remover radicais livres

parece não incluir o radical hidroxil produzido via reação de Fenton, assim mais uma vez, a associação NAC + DFX poderia melhorar o efeito terapêutico da NAC, como se confirmou nos resultados do segundo estudo.

Estes resultados abrem a perspectiva de estudar os efeitos da NAC + DFX em associação com tratamento de suporte e outros tratamentos experimentais descritos na literatura. Esperamos iniciar o uso combinado de NAC + DFX com inosina e frutose 1,6 bifosfato além de suporte básico e avaliar o impacto destas intervenções na mortalidade em modelo animal de CLP. Além disto, existe trabalho em andamento para avaliar os marcadores plasmáticos aqui descritos em pacientes com sepse, sepse grave ou choque séptico.

CAPÍTULO V

CONCLUSÕES

1 - Dano oxidativo e produção de superóxido mitocondrial é observado nos principais órgãos envolvidos na resposta séptica, especialmente nos animais não-sobreviventes;

2 - Um desequilíbrio entre SOD e CAT é observado nos principais órgãos envolvidos na resposta séptica, especialmente nos animais não-sobreviventes;

3 - O uso combinado de NAC + DFX atenua os danos oxidativos, produção de superóxido mitocondrial e ativação de mieloperoxidase nos principais órgãos envolvidos na resposta séptica;

4 - O uso combinado de NAC + DFX reduz a mortalidade em modelo animal de CLP, em uma magnitude similar a observada com o uso de antibióticos e ressucitação volêmica.

CAPÍTULO VI

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