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Efeito Terapêutico da N-acetilcisteína e Frutose-1,6-bisfosfato no Tratamento da Sepse Experimental

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Projeto de Pesquisa:

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SUMÁRIO

LISTA DE ABREVIATURAS

ALT - Alanina Aminotransferase

AMs - Antimicrobianos

AST - Aspartato Aminotransferase

ATP - Adenosina Trifosfato

CAT - Catalase

CK-Total - Creatinofosfoquinase

c-NOs - Óxido Nítrico Sintase Constitutiva

DNA - Ácido Desoxirribonucléico

DP - Desvio Padrão

ERN - Espécies Reativas de Nitrogênio

ERO - Espécies Reativas de Oxigênio

FBP - Frutose-1,6-Bisfosfato

FiO2 - Fração Inspirada de Oxigênio

GPX - Glutationa Peroxidase

H_2O_2 - Peróxido de Hidrogênio

IL-1 – Interleucina-1

IL-10 – Interleucina-10

IL-13 – Interleucina-13

IL-4 – Interleucina-4

IL-6 – Interleucina-6

IL-8 – Interleucina-8

i-NOs - Óxido Nítrico Sintase Induzível

INR - International Normalized Ratio

KTTP - Tempo de Tromboplastina Parcial

LDH - Lactato Desidrogenase

MCP-1 - Proteína Quimiotática de Monócitos-1

MTT - 3-[4-5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

NAC - N-acetilcisteína

NADP - Nicotinamida Adenina Dinucleotídeo Fosfato

NO - Óxido Nítrico

NOs - Óxido Nítrico Sintase

$O_2^{\cdot -}$ - Radical Superóxido

$OH^{\cdot -}$ - Radical Hidroxila

PAF – Fator Ativador Plaquetário

PAM - Pressão Arterial Média

PaO2 - Pressão Parcial de Oxigênio Arterial

PAS - Pressão Arterial Sistólica

PBMCs - Células Mononucleares de Sangue Periférico

RLs - Radicais Livres

SOD - Superóxido Dismutase

SRIS - Síndrome da Resposta Inflamatória Sistêmica

TGF-β - Fator de Crescimento Tumoral Beta

TNF-α - Fator de Necrose Tumoral Alfa

UTI - Unidade de Terapia Intensiva

RESUMO

A sepse é uma síndrome complexa ocasionada pela resposta inflamatória sistêmica descontrolada do indivíduo, que representa um grave problema epidemiológico em todo mundo. Sendo que, as citocinas inflamatórias representam o papel central na patogênese do choque séptico. O objetivo deste estudo foi investigar o efeito de duas drogas, N-acetilcisteína (NAC), um tiol-composto precursor da glutationa e a Frutose-1,6-bisfosfato (FBP), um metabólito glicolítico de alta energia, no tratamento da sepse experimental, através da avaliação do efeito imunomodulador (*in vitro*) e efeito terapêutico (*in vivo*), com o intuito de se obter drogas alternativas para o tratamento do choque séptico.

O modelo experimental escolhido para o estudo *in vitro*, foi à cultura de células mononucleares do sangue periférico isoladas de humanos saudáveis através de gradiente de centrifugação, Os linfócitos T foram estimulados durante 96 h com fitohemaglutinina, sendo utilizadas concentrações isoladas de NAC nas concentrações de 1, 5, 10 e 15 mM e associadas à FBP na concentração de 1,25 mM. Os resultados sugerem que tanto a NAC como sua associação com a FBP inibem a proliferação celular, agindo como potentes agentes na imunomodulação, sugerindo o uso destas contra doenças inflamatórias.

Para a experimentação in vivo, utilizaram-se ratos Wistar machos, divididos em cinco grupos experimentais: (i) controle normal (não induzidos); (ii) controle séptico (induzidos com uma cápsula com conteúdo fecal não estéril e *E. coli* (1,5 x 10^9 C.F.U.); (iii) induzidos e tratados com FBP (500 mg/kg I.P.); (iv) induzidos e tratados com NAC (150 mg/kg I.P.); (v) induzidos e tratados com a associação da FBP (500 mg/kg I.P.) com NAC (150 mg/kg I.P.). Na tentativa de se explicar o mecanismo de ação, principalmente o resultados do grupo séptico e tratado com NAC, foram realizadas análises hematológicas, dosagens bioquímicas, além da verificação do estresse oxidativo tecidual. Também foram realizadas hemoculturas, a fim de se verificar se houve indução séptica. Nossos resultados sugerem que a NAC impediu a mortalidade dos animais após a indução séptica. Estes dados comprovam a validade do uso da NAC no tratamento da sepse, possivelmente pela sua propriedade de ser um precursor da glutationa, pela sua capacidade hepatoprotetora e possível proteção renal. Nossos dados também demonstram que a ação sinérgica com FBP não melhora o quadro, esta em parte explicada pelo aumento do TBARS renal e pela diminuição da glutationa hepática.

Palavras chaves: Sepse, Estresse Oxidativo, Imunomodulação, N-acetilcisteína, Frutose-1,6-bisfosfato

ABSTRACT

Sepsis is a syndrome caused by uncontrolled systemic inflammatory response of the individual, which represents a serious epidemiological problem worldwide. Since inflammatory cytokines represent a central part in the septic shock pathogenesis. The aim of this study was to investigate the effect of two drugs, N-acetylcysteine (NAC), a thiol-compound precursor of glutathione and fructose-1,6-bisphosphate (FBP), a high energy glycolytic metabolite, in the treatment of experimental sepsis. by evaluating the immunomodulatory effect (in vitro) and therapeutic (in vivo), in order to obtain alternative drugs for the treatment of septic shock.

The experimental model chosen for the in vitro study was to culture of peripheral blood mononuclear cells were isolated from healthy humans through gradient centrifugation. T-Lymphocytes were stimulated for 96 h with phytohemagglutinin, being isoled from NAC used in concentrations of 1,5,10,15 mM or associated with 1.25 mM FBP were tested. The results suggest that either NAC itself or association with FBP inhibits cellular proliferation, acting as potent immunomodulatory agents, suggesting their use in inflammatory diseases.

For in vivo experiments, we used male Wistar rats that were divided into five experimental groups: (i) normal control (not induced), (ii) septic control (induced using a capsule with non sterile fecal content and E. coli ($1,5 \times 10^9$ CFU), (iii) induced and treated with FBP (500 mg/kg IP), (iv) induced and treated with NAC (150 mg/kg IP), (v) induced and treated with the combination of FBP (500 mg/kg IP) with NAC (150 mg/kg IP). In attempting to explain the possible mechanism of action, mainly the results of the septic group and treated with NAC, complete blood analysis, biochemical measurements were performed, besides the verification of tissue oxidative stress. Were also performed blood cultures and in the different groups in order to check whether there was induction septic. Our results show that NAC prevented the mortality of animals after septic induction. These data confirm the validity of the use of NAC in the treatment of sepsis, possibly due to its property to be a precursor to glutathione, by their hepatoprotective ability and possible renal protection. Our data also show that the synergistic action with FBP does not improve the picture, and this can be partly explained by heightened activation of the immune (defense) or a decrease of hepatic glutathione.

Keywords: Sepsis, Oxidative Stress, Immunomodulation, N-acetylcysteine and Fructose-1,6-bisphosphate.

1. CAPÍTULO 1

1.1 INTRODUÇÃO

1.1.1 Definição

A sepse é uma síndrome complexa ocasionada pela resposta inflamatória sistêmica descontrolada do indivíduo, de origem infecciosa, caracterizada por manifestações múltiplas e que pode determinar disfunção ou até mesmo a falência de um ou mais órgãos, e consequentemente sua morte. Seus fatores fisiopatológicos incluem, principalmente, o local da infecção, sendo os sistemas da coagulação, fibrinolítico e inflamatório os determinantes de sua evolução.[1]

O termo *Sepse* vem sendo usado desde a Grécia antiga, para descrever casos onde havia putrefação, associado com doença e morte.[2] Porém, esta patologia só foi descrita cientificamente em 1973 por Tilney *et al.,*[3] como “falência sistêmica sequencial”, abrangendo três pacientes que evoluíram para óbito por falência orgânica. Em 1975, Baue[4] descreveu a mesma patologia como “falência orgânica sistêmica progressiva, múltipla ou sequencial”.

Devido à grande quantidade de termos sinônimos para designar a mesma condição clínica e a sua gravidade, em agosto de 1991, uma nova definição foi estabelecida pelo *American College of Chest Physicians* e a *Society of Critical Care Medicine*, determinando assim um consenso sobre as definições e os critérios para o diagnóstico da sepse.[5] Em 2001, a *International Sepsis Definitions Conference* (Tabela 1), congregando um maior número de pesquisadores e peritos de várias partes do mundo, optou por não modificar as definições vigentes e sim por ampliar a lista de sinais e sintomas da sepse.[6]

Tabela 1 - Critérios diagnósticos para a sepse

Infecção documentada ou suspeita e algum dos seguintes critérios:
– Variáveis gerais
Febre (temperatura central > 38,3º C)
Hipotermia (temperatura central < 36º C)
Freqüência cardíaca > 90 bpm ou > 2 DP acima do valor normal para a idade
Taquipnéia
Alteração de sensório
Edema significativo ou balanço hídrico positivo (> 20 ml/kg/24 horas)
Hiperglicemia na ausência de diabete (glicemia > 120 mg/dl)
– Variáveis inflamatórias
Leucocitose (contagem leucócitos totais > 12.000 / mm³)
Leucopenia (contagem leucócitos totais < 4.000 / mm³)
Contagem de leucócitos totais normal com > 10% de formas imaturas
Proteína C- reativa no plasma > 2 DP acima do valor normal
Procalcitonina plasmática > 2 DP acima do valor normal
– Variáveis hemodinâmicas
Hipotensão arterial (PAs < 90 mmHg, PAM < 70 mmHg, ou
Redução da PAs > 40 mmHg em adolescentes, ou PAs / PAM < 2 DP abaixo do normal para idade)
Saturação de oxigênio venoso misto > 70% (não válido para crianças)
Índice cardíaco > 3,5 L/min (não válido para crianças)
– Variáveis de disfunção de órgãos
Hipoxemia arterial (PaO2 / FiO2 < 300)
Oligúria aguda (diurese < 0,5 mL/kg/h)
Creatinina > 0,5 mg/dl
Alterações de coagulação (INR > 1,5 ou KTTP > 60 s)
Íleo (ausência de ruídos hidroaéreos)
Trombocitopenia (contagem de plaquetas < 100.000 / mm³)
Hiperbilirrubinemia (Bilirrubina total > 4 mg/dl)
– Variáveis de perfusão tecidual
Hiperlactatemia (> 1 mmol/l)
Enchimento capilar reduzido ou moteamento

Modificado de Levy e cols., 2001, International Sepsis Definitions Conference.
DP: desvio padrão, PAs: pressão arterial sistólica, PAM: pressão arterial média, PaO2: pressão parcial de oxigênio arterial, FiO2: fração inspirada de oxigênio, INR: international normalized ratio, KTTP: tempo de tromboplastina parcial.

1.1.2 Epidemiologia

A sepse tem representado um grave problema epidemiológico para os sistemas de saúde em todo o mundo, tanto do ponto de vista econômico como social. De acordo com um estudo epidemiológico nos EUA, a incidência da sepse aumentou de 82,7 para 240,4/100 mil habitantes, bem como as mortes relacionadas

a ela, ainda que a taxa de mortalidade geral entre os pacientes com sepse tenha sido reduzida nesse período.[7]

Nas últimas décadas o aumento nas taxas de incidência e de morbimortalidade relacionadas à sepse, está diretamente relacionado aos avanços médicos, ou seja, cada vez mais são tratados pacientes gravemente doentes e internados nas Unidades de Terapia Intensiva (UTIs), ocasionando sepse secundária, seja ela decorrente do comprometimento imunológico e/ou pelas condutas e procedimentos médicos.[8]

A incidência da sepse relatada na literatura pode variar de acordo com as características de cada região e local. Nos EUA e Europa, a sepse é responsável por 2-11% das internações em UTI.[9] Análise retrospectiva de Jacobs *et al.,*[10] em mais de 2.000 admissões de uma UTI pediátrica, identificou 42,5% de pacientes com doença infecciosa, dos quais 63% destes evoluíram para o estado de choque séptico. Proulx *et al.,*[11] avaliando 1.058 admissões em UTI pediátrica do hospital universitário canadense, identificaram 82% de síndrome da resposta inflamatória sistêmica (SRIS), sendo 23% de etiologia infecciosa (sepse), das quais 2% com choque séptico. No Brasil a incidência da mortalidade provocada pela sepse e suas conseqüências varia de 40 a 45%, conforme dados do *Brazilian Sepsis Epidemiological Study.*[12]

1.1.3 Resposta do Organismo à Infecção na Sepse

A inflamação é uma resposta normal do hospedeiro contra agentes infecciosos. A sepse é caracterizada pela produção excessiva de mediadores

inflamatórios, assim como pela expressiva ativação de células inflamatórias, resultando em uma desregulação metabólica.[13]

Quando a infecção ou bacteremia ocorre, a primeira linha de defesa do hospedeiro é realizada por células fagocitárias (macrófagos, monócitos e granulócitos polimorfonucleares) e pela via alternativa do complemento, agindo de maneira não específica. Posteriormente, as imunoglobulinas e as células imunocompetentes iniciam uma resposta imune específica.[13,14]

Os componentes da parede bacteriana são os principais ativadores desta resposta do hospedeiro. As endotoxinas (lipopolissacárideos) dos microorganismos gram-negativos (principalmente o lipídio A) e o ácido teicóico dos microorganismos gram-positivos, desencadeiam indiretamente a cascata inflamatória, pela indução da produção de citocinas pelos macrófagos e monócitos, que quando ativados, produzem sequencialmente: Fator de Necrose Tumoral Alfa (TNF-α), Interleucina-1 (IL-1), Interleucina-6 (IL-6) e a Interleucina-8 (IL-8) que interagem com outras células e elementos celulares (polimorfonucleares, células endoteliais, fibroblastos, plaquetas e os próprios monócitos), induzindo a produção e liberação de mediadores secundários, contribuindo para uma resposta inflamatória tardia.[14,15]

Paralelamente à liberação das citocinas pró-inflamatórias, o organismo responde a agentes infecciosos, liberando citocinas antiinflamatórias como Interleucina 4 (IL-4), Interleucina 10 (IL-10), Interleucina 13 (IL-13), Fator de Crescimento Tumoral Beta (TGF-β), entre outras. Estes mediadores parecem tanto contrabalançar as ações dos mediadores pró-inflamatórios, através da redução da síntese e da liberação desses mediadores, quanto antagonizar seus efeitos.[16,17]

As células endoteliais possuem um importante papel na homeostasia, regulação do tônus vascular e fibrinólise.[18,19] E quando ativadas diretamente pelas endotoxinas ou pelas citocinas, adquirem uma função pró-coagulante e

protrombótica, provocadas pela liberação de tromboplastina, inibidor do ativador do plasminogênio e do fator ativador plaquetário (PAF), além da diminuição da produção de trombomodulina. Elas também produzem mediadores inflamatórios, tais como as Interleucina (IL-1, IL-6 e IL-8), prostaciclina, endotelina (capaz de aumentar o tônus vascular) e o óxido nítrico.[20,21]

A destruição local do endotélio pela aderência de polimorfonucleares ativos causa um aumento da permeabilidade e edema tecidual, que contribui para a ampliação da reação inflamatória.[19]

Alterações nas dimensões dos pequenos vasos, juntamente com alterações bioquímicas e fisiológicas sangüíneas, prejudicam a homeostasia da microcirculação durante o choque séptico, sendo esse o principal sítio de ataque, podendo tornar-se uma área fértil para o crescimento bacteriano descontrolado.[18] Um importante fator precipitante é a diminuição da deformidade das hemácias, que depende das propriedades viscoelásticas da membrana celular, viscosidade do citoplasma e da razão entre a área de superfície corpórea e o seu volume, podendo estar todos estes fatores alterados, devido à acidose, hipotermia e alterações na geometria da hemácia.[18]

1.1.4 Radicais Livres (RLs) e Estresse Oxidativo na Sepse

Os radicais livres (RLs) são definidos como qualquer espécie química capaz de existir de forma independente e que contenha um ou mais elétrons desemparelhados.[22] São espécies instáveis que reagem rapidamente, ocasionando danos nas células epiteliais, formação de fatores quimiotáticos, recrutamento de

neutrófilos, oxidação e peroxidação de lipídeos, dano ao ácido desoxirribonucléico (DNA), liberação de TNF-α e IL-1 e formação de peroxinitrito.[23]

Dentre os RLs, pode se destacar dois grupos: as espécies reativas de oxigênio (ERO) e as espécies reativas de nitrogênio (ERN). As ERO mais importantes são: o radical superóxido ($O_2^{\cdot -}$), radical hidroxila ($OH^{\cdot -}$) e peróxido de hidrogênio (H_2O_2). O óxido nítrico e o peroxinitrito constituem as principais ERN.[22]

Os RLs são gerados em processos de oxidação biológica e exercem funções importantes no organismo. A redução do oxigênio à água forma radicais livres, sendo o $O_2^{\cdot -}$ o primeiro radical livre formado nesse processo. A geração de RLs, como o $O_2^{\cdot -}$, é outro importante mecanismo de lesão utilizado pelos polimorfonucleares, principalmente pelos neutrófilos. Na cadeia respiratória mitocondrial, 5% do oxigênio utilizado não é completamente reduzido à água, e ocorre a formação de $O_2^{\cdot -}$.[22] Segundo Halliwell,[24] um desequilíbrio mitocondrial provocado pela sepse pode levar a um aumento de cálcio intracelular, aumentando a produção de espécies reativas de oxigênio.

O H_2O_2, apesar de ser considerado um oxidante estável, possui um papel importante na fisiopatologia da sepse. Este pode ser metabolizado por duas enzimas antioxidantes, a glutationa peroxidase (GPX) e a catalase (CAT), mas quando em presença de metais de transição (Fe^{++}), é transformado pela reação de Fenton em radical hidroxil, este altamente tóxico e reativo. Os danos às células musculares e a acidose aumentam a quantidade de ferro liberado da mioglobina e hemoglobina, facilitando esta reação.[25] Wizoerek *et al.,*[26] demonstraram que alterações do metabolismo de ferro podem estar relacionadas com a mortalidade em modelos animais de sepse.

Os organismos vivos dispõem de defesas enzimáticas e não-enzimáticas capazes de protegê-los contra os efeitos adversos dos RLs. A defesa endógena é

realizada pelas enzimas catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPX). Quando os RLs são produzidos em taxas que superam a capacidade antioxidante dos organismos, ocasionam uma situação de estresse oxidativo.[27] Portanto, o estresse oxidativo ocorre quando existe um desequilíbrio entre a geração de ERO e as defesas antioxidantes, ocasionando um potencial dano oxidativo.[28]

1.1.5 Tratamento

A resposta inflamatória sistêmica da sepse, pode se restringir a um fenômeno auto-limitado ou pode progredir para quadros de maior gravidade, como sepse grave, choque séptico e disfunção ou falência de um ou mais órgãos. Apesar da grande quantidade de investigações e de relatos sobre sepse e síndromes correlatas nos últimos anos, o controle definitivo do foco infeccioso é imperativo no tratamento, sendo a primeira prioridade. Contudo, além das medidas de suporte de vida, quando indicadas, outras medidas devem ser tomadas de acordo com a gravidade de apresentação da respectiva síndrome.[29]

Os antimicrobianos (AMs) são os agentes mais específicos e acessíveis para o tratamento do paciente com infecção, embora representem uma abordagem somente parcial do problema. Nas últimas quatro décadas, os estudos sobre efeito do uso de AMs nas infecções graves por germes gram-positivos ou gram-negativos têm demonstrado uma considerável redução da morbidade e da mortalidade.[29] Os AMs podem ser mais úteis no tratamento de estágios clínicos precoces da sepse, antes que a produção seqüencial dos mediadores do hospedeiro determine estágios mais adiantados na cascata inflamatória, com eventuais danos teciduais graves.[30]

Entretanto, alguns autores sustentam a idéia de que os AMs podem exacerbar a resposta inflamatória devido à lise dos microrganismos, com liberação de material de sua parede celular e consequente produção de mediadores inflamatórios endógenos.[31]

Atualmente, vêm sendo testadas estratégias para modular a excessiva geração ou ação de mediadores na sepse. A intervenção em qualquer passo da sequência dos eventos fisiopatológicos que caracterizam a resposta inflamatória sistêmica da sepse, no sentido de modificar (modular) essa reação do hospedeiro, parece ser a estratégia terapêutica com maiores perspectivas de mudar os resultados na terapia da sepse. Infelizmente, o uso clínico de terapias bloqueadoras de mediadores individuais tem falhado em reduzir a mortalidade geral associada à sepse. Contudo, a interrupção da sequência, na patogênese, em múltiplos pontos, é a melhor chance na redução da alta mortalidade atual desta patologia.[29]

1.1.6 Frutose-1,6-bisfosfato (FBP)

A frutose-1,6-bisfosfato (FBP) é um dos metabólicos encontrados na rota glicolítica, apresentando estruturas estáveis anoméricas: α e β furanose. Este açúcar bisfosforilado, além de ser um subproduto da via glicolítica também exerce papel importante junto a diversas rotas metabólicas do organismo. Entre as ações como regulador aparece a sua capacidade de alterar o metabolismo de carboidratos estimulando a glicólise e inibindo a gliconeogênese.[32]

A FBP tem demonstrado efeitos terapêuticos em várias situações patológicas como: isquemia, choque e lesões tóxicas.[33] Também foram documentados os efeitos

benéficos de FBP em deficiências orgânicas cardíacas, renais, cerebrais, hepáticas e intestinais (intestino delgado).[34,35]

Os mecanismos pelos quais FBP protege os neurônios do cérebro ainda não são claros. Um possível mecanismo de proteção inclui o metabolismo anaeróbio da FBP para gerar adenosina trifosfato (ATP)[36] ou reduzir a sua perda,[37] e/ou pela sua propriedade quelante de cálcio.[38] Esta capacidade da FBP em diminuir a quantidade de cálcio extracelular, melhora o rendimento mecânico e respiratório do coração isquêmico,[34] este mediado pela ativação de fosfoquinase-C que modula a atividade intracelular do cálcio.[39] A FBP também aumenta a captação celular de potássio que resulta em uma diminuição intracelular da concentração de sódio, reduzindo assim, o edema de celular citotóxico.[40]

O mecanismo pelo qual a FBP reduz a formação de $O_2^{\cdot -}$ pode ser decorrente do aumento nos níveis de ATP, tendo em vista de que este pode ser o regulador fisiológico da atividade catalítica da enzima nicotinamida adenina dinucleotídeo fosfato (NADP) oxidase, uma das enzimas responsáveis pela produção destes radicais.[41] A FBP inibe a formação de ROS e a ativação de neutrófilos,[42] além de reduzir a proliferação e a viabilidade de linfócitos T.[43] A inibição de ROS pode ser mediada em parte pela estabilização de glutationa intracelular.[44] Segundo, Nunes *et al.,*[45] a utilização da FBP em animais com a sepse experimental aumentou a taxa de sobrevivência dos mesmos.

1.1.7 N-acetilcisteína (NAC)

Antioxidantes são substâncias que, quando presentes em baixas concentrações comparadas com o agente oxidante, de modo significativo, reduzem

ou previnem a oxidação do substrato (molécula). Diferentes antioxidantes são necessários para proteção contra o estresse oxidativo.[46]

A N-acetilcisteína (NAC), um tiol-composto, tem sido usado terapeuticamente devido à sua propriedade de ser um precursor da glutationa,[47] possuindo, portanto, um papel-chave na homeostasia celular, visto que a depleção de glutationa pode causar morte celular devido à peroxidação lipídica e declínio nos níveis de tiol-proteínas.[48]

A NAC tem sido utilizada largamente como antioxidante *in vivo* e *in vitro.*[49] Ela também é eficiente no tratamento de algumas situações de overdose de alguns fármacos, como paracetamol.[50]

A NAC pode atuar no metabolismo mitocondrial influenciando a fosforilação oxidativa, através de dois mecanismos: protegendo proteínas da fosforilação oxidativa contra o dano oxidativo através da manutenção dos grupos SH que são essenciais para a atividade enzimática e evitando a peroxidação lipídica das membranas mitocondriais, o que poderia diminuir a atividade dos complexos.[51]

Wan *et al*.,[52] relataram que NAC inibe a formação de radical hidroxila após infusão de d-anfetamina (d-AMPH) em ratos estriados, sugerindo que NAC poderia proteger contra o estresse oxidativo induzido pela d-AMPH. A capacidade antioxidante da NAC quando em conjunto com a desferoxamina (quelante de Fe^{++}) reduz a mortalidade em ratos submetidos à indução de sepse.[53]

1.2 JUSTIFICATIVA

Nos últimos 10 anos, progressos em biologia celular e molecular mostraram que a agressão bacteriana ou de seus subprodutos (endotoxinas e ácido teicóico), não são os únicos responsáveis pela deterioração clínica dos pacientes em choque séptico. A resposta do hospedeiro desempenha papel importante nos diferentes tipos de agressões, quer infecciosas ou não.

A identificação de mediadores e dos mecanismos envolvidos na produção das alterações fisiológicas, metabólicas e celulares é de grande interesse, pois estão envolvidos na perda da capacidade de homeostasia celular do organismo.

A maioria dos pesquisadores concorda que melhores taxas de sobrevida em pacientes com sepse grave só poderão ser atingidas com terapias adicionais às terapias antimicrobianas convencionais. Quanto mais se conhece a complexidade e a interdependência dos mecanismos fisiopatológicos da sepse, mais se buscam estratégias terapêuticas com base em substâncias que modulem ou interrompam os efeitos dos mediadores endógenos e exógenos da sepse. Portanto, a interrupção da sequência, na patogênese, em múltiplos pontos, é a melhor chance na redução da alta mortalidade desta patologia.

Portanto, nesta investigação avaliamos o efeito imunomodulador (*in vitro*) e terapêutico (*in vivo*) da N-acetilcisteína e/ou Frutose-1,6-bisfosfato no tratamento da sepse por *Escherichia coli*.

1.3 OBJETIVOS

1.3.1 Objetivo Geral

Avaliar o efeito imunomodulador (*in vitro*) e terapêutico (*in vivo*) da N-acetilcisteína e/ou Frutose-1,6-bisfosfato no tratamento da sepse.

1.3.2 Objetivos Específicos

***in vitro*:**

1.3.2.1 O objetivo deste estudo foi avaliar o uso da N-acetilcisteína e sua associação com a Frutose-1,6-bisfosfato na proliferação de linfócitos T e nos níveis de Interleucina-1β (IL-1β), Interleucina-6 (IL-6) e Proteína Quimiotática de Monócitos-1 (MCP-1) que estão envolvidos no processo que desencadeia o choque séptico.

***in vivo*:**

1.3.2.2 Investigar o efeito de duas drogas, a N-acetilcisteína e a Frutose-1,6-bisfosfato no tratamento da sepse experimental.

1.4 ASPECTOS ÉTICOS

Este projeto foi aprovado pelo Comitê Científico da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul, protocolado sob o nº OF.CEP 151/09.

2. CAPÍTULO 2

2.1 ARTIGO CIENTÍFICO Nº 1

N-acetylcysteine and Fructose-1,6-bisphosphate: Immunomodulatory Effects in Mononuclear Cells Culture

Submetido ao periódico: **Inflammation Research**

Your manuscript entitled "N-ACETYLCYSTEINE AND FRUCTOSE-1,6-BISPHOSPHATE: IMMUNOMODULATORY EFFECTS IN MONONUCLEAR CELLS CULTURE" has been successfully submitted online and is presently being given full consideration for publication in Inflammation Research.

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N-acetylcysteine and Fructose-1,6-bisphosphate: Immunomodulatory Effects in Mononuclear Cells Culture

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ABSTRACT

Sepsis is a complex syndrome caused by an uncontrolled systemic inflammatory response of the individual. Inflammatory cytokines represent a central part in the septic shock pathogenesis. The objective of the present study was to investigate the therapeutic effect of two drugs, N-acetylcysteine (NAC), a thiol-compound that is a glutathione precursor, and its association with Fructose-1,6-bisphosphate (FBP), a high energy glycolytic metabolite, on the T-lymphocytes proliferation, interleukin-1β (IL-1β), interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) levels. Peripheral blood mononuclear cells were isolated from healthy humans through gradient centrifugation. T-Lymphocytes were stimulated for 96 h with phytohemagglutinin and different concentrations of NAC alone or associated with 1.25 mM FBP were tested. NAC alone (10 and 15 mM) and NAC (15 mM) associated with FBP, significantly reduced the T-lymphocytes proliferation. NAC (1mM) also reduced the cellular viability when associated with FBP. The IL-1β levels increased with NAC (15 mM) isolated and associated with FBP (1.25 mM), and IL-6 levels reduced with NAC (15 mM) isolated and associated to FBP (1.25 mM). The MCP-1 levels were significantly reduced only by NAC (15 mM) associated with FBP (1.25 mM). The results suggest that either NAC itself or association with FBP inhibits cellular proliferation, acting as potent immunomodulatory agents, suggesting their use in inflammatory diseases.

Key-Words: Immunomodulation, Fructose-1,6-bisphosphate, N-acetylcysteine, Sepsis.

INTRODUCTION

Sepsis is a complex syndrome caused by an uncontrolled systemic inflammatory response of the individual, with an infectious origin and characterized by multiple manifestations that can determine dysfunction or even failure of one or more organs.[1,2] The main pathophysiological factors include the local of the infection and the coagulation, fibrinolytic and inflammatory systems are determinants on its evolution.[3] Sepsis has been considered a serious epidemiological problem for health systems all over the world, both in an economical as well as social point of view. Previous study conducted in the USA showed that the incidence of Sepsis increased from 82.7 to 240.4/100 thousand inhabitants.[4] In Brazil, the mortality rate caused by Sepsis and its consequences varies between 40 to 45%.[5]

The septic shock represents an example of the increased inflammatory response. Its systemic effects are caused by an excessive production of inflammatory mediators (endogenous cytokines), as well as for the intense activity of inflammatory cells, resulting in a metabolic disequilibrium.[6] The shock complications are mainly linked with the release of bacterial wall components. Endotoxins (lipopolysaccharides) from gram-negative microorganisms (lipid A, mainly) and the teichoic acid from gram-positives microorganisms, indirectly induce an inflammatory cascade by increasing the production of cytokines by macrophages and monocytes. Their activation sequentially produces tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-8 (IL-8), that interacts with other cells and cellular elements (polymorphonuclear, endothelia cells, fibroblast cells, platelets and monocytes), inducing the production and release of secondary mediators, contributing to a delayed inflammatory response.[7,8] However, the

overproduction or inappropriate expression of these factors can lead to a variety of pathological conditions, including systemic toxicity and septic shock.[9,10]

Therefore, therapeutical strategies in order to modulate the excessive generation or function of sepsis mediators have been tested. The intervention on any step of the pathophysiological event sequence that characterize the systemic inflammatory sepsis response, in order to modify/modulate the host reaction, seems to be the most likely therapeutical approach to change its evolution in the sepsis therapy. Unfortunately, the clinical use of individual mediator blockers have failed to reduce the mortality associated to sepsis. However, the interruption of the pathophysiological response sequence, in many levels, seems to be the best chance of reducing the high mortality of this pathology.[11]

The fructose-1,6-bisphosphate (FBP) is a high-energy glycolytic metabolite that is believed to have a protective effect against toxic agents.[8] In a recent study, it has been demonstrated that FBP in concentrations of 1.2 to 10 mM reduced the interleukin-2 (IL-2) soluble receptor levels, suggesting that FBP has an immunomodulatory effect.[12] The possible mechanism involved could be related to the interaction of FBP with cellular membranes, leading to changes in the ionic permeability. In the presence of FBP, there is a reduced K^+ efflux by passive and active channels in hepatocytes.[13] The K^+ conductance through the cell membrane is the main determinant for the T-lymphocyte electric potential, where changes can lead to mitogenesis in these cells.[14]

The N-acetylcysteine (NAC), a thiol-compound, has been therapeutically used due to its property of being a glutathione precursor,[15] assuming a key-role in the cellular homeostasis, since the glutathione depletion can cause cellular death due to lipidic peroxidation and decrease the thiol-protein levels.[16] NAC has been widely used both *in vitro* and *in vivo* as an antioxidant.[17] It is also efficient in the treatment of

some pharmacological overdoses, as in the one caused by paracetamol.[18] NAC can also act in the mitochondrial metabolism influencing the oxidative phosphorylation through two mechanisms: protecting oxidative phosphorylation proteins from the oxidative damage through the maintenance of the thiols groups, which are essential for the enzymatic activity, and preventing the lipidic peroxidation of mitochondrial membranes, that could reduce the mitochondrial respiratory chain activitiy.[19] Wan *et al*.[20] showed that NAC inhibits the hydroxyl radical formation after d-amphetamine infusion (d-AMPH) in rats, suggesting that NAC could protect against induced oxidative stress.

Thus, the objective of the present study is to evaluate the use of alternative drugs, such as N-acetylcysteine and its association with fructose-1,6-bisphosphate in the T-lymphocytes proliferation and in the interleukin-1β (IL-1β), interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) levels, which are involved in the process that can induce the septic shock.

MATERIAL AND METHODS

Peripheral blood mononuclear cells (PBMCs) preparation

PBMCs were isolated from the blood of healthy humans through gradient centrifugation. A total of 15 mL of heparinized blood, separated from plasma, was diluted (1:2) in RPMI 1640 medium (Gibco). Each 3 mL of Ficol (Amersham Biosciences) was added to 7 mL of the previous dilution and then centrifuged 800xg at room temperature for 20 minutes. PBMCs, including T-lymphocytes, were collected from the interface induced after samples were centrifuged, with a sterile Pasteur pipette, and washed twice in 10 mL of saline phosphate buffer. After that, the

cells were re-suspended in RPMI 1640 medium supplemented with 2,7 mg/mL garamycin (Schering-Plough) and 20% of autologous serum, with a final concentration of 1,6 x 10^6 cells/mL. The contamination of the solution was less than 1% and the cellular viability determined by exclusion using Trypan blue (Sigma) was uniformly equal or superior to 90%.

Lymphoproliferation

Phytohemagglutinin (PHA) (Invitrogen) was used to assess the T-lymphocyte proliferation. The NAC and FBP solutions used were dissolved in supplemented RPMI 1640 medium. Plates of 96 wells (Nunc) were used for the PBMCs (1.6 x 10^5 cells/well) culture stimulated with PHA (10 mg/mL). The cells were treated with either different isolated NAC concentrations (1 mM, 5 mM, 10 mM and 15 mM) or associated with FBP (1.25 mM) (n=6 for each concentration)[12] and kept for 96 h in 5% CO_2 with a constant temperature of 37℃.

The lymphocytes proliferation was determined by 3-[4-5-dimetiltiazol-2-yl]-2,5-difeniltetrazolium bromide (MTT) technique, as described previously.[21] MTT (Acros Organic) was dissolved in RPMI 1640 medium at 5 mg/mL, added to all plate wells and then incubated at 37℃ for 4 h. After the incub ation, dimethylsulfoxide (DMSO) was added to all supernatants in order to dissolve the dark blue crystals formed by MTT. After 5 minutes, the results were analyzed through a Hyperion MicroReader using a 540 nm wave length and a filter reference of 650 nm. All experiments were conducted in triplicate.

Cytotoxicity analysis

Plates of 96 wells were used to incubate PBMCs (1.6 x 10^5 cells/200µL) with NAC and FBP dissolved in RPMI 1640 medium. The cells were treated with either

isolated NAC concentrations (1 mM, 5 mM, 10 mM and 15 mM) or associated with FBP (1.25 mM) (n=6 for each concentration)[12]. The plates were kept for 96 h at 37℃ in a 5% CO_2 environment. The cellular viability was measured through cell counting, with a Neubauer chamber, using the exclusion technique with Trypan blue (Sigma). Viabilities equal or superior to 90% were accepted and the results were expressed in absolute values. The experiments were conducted in triplicate.

Pro-Inflammatory cytokine analysis

Cytokines production was evaluated in the supernatants of PBMCs (1.6 x 10^5 cells/well) incubated in a culture medium for 96 h in 5% CO_2 at 37ºC. The cells were incubated (n=6) in 96 wells plate and received the following treatments: (I) not stimulated; (II) stimulated with PHA; (III) stimulated with PHA plus a 15 mM NAC solution; (IV) stimulated with PHA plus a 15 mM NAC solution associated with a 1.25 mM FBP solution; (v) not stimulated, but treated with a 15 mM NAC solution; (vi) not stimulated, but treated with a 15 mM NAC solution associated with a 1.25 mM FBP solution. These concentrations were chosen based in the suppressive effect demonstrated in the lymphoproliferation assay. After the incubation period, the plates were centrifuged 900xg for 20 minutes and the supernatants were removed and stored at -70ºC until processed. Commercial ELISA kits (Biosource) were used to measure IL-1β, IL-6 and MCP-1 concentrations. All readings were performed in the ELISA (Biorad, UK) reader using a 570 nm wave length and a reference filter of 650 nm. All samples and standards were tested in triplicate. The results were expressed in pg/mL.

Statistical analysis

The results are presented using descriptive statistics. The differences between groups were evaluated through a one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test. A significance level of $P<0.05$ was used. All statistical analysis was performed using the SPSS® 15.0 (SPSS Inc. Ohio, USA) software.

RESULTS

N-acetylcysteine and Fructose-1,6-Bisphosphate immunomodulatory effect in PHA stimulated T-Lymphocytes

The immunomodulatory effects of NAC and FBP in PHA (10 mg/mL) stimulated T-lymphocytes were evaluated. Figure 1 shows that NAC in concentrations of 10 mM and 15 mM significantly reduced ($P<0.05$ and $P< 0.001$, respectively) the lymphocytic proliferation. On the other hand, when associated with FBP (1.25 mM), NAC significantly reduced the lymphocytic proliferation in concentrations of 1 mM and 15 mM ($P<0.05$), as demonstrated in figure 2.

N-acetylcysteine and Fructose-1,6-bisphosphate cytotoxic effects in PBMCs

In order to evaluate if the lymphoproliferative inhibitory effect of NAC alone and associated with FBP could be a result of cellular death, which would be a toxic effect, the cellular viability was also evaluated. Figure 3 show that when the same concentrations of NAC alone were used, no cellular cytotoxicity was demonstrated. However, when 1.25 mM of FBP was associated with 1 mM of NAC, a significant reduction ($P<0.01$) in the cellular viability was demonstrated (Figure 4), excluding its use in the lymphoproliferative evaluation.

Evaluation of IL-1β, IL-6 and MCP-1 levels

In order to evaluate a possible mechanism for the NAC (15 mM) immunomodulatory effects, either isolated or associated with 1.25 mM FBP, the levels of IL-1β, IL-6 and MCP-1 were measured with or without PHA stimulation. As showed in Figure 5, both NAC used isolated or associated with FBP, significantly increased IL-1β levels with or without PHA stimulation ($P<0.001$). However, only NAC administrated alone or associated to FBP, but without PHA stimulation, induced a significant reduction in the IL-6 levels (Figure 6). When the MCP-1 levels were evaluated, a significant reduction ($P<0.001$) was demonstrated when NAC associated with FBP, but without PHA stimulation, was tested (Figure 7).

DISCUSSION

The objective of the present study was to investigate the possible effects of both NAC alone or associated with FBP, that already has a well known immunomodulatory effect [12], on the proliferation of T-Lymphocytes, which could help in the development of alternative drugs for the septic shock treatment. Moreover, it also aims at explaining possible immunomodulatory mechanisms, through the evaluation of pro-inflammatory cytokine (IL-1β, IL-6 and MCP-1).

Our results demonstrate that 10 and 15 mM NAC administrated alone and 15 mM associated with 1.25 mM FBP presented an important immunomodulatory effect in cells. However, Hadzic *et al*.,[22] showed that in an inhibited lymphocyte proliferation model induced by a glutathione (GSH) blockage, NAC induced an increase in the T-lymphocytes proliferation, suggesting that the thiol groups would participate in the T-lymphocytes proliferation regulation. On the other hand, 1mM NAC associated with

FBP significantly reduces the cellular viability, suggesting that this association would have a cellular toxic effect. Furthermore, when NAC concentrations are increased, either isolated or associated with FBP, a proportional reduction in the T-lymphocytes proliferation was seen.

Extensive evidence in the literature demonstrates a correlation between the increased proinflammatory cytokines production and the sepsis mortality rate, both in humans and experimental models, being the IL-1 and IL-6 cytokines suggested to play a key role. Therefore, many recent clinical therapies are evaluating the security and effectiveness of the anti-cytokine use either isolated or in association with other compounds.[23]

IL-1 is formed by two different molecules: IL-1α and IL-1β. It induces an increase in the concentration of colony-stimulating factors, IL-6, MCP-1, acute phase hepatic proteins, bone reabsorption, collagen synthesis and lipoprotein lipase inhibition.[24,25] The IL-1 natural mechanism of inhibition involves blocking the receptor binding trough the use of cytokines receptor antagonists, as IL-1Ra. IL-1Ra is a protein from the interleukins family, originally described as a molecule secreted by monocytes and macrophages, that modulate many immune and inflammatory responses related to the IL-1.[26,27] The IL-1participation in the sepsis physiopathology was mostly studied trough the use of this antagonist (IL-1Ra), which reduces the mortality caused by endotoxin administration[28] or *E. Coli*.[29] Present study shows an increase in the IL-1β levels, either when isolated NAC or in association with FBP were used with or without PHA stimulation, suggesting that the NAC (and/or its association with FBP) protective role may be due to its immunomodulatory effect and not to its antiinflammatory effect. However, some studies in the literature, have shown a reduction in the IL-1β levels after NAC treatment.[30,31]

IL-6 is a part of the innate and acquired immunity process and is produced in response to TNF, IL-1, and some activated T-cells. In the innate response, IL-6 stimulates hepatic protein synthesis, for instance amyloid A protein and fibrinogen, which are a part of the acute phase systemic inflammation response. In the acquired immunity, IL-6 stimulates the development of the antibody-producing B-lymphocyte. This effect can be seen in the myeloma neoplasic plasmocytes and in the monoclonal antibody producing neoplasic cells (hybridomas).[32,33] Although its effects in sepsis are not clear, this cytokine presents a strong correlation with mortality in both experimental models and patients with sepsis, that is, the higher the IL-6 plasma levels are, the greater the probability of a fatal outcome.[34,35] Our results show a reduction in the IL-6 levels when compared to the control group, either when NAC was used isolated or associated with FBP without PHA stimulation. These results also demonstrate that the immunomodulatory responses seen in the PHA stimulated cells are not involved with an IL-6 effect.

MCP-1 has the ability to attract circulating monocytes to become macrophages in the adipose tissue. These macrophages are a source of cytokines with inflammatory activity. Pre-adipocytes and adipocytes produce MCP-1 (among others) in response to several stimuli: nitric oxide (NO) and TNF-α, IL-1β, IL-4 and Interferon-γ (IFN-γ).[36] Our study demonstrate, in spite of the IL-1β increase, a MCP-1 reduction when NAC was used in association with FBP without PHA stimulation, suggesting that this would probably be a FBP effect, since when NAC was administered alone no significant differences were demonstrated. However, Maruno *et al*.[37] showed a reduction in the MCP-1 levels when NAC treatment was used after vascular endothelial growth factor induction, indicating that NAC itself may have an effect on this cytokine production.

Taken together, our results suggest that either NAC isolated or in association with FBP can inhibit the cellular proliferation, acting as major immunomodulatory agents and suggesting their use in inflammatory processes, including sepsis. The IL-1β, IL-6 and MCP-1 production doesn't explain the immunomodulatory action in T-Lymohocytes cell cultures stimulated with PHA, since no reductions in their levels were seen. On the other hand, the N-acetylcysteine and its association with Fructose-1,6-bisphosphate in T-Lymohocytes cell cultures without PHA stimulation, reduced the IL-6 and MCP-1 levels, suggesting that there is a modulation in the interleukins synthesis and that this response can be related to its immunomodulatory effect.

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Figure legends

Figure 1: N-acetylcysteine immunomodulatory effects in PHA stimulated T-Lymphocytes. The results were evaluated by optical density in triplicate cultures and expressed as mean ± SEM. * indicates a significant difference compared to PHA ($P<0.05$). ** indicates a significant difference compared to the PHA group ($P<0.001$).

Figure 2: N-acetylcysteine associated with Fructose-1,6-bisphosphate (1,25 mM) immunomodulatory effects of in PHA stimulated T-Lymphocytes. The results were evaluated by optical density in triplicate cultures and expressed as mean ± SEM. * indicates a significant difference compared to the PHA group ($P<0.05$).

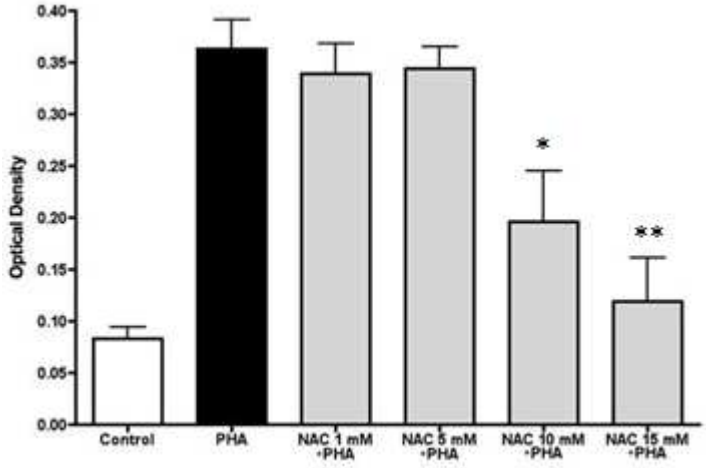
Figure 3: N-acetylcysteine cytotoxic effects in PBMCs. The cellular viability was verified by trypan blue exclusion method. The results are presented in absolute values (mean ± SEM). Cultures were performed in triplicates. No significant differences were identified.

Figure 4: N-acetylcysteine associated with Fructose-1,6-bisphosphate (1,25 mM) cytotoxic effects in PBMCs. The cellular viability was verified by trypan blue exclusion method. The results are presented in absolute values (mean ± SEM). Cultures were performed in triplicates. * indicates a significant difference when compared to the control group ($P<0.01$).

Figure 5: Effects of N-acetylcysteine (15 mM) and Fructose-1,6-bisphosphate (1,25 mM) in the IL-1β levels. The results are presented in absolute values (mean ± SEM). Cultures were performed in triplicates. * indicates a significant difference compared to the control group ($P<0.001$). # indicates a significant difference when compared to the PHA group ($P<0.001$).

Figure 6: Effects of N-acetylcysteine (15 mM) and Fructose-1,6-bisphosphate (1,25 mM) in the IL-6 levels. The results are presented in absolute values (mean ± SEM). Cultures were performed in triplicates. * indicates a significant difference compared to the control group ($P<0.05$). ** indicates a significant difference when compared to the control group ($P<0.01$).

Figure 7: Effects of N-acetylcysteine (15 mM) and Fructose-1,6-bisphosphate (1,25 mM) in the MCP-1 levels. The results are presented in absolute values (mean ± SEM). Cultures were performed in triplicates. * indicates a significant difference compared to the control group ($P<0.001$). # indicates a significant difference when compared to the PHA group ($P<0.001$).



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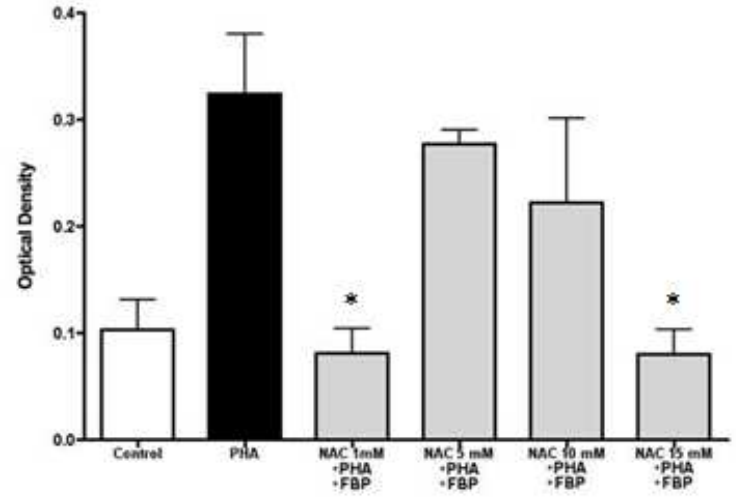

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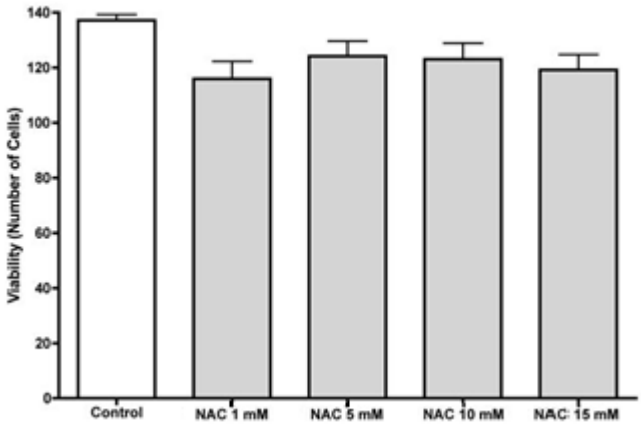

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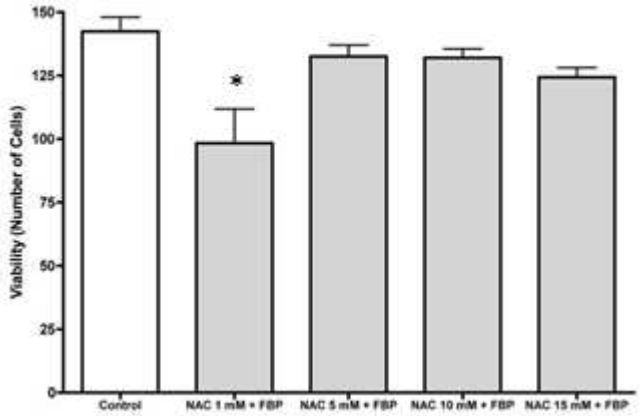

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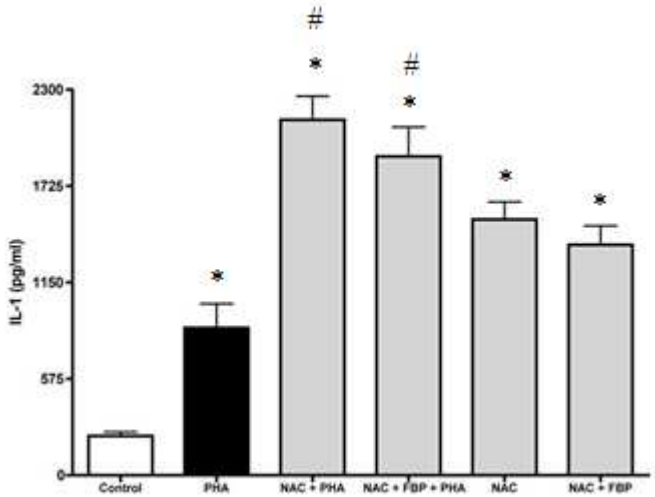

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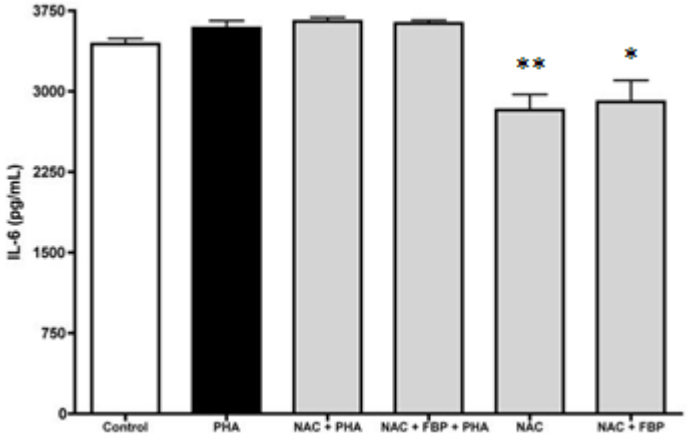

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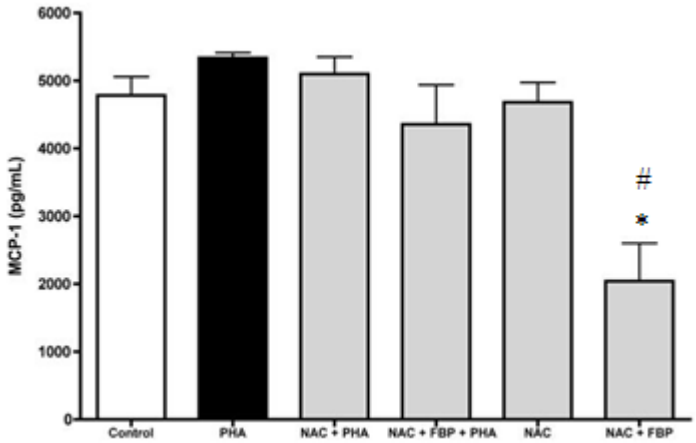

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3. CAPÍTULO 3

3.1 ARTIGO CIENTÍFICO Nº 2

Effect of N-acetylcysteine and Fructose-1,6-bisphosphate in the Treatment of Experimental Sepsis

Submetido ao periódico: **Inflammation**

Thank you for submitting your manuscript, IFLA-470 "EFFECT OF N-ACETYLCYSTEINE AND FRUCTOSE-1,6-BISPHOSPHATE IN THE TREATMENT OF EXPERIMENTAL SEPSIS", to Inflammation.

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Effect of N-acetylcysteine and Fructose-1,6-bisphosphate in the Treatment of Experimental Sepsis

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ABSTRACT

Sepsis is a syndrome caused by uncontrolled systemic inflammatory response of the individual, which represents a serious epidemiological problem worldwide. The aim of this study was to investigate the effect of two drugs, N-acetylcysteine (NAC), a thiol-compound precursor of glutathione and fructose-1,6-bisphosphate (FBP), a high energy glycolytic metabolite, in the treatment of experimental sepsis . We used male Wistar rats that were divided into five experimental groups (n=6): (i) normal control (not induced), (ii) septic control (induced using a capsule with non sterile fecal content and E. coli (1,5 x 10^9 CFU), (iii) induced and treated with FBP (500 mg/kg IP), (iv) induced and treated with NAC (150 mg/kg IP), (v) induced and treated with the combination of FBP (500 mg/kg IP) with NAC (150 mg/kg IP). In the group treated with NAC, 16.68% of the mice survived, the FBP reduced the mortality of mice during the acute stage of the disease and increased the animals survival time in 33.34%, whereas the combination of drugs had no effect. In attempting to explain the possible outcome and/or mechanism of action, mainly the results of the septic group and treated with NAC, complete blood analysis, biochemical measurements were performed, besides the verification of tissue oxidative stress. Were also performed blood cultures and in the different groups in order to check whether there was induction septic. Our results show that NAC prevented the mortality of animals after septic induction. These data confirm the validity of the use of NAC in the treatment of sepsis, possibly due to its property to be a precursor to glutathione, by their hepatoprotective ability and possible renal protection. Our data also show that the synergistic action with FBP does not improve the picture, and this can be partly explained by heightened activation of the immune (defense) or a decrease of hepatic glutathione.

Keywords: Sepsis, Oxidative Stress, Fructose-1,6-bisphosphate and N-acetylcysteine.

INTRODUCTION

Sepsis is an inflammatory response characterized by significant activation of inflammatory cells as well as by excessive production of inflammatory mediators, resulting in a metabolic imbalance.[1,2] The incidence of sepsis reported in the literature can vary according to each region characteristics. In the USA and Europe, sepsis accounts for 2-11% of intensive care units admissions[3] and it is also a leading cause of death in the USA[4] and Brazil.[5]

Changes in the small vessels size, along with biochemical and physiological blood alterations, affects the microcirculatory homeostasis during the septic shock, being this the main site of alterations.[6,7] These hemodynamic changes are due to the release of bacterial wall components (endotoxins and teichoic acid), which induces a sequence of inflammatory changes, including the activation of effectors cells and the release of several mediators, including cytokines, adhesion molecules, endothelial cells, platelet activating factor and nitric oxide (NO).[8.9] These events induce the local destruction of the endothelium, resulting in an increased capillary permeability and vasodilatation, resulting in tissue edema and contributing to decrease the tissue oxygen supply.[7,10] As a result, there is an anaerobic glycolysis predominance, increasing the lactic acid (lactic acidosis) and the cellular damage.[11]

Another important primary factor is the erythrocyte deformity reduction, which depends on the viscoelastic properties of the cell membrane, cytoplasm viscosity as

well as the body surface area and volume ratio. All these factors could altered due to acidosis, hypothermia and erythrocyte geometry changes.[6] These events lead to a gradual decrease in the circulatory flow, decreasing the oxygenation of the inflammation site[12] and the cellular energy production, which can lead to cellular death.[13]

The univalent reduction of the oxygen is a characteristic way of generating reactive oxygen species (ROS), which may contribute to the formation of other free radicals (FR). The FR are unstable species that react rapidly causing epithelial cells damage, chemotactic factors formation, neutrophil recruitment, oxidation and lipid peroxidation, damage to deoxyribonucleic acid (DNA), release of TNF-α and IL-1 and peroxynitrite formation.[14] The FR are generated in biological oxidation processes and play key functions in the body. The oxygen reduction to water forms free radicals and the superoxide anion is the first free radical formed in this process.[15] However, when FR are produced at rates that exceed the organism antioxidant capacity, a situation of oxidative stress is created, leading to a potential oxidative damage.[16]

Despite therapeutic efforts, sepsis has been considered a serious worldwide epidemiological issue for healthcare systems. New strategies are being tested to modulate the excessive generation or action of many sepsis mediators. The interruption of the inflammatory response event sequence at multiple points is considered to be one of the best ways to reduce the high sepsis mortality rate.[17] One possible strategy is the use of new drugs, such as fructose-1,6-bisphosphate (FBP), which is a high energy glycolytic metabolite and has shown to have therapeutic effects in several pathological conditions such as ischemia, shock and toxic injuries.[18] There is also evidence of FBP beneficial effects in cardiac, kidney, brain, liver and bowel (small intestine) dysfunctions.[19,20] The FBP also increases the potassium cellular uptake, resulting in a decrease of the intracellular sodium concentration and

reducing the cytotoxic cellular edema.[21] FBP also inhibits the ROS formation, the neutrophils activation[22] and reduces the T-lymphocytes proliferation.[23] Indeed, Nunes et al.[24] demonstrated that the use of FBP in animals with experimental sepsis increased the survival rate after the disease.

N-acetylcysteine (NAC) is a thiol-compound that has been therapeutically used due to its ability to be a glutathione precursor,[25] playing a key role in the cellular homeostasis, whereas the glutathione depletion can cause cell death due to lipid peroxidation and decrease in the thiol proteins levels.[26] NAC also has been related to the biological effect intensification when combined to NO, forming S-nitrosothiol, which is considered the most stable form of NO.[27] It is also effective in the treatment of some drug overdose cases, such as paracetamol.[28] NAC can also act in the mitochondrial metabolism influencing the oxidative phosphorylation through two mechanisms: protecting oxidative phosphorylation proteins against oxidative damage by maintaining the thiol groups, that are essential for the enzyme activity, and preventing lipid peroxidation of mitochondrial membranes, which could decrease some complexes activity.[29] The NAC antioxidant capacity, when combined with deferoxamine (DFX) (ferrous chelator), reduces mortality in septic-induced mice.[30]

Thus, the aim of this study was to evaluate the effects of the combined administration of N-acetylcysteine and fructose-1,6-bisphosphate in the treatment of experimental sepsis. We hypothesized to minimize the morbidity and mortality resulting from the septic process.

MATERIAL AND METHODS

Animals

A controlled experimental study was conducted using male Wistar albino rats (Rattus norvegicus), weighing 180 to 220g, obtained from Fundação Estadual de Pesquisa e Produção em Saúde, Porto Alegre, Brazil. The animals were housed in a number of three per cage and a two week adaptation period in the School of Biosciences (PUCRS) vivarium was used before the experiments were performed. Animals were treated in accordance with the ethical principles of the Brazilian College of Animal Experimentation (COBEA). Both diet (Nuvital-Nuvilab®) and water were provided *ad libitum*. The animals were kept on shelves with ventilated cages that provide 60 air cycles per hour, a relative humidity ranging between 55-65%, a 12 hour light-dark cycle and a mean temperature of 22°C ± 2℃. Both cages and drinking bottles were replaced for clean sterile ones every two days.

Experimental Sepsis Induction and Treatment

The animals were weighed and then anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (50 mg/kg) intraperitoneally (IP). All procedures were performed using sterile surgical instruments. Sepsis was induced by introducing in the peritoneal cavity a sterile capsule size "0" containing another sterile capsule size "00" with the *Escherichia coli* (ATCC 25922) suspension and a non-sterile fecal content. The E. coli suspension concentration was measured by spectrophotometry (650 nm), resulting in an OD between 1180 and 1200, which resulted in a total of 1.5 x 10^9 CFU (colony forming units/mL).[31]

The animals were then divided into five groups (six animals per group) as follows: (i) normal control (unhandled), (ii) septic control (induced and untreated), (iii)

induced and treated with FBP (500 mg/kg IP at the time of induction), (iv) induced and treated with NAC (150 mg/kg IP at induction), (v) induced and treated with FBP (500 mg/kg IP at induction) and NAC (150 mg/kg IP at the time of induction). Blood samples were collected through cardiac puncture 12 hours after the sepsis induction.

Experimentation Protocol

A survival curve in the different experimental groups was performed. After seven days, animals that were still alive were anesthetized with an IP solution of ketamine (100 mg/kg) and xylazine (50 mg/kg) and decapitated. Subsequently, the sepsis induction was evaluated trough blood cell analysis, blood cultures and biochemical analysis from animals in the different experimental groups. The kidneys, heart, liver and lungs were also removed, properly processed and stored in a -70ºC freezer until the oxidative stress assay day.

Blood culture

A blood sample of 1mL from each animal was collected to perform the blood culture. The blood collection was performed using a tube filled with a nutrient medium (Becton Dicknson®). After this, the tubes were placed in an oven at 37ºC for a period of 24 hours. The bacterial growth analysis was performed according to the turbidity and pellicle formation in the nutrient medium. The positive blood cultures were sent to a Clinical Diagnostic Center for the microorganism analysis and characterization. Blood cultures were incubated in six days and then transferred from the culture medium to Petry plates divided into "Hembi & Azide" (solid culture medium - Merck®). The biochemical tests for the bacterial identification were also performed. The blood culture analysis aimed at providing accurate information on the presence or absence of microorganisms involved in the sepsis process in the samples.

Hematologic Analysis

Blood samples were collected in tubes filled with anticoagulant (EDTA). After that, a blood cell count was performed (complete hemogram) using an automated equipment (Cell-Dym® 1700) and the differential was manually performed using an optical microscopy (Nikon® E200 Eclypise).

Biochemical Analysis

Biochemical analysis were performed from whole blood samples collected in tubes without anticoagulant, centrifuged (1000G for 5 minutes) after the clot retraction and the serum frozen at -70℃ until analysis. Serum levels of urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, lactate dehydrogenase (LDH), glucose and iron were evaluated using Cobas Mira Plus® automated device.

Oxidative Stress

The oxidative stress analysis was performed by measuring the thiobarbituric acid reactive substances (TBARS)[32], the superoxide dismutase (SOD),[33] catalase (CAT)[34] and reduced glutathione (GSH)[35] levels in the following organs: kidneys, heart , liver and lungs. The organs were properly processed and stored in a -70ºC freezer until the oxidative stress assays.

Statistical Analysis

The results were presented using descriptive statistics. Differences between groups were evaluated using a one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test. The level of significance accepted was $P<0.05$. All analyses were performed using SPSS® 15.0 (SPSS Inc. Ohio, USA).

RESULTS

Septic animals treated with NAC showed a 16.68% survival rate, whilst animals treated with FBP presented a 33.34% increase in the survival time, although there was no survivors. On the other hand, the association of NAC with FBP had no effect in the animal's survival, as shown in Figure 1. Blood cultures were also performed in order to check whether there was proper sepsis induction. Blood cultures were 100% positive in all septic groups and all showed bacterial growth of Escherichia coli in the culture medium. In order to explain the possible mechanism of action, especially regarding the septic and NAC treated groups, hematological, biochemical and tissue oxidative stress were analyzed.

Hematologic Analysis

The hematological analysis of all sepsis-induced groups showed a decrease in the leukocyte levels and an increase of approximately 10% in the number of immature neutrophil evaluated through the differential blood cell count when compared to the control group, as shown in Figures 2 and 3, respectively. These results confirm the induction technique accuracy in all septic groups. Another interesting hematological finding is that FBP preserved the total number of platelets compared to the septic group ($P<0.05$), as shown in Figure 4.

Biochemical Analysis

In order to explain the previous results found in the different experimental groups, serum levels of urea, creatinine, AST, ALT, albumin, lactate dehydrogenase, glucose and iron were measured. To evaluate the possible kidney damage, urea and creatinine serum levels were measured and only the urea levels showed statistical

significance (P<0.001 vs Control), as demonstrated in figures 5 and 6, respectively. When the urea/creatinine ratio was calculated, we observed a significant increase in all septic groups compared to the control group, as shown in Figure 7. However, this finding was not able to explain the possible outcome of the group induced and treated with NAC, because it was not able to reverse this situation.

However, when the liver function (AST and ALT) parameters were evaluated, the NAC treated group presented a reduction in the AST plasma levels (Figure 8) compared to the untreated septic group (P<0.01 vs Sepsis), demonstrating a hepatoprotective function. However, there were no statistical differences in relation to the TGP levels in the different experimental groups (Figure 9).

We have also demonstrated the NAC ability to prevent the formation of tissue damage, evaluated through the LDH analysis (P<0.01 vs Sepsis), as described in Figure 10. When serum glucose levels were analyzed, we observed a significant decrease in all septic groups (P<0.001 vs Control), as shown in Figure 11. This difference was also observed in the iron levels measurement (P<0.001 vs Control), where the septic group treated with FBP presented an important decrease when compared to the untreated septic group (P<0.05 vs Sepsis), as shown in Figure 12.

Oxidative Stress

The tissue oxidative stress was analyzed by the evaluation of the thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) and catalase (CAT) levels in the kidney, heart, liver and lung, as well as the reduced glutathione (GSH) levels in the liver. It was observed that the kidney was the only organ that showed significant changes in the TBARS levels (Figure 13), showing that both septic groups treated with FBP or FBP plus NAC presented an increase in the renal tissue damage when compared to the septic group. Lung, liver and heart showed no significant

changes in the TBARS levels between the different groups (data not shown). In relation to the SOD activity, no significant differences between the groups in the different tissues were demonstrated (data not shown).

However, when both renal and hepatic catalase levels were analyzed, the FBP-treated septic groups, as well as those treated with the combination of NAC and FBP, showed a significant increase in its levels ($P<0.001$ vs Control/Sepsis), compared to the control group (Figure 14 and 15, respectively). In the lung, there was a significant decrease in the catalase levels compared to the septic group ($P<0.05$ vs Sepsis), as shown in Figure 16. No significant differences between groups were observed when the heart was evaluated (data not shown). Figure 17.shows that both groups treated with FBP, as well as those treated with the combination of NAC and FBP, decreased the hepatic levels of glutathione ($P<0.01$ vs Control and $P<0.001$ vs Sepsis).

DISCUSSION

Sepsis has been considered a serious epidemiological problem for healthcare systems worldwide and it is characterized as a complex syndrome resulting from an imbalance between pro and anti inflammatory responses.[36] The body's inflammatory response occurs through the activation of cellular and humoral components, that can cause mitochondrial dysfunction. The mitochondrial damage and the energy deficit seems to be the main stimulus for the modification of mitochondrial proteins.[37] These changes may decrease the heme synthesis and the Fe-S clusters biosynthesis.[38] The formation of these clusters is essential to prevent the accumulation of iron and oxidative stress.[39]

Evidences in the literature have shown that the ROS play an important role in the development of multiple organ failures and septic shock.[40] When the release of ROS occurs into the extracellular matrix, cell injury may involve mechanisms such as degradation of some matrix components (collagen and hyaluronic acid), the cell membrane destruction by lipid peroxidation, organelles membrane disruption, interference in key enzyme systems and the induction of excessive cell oxygen intake.[41]

Treatments that reduce the generation or prevent the effects of ROS have shown beneficial effects in several experimental models of septic shock.[42, 43] NAC has been used in therapeutic interventions as an antioxidant, however, its use alone may have some limitations due to pro-oxidant effects, probably through its interaction with the iron.[44] The biological effects of NAC can be intensified by combining it with NO to form S-nitrosothiol, which can cause mitochondrial complex I inactivation. This complex is an important regulator of the ROS, besides being involved in the mitochondrial permeability.[45]

The results of the present study revealed that the septic animals treated with NAC showed a reduced mortality of 50% in 15 hours and 16.68% of the animals survived, while in the untreated septic group the percentage of mortality reached 100% in the same period. The mortality of the septic animals treated with FBP was reduced to 66.66% in 15 hours, but no animals survived, unlike the study of Nunes et al.,[20] that found 50% of survivors in septic rats when treated with FBP. On the other hand, when animals were treated with the combination of NAC and FBP the mortality was 100% in 15 hours.

Blood cultures were 100% positive for *Escherichia Coli* in all groups, indicating a proper sepsis induction. The hematological analysis in the septic-induced animals showed a decrease in the leukocyte levels and an increase of approximately 10% in

the number of lashes in the differential blood cell count, when compared to the control group, indicating an acute infection. These results also confirm the sepsis induction, as established by the International Sepsis Definitions Conference, [46] and suggest that the animal's survival is not due to the bacterial infection decrease or due to the lymphocyte defenses increase. Another important hematological finding is that FBP prevents the septic-induced platelet decrease, indicating that FBP can act as an important platelet aggregation inhibitor, as previously demonstrated by Oliveira et al.[47] The inhibition of the platelet aggregation can to be very important, since this can improve the tissue perfusion, that is harmed in the septic shock for the formation of clots.[47]

In order explain the survival curve results, creatinine and urea serum levels were measured and a significantly elevated urea level was demonstrated in all septic groups (treated and untreated) when compared to the control group. However, no differences were demonstrated in the creatinine levels between groups and when the urea/creatinine ratio was calculated, a significant increase was observed, characterizing a pre-renal azotemia that could be partly explained by the sepsis-induced hypotension.[6] These results suggests that the increased urea serum levels are due to an increased renal reabsorption and not a consequence of a kidney lesion. It was also observed that the kidney was the only organ that showed significant changes in the TBARS levels, indicating that the treatment of septic animals with FBP and its combination with NAC resulted in a renal tissue damage increase. However, Andrade et al.[48] demonstrated that rats treated with NAC improved the kidney function after ischemic acute renal failure induction and present results suggest that this increased damage is caused by FBP or its association with NAC, but not NAC alone, since no alterations in the TBARS levels were seen in this group.

When the liver lesion parameters (AST and ALT) were analyzed, only the NAC-treated group presented a reduction in the AST plasma levels compared to the septic group, demonstrating its hepatoprotective role. This protective ability has been previously described in the literature, using carbon tetrachloride (CCl_4)-induced cirrhotic animals, suggesting a protective effect related to the glutathione peroxidase, an endogenous antioxidant enzyme key in combating the ROS.[49] To verify that NAC would increase the cellular antioxidant defenses, we have also measured hepatic reduced glutathione levels. Our results showed that the reduced glutathione levels have not increase by treatment with NAC when compared the septic group, discarding this theory. Also was observed that the FBP, with as well as the association with NAC, reduced the expression the of glutathione tissue levels, what in part it could explain the evil prognostic synergic action of the drugs. No significant differences between the experimental groups were demonstrated for the ALT levels. Another important finding is the NAC ability to prevent tissue damage observed through the reduction of serum LDH, which could be partially explained by the NAC protective effect against tissue oxidative damage.

We have also showed that the iron serum levels significantly decreased in all septic groups when compared to the control, although the induced group treated with FBP presented a larger decrease when compared to the septic group. However, the relationship between iron and immunity is controversial. While some authors claim that iron deficiency predisposes to infection, others suggest that excessive iron may increase the risk of infections and their severity, because microorganisms need iron for the development of vital functions such as DNA synthesis and transport of eletrons.[50,51] According to Kent et al.,[52] chronic disease anemia represents a body's defense against the proliferation of microorganisms and neoplastic cells and may be involved, along with fever, as a complementary strategy that the body uses to protect

from disease, suggesting that the low level of plasma iron inhibits bacterial growth. Thus, the abnormalities in the iron metabolism may represent an evolved mechanism of host defense against bacterial invasion (nutritional immunity), in order that the accumulation or excessive iron is extremely harmful to the tissues, since the free iron promotes the synthesis of ROS, which are toxic and damage proteins, lipids and DNA.[53, 54] our results show that reduction of iron by FBP could be involved in its protective mechanism and that would act independently of the metabolism of iron ion.

In spite of the fact that hyperglycemia is commonly involved in the sepsis process,[55] a hypoglycemia in the septic groups was observed when compared to the control animals, which could be partially explained by the oxygen supply decrease and the bacterial use of glucose as an energy source during the time course measured in our experiments.[12,13]

In order to evaluate the antioxidant capacity, the levels of CAT and SOD, that are responsible for the organism endogenous defenses, have been determined. Several studies have demonstrated the relationship between extracellular SOD and neutrophils recruitment reduction,[56,57] as well as reduction in the expression of adhesion molecules. A mechanism by which extracellular SOD could modulate the neutrophils inflammation would be the reduction of the cytokine production by macrophages,[56] suggesting that the extracellular SOD could be considered as an anti-inflammatory and antioxidant enzyme. Our results have shown no changes in the SOD levels. However, Ritter et al.[58] showed an increased SOD activity in septic rats treated with NAC associated to DFX, suggesting that the protective effect of NAC plus DFX could be secondary to its effect on the SOD activity. In addition, it demonstrates the limitations of treating the NAC due to its pro-oxidant effect.

When both renal and liver CAT levels were evaluated, the FBP-treated septic groups, as well as the NAC and FBP combination, a significant increase compared to

control group was demonstrated. This exacerbation in the CAT levels could be a consequence of the renal damage evidenced by the TBARS increase. On the other hand, there was a significant CAT level decrease in the animals treated with both NAC and FBP when compared to the septic group in the lung. Ritter et al.[58] also showed a decrease in the pulmonary CAT levels in septic rats treated with NAC associated to DFX, confirming this finding.

Taken together, our results demonstrate that NAC prevented the mortality in septic-induced animals. These data confirm an important role for NAC in the treatment of sepsis, suggesting its ability as a glutathione precursor, as a hepatoprotective agent and possible renal protection. Our data also show that the combination of NAC and FBP does not improve the animal survival, which could be partially related to the high activation of the immune defense and a hepatic glutathione decrease. Further studies will be necessary to address some of these issues.

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Figure legends

Figure 1: Survival Curve. NAC treatment prolongs the survival of rats with sepsis-induced (Kaplan-Meier survival curve). FBP: sepsis treated with FBP; NAC: sepsis treated with NAC.

Figure 2: Leukocyte absolute values. The results were evaluated by an automated cell counter, expressed in mean ± SEM and performed in triplicate. * Indicates significant difference compared to the control ($P<0.01$). ** Indicates significant differences compared to the control ($P<0.001$).

Figure 3: Hematologic analysis. The results were manually evaluated in an optical microscopy, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control ($P<0.01$).

Figure 4: Platelet absolute values. The results were evaluated by an automated cell counter, expressed in mean ± SEM and performed in triplicate. * Indicates significant difference compared to the control ($P<0.05$). # Indicates significant difference compared to the sepsis group ($P<0.05$).

Figure 5: Urea serum levels. The results were evaluated by an automated device expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control ($P<0.001$).

Figure 6: Creatinine serum levels. The results were evaluated by an automated device expressed in mean ± SEM and performed in triplicate. No significant differences were demonstrated.

Figure 7: Urea/Creatinine serum levels ratio. The results were evaluated by an automated device expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control ($P<0.001$).

Figure 8: AST serum levels. The results were evaluated by an automated device expressed in mean ± SEM and performed in triplicate. * Shows significant difference compared to the control ($P<0.001$). ** Indicates significant difference compared to the control ($P<0.05$). *** Indicates significant difference compared to the control ($P<0.01$). # Indicates significant difference compared to the sepsis group ($P<0.01$).

Figure 9: ALT serum levels. The results were evaluated by an automated device. expressed in mean ± SEM and performed in triplicate. No significant differences were demonstrated.

Figure 10: LDH serum levels. The results were evaluated by an automated device. expressed in mean ± SEM and performed in triplicate. * Indicates significant difference compared to the control ($P<0.001$). ** Indicates significant difference compared to the control ($P<0.01$). *** Indicates significant difference compared to the control ($P<0.05$). # Indicates significant difference compared to the sepsis group ($P<0.01$).

Figure 11: Glucose serum levels. The results were evaluated by an automated device, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control ($P<0.001$).

Figure 12: Iron serum levels. The results were evaluated by an automated device, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control ($P<0.001$). # Indicates significant differences compared to the sepsis group ($P<0.05$).

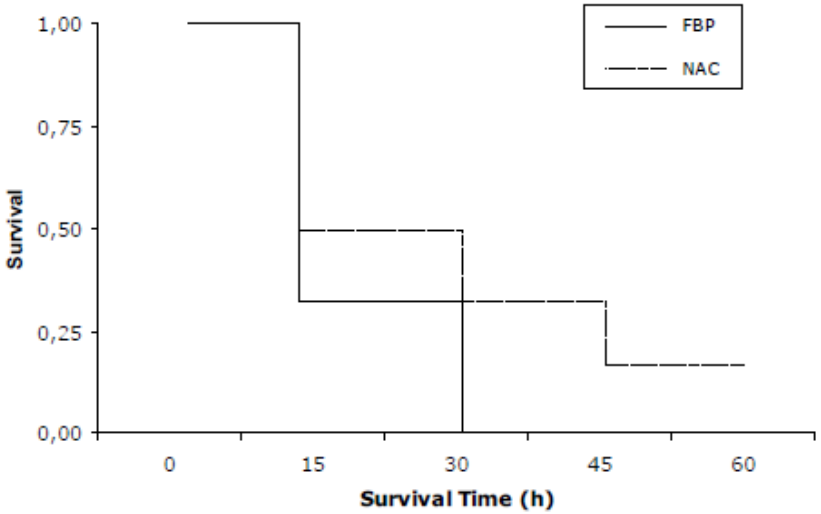
Figure 13: Renal thiobarbituric acid reactive substances. The results were evaluated by spectrophotometry, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control group ($P<0.01$). # Indicates significant differences compared to the sepsis group ($P<0.01$).

Figure 14: Renal catalase levels. The results were evaluated by spectrophotometry, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control group ($P<0.001$). # Indicates significant differences compared to the control sepsis group ($P<0.001$).

Figure 15: Liver catalase levels. The results were evaluated by spectrophotometry, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control and control group ($P<0.001$). # Indicates significant differences compared to the control and sepsis group ($P<0.001$).

Figure 16: Pulmonary catalase levels. The results were evaluated by spectrophotometry, expressed in mean ± SEM and performed in triplicate. # Indicates significant difference compared to the sepsis group ($P<0.05$).

Figure 17: Liver glutathione levels. The results were evaluated by spectrophotometry, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control ($P<0.01$). # Indicates significant differences compared to the sepsis group ($P<0.001$).



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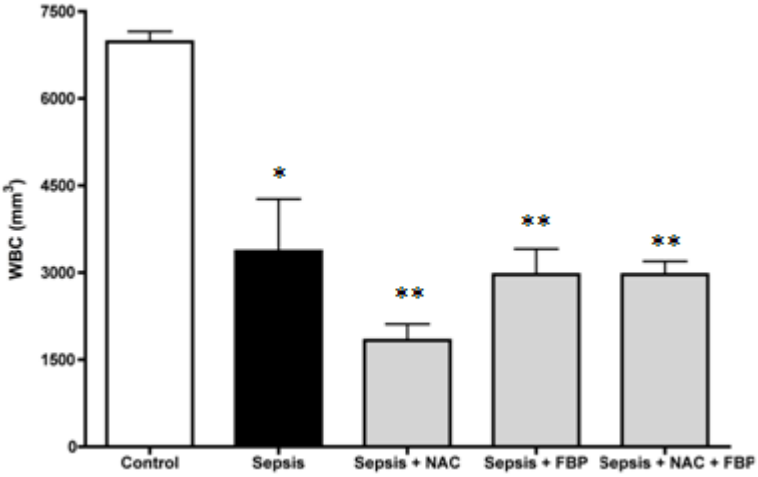

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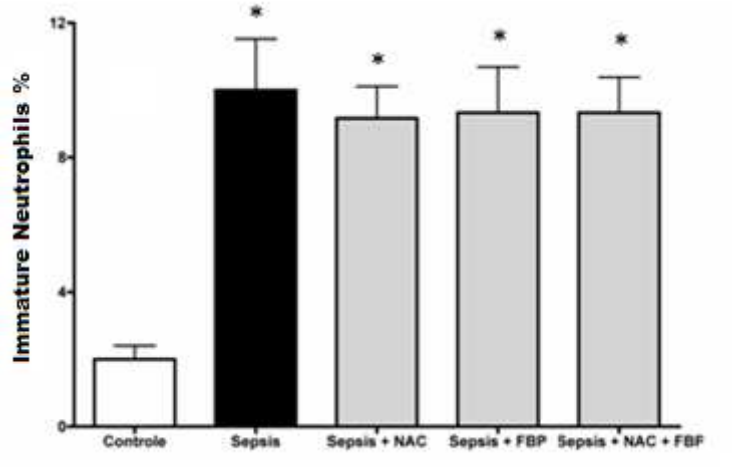

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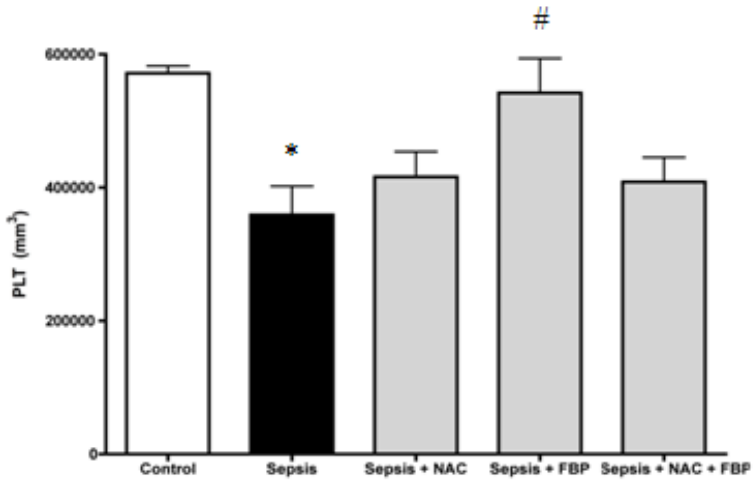

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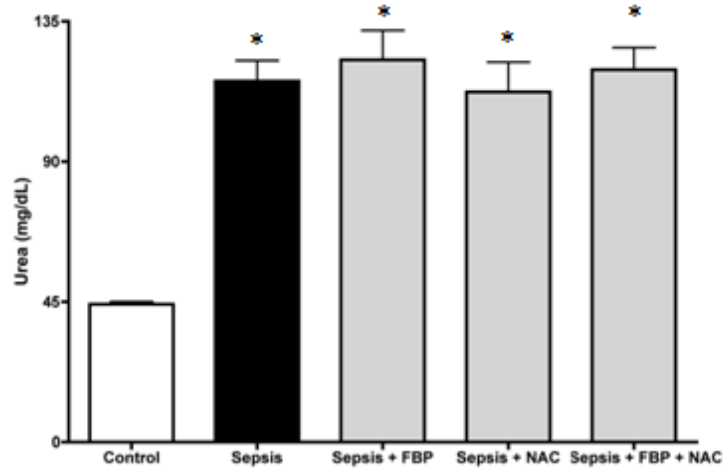


Figure 5: Urea serum levels. The results were evaluated by an automated device expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control (P<0.001).

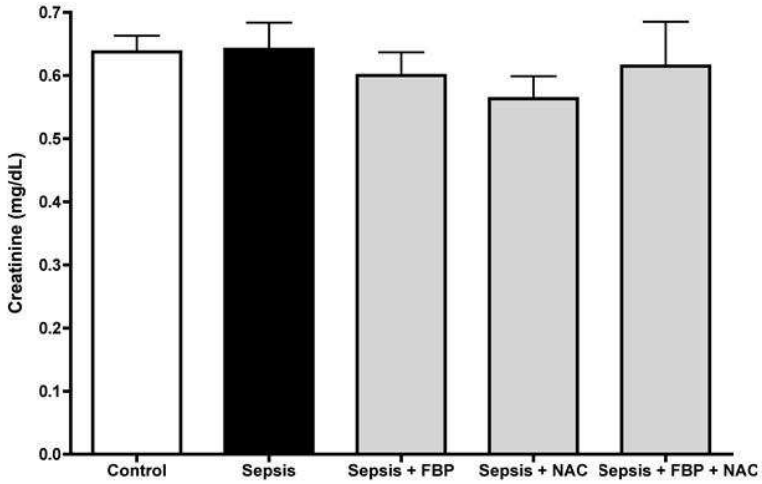
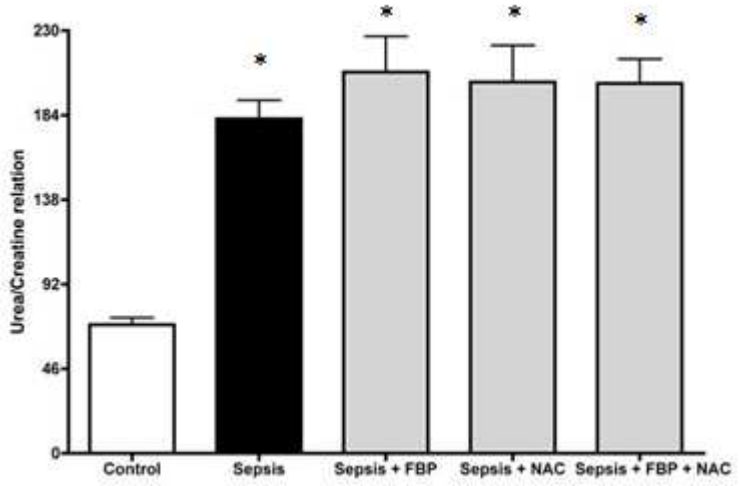


Figure 6: Creatinine serum levels. The results were evaluated by an automated device expressed in mean ± SEM and performed in triplicate. No significant differences were demonstrated.



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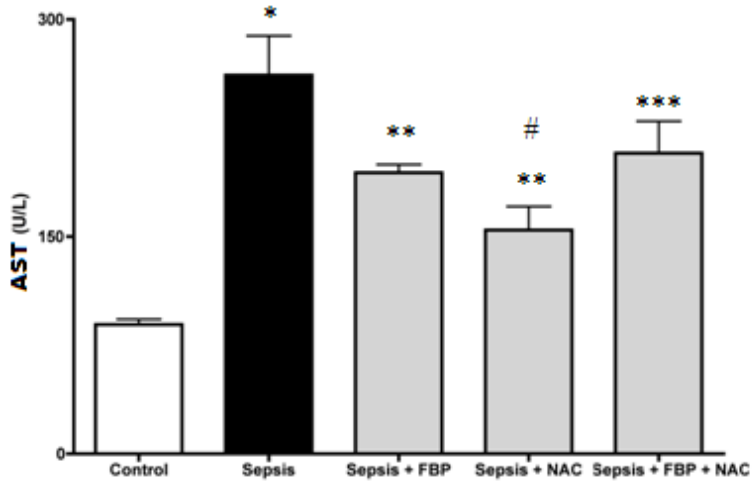

Figure 8: AST serum levels. The results were evaluated by an automated device expressed in mean ± SEM and performed in triplicate. * Shows significant difference compared to the control (P<0.001). ** Indicates significant difference compared to the control (P<0.05). *** Indicates significant difference compared to the control (P<0.01). # Indicates significant difference compared to the sepsis group (P<0.01).

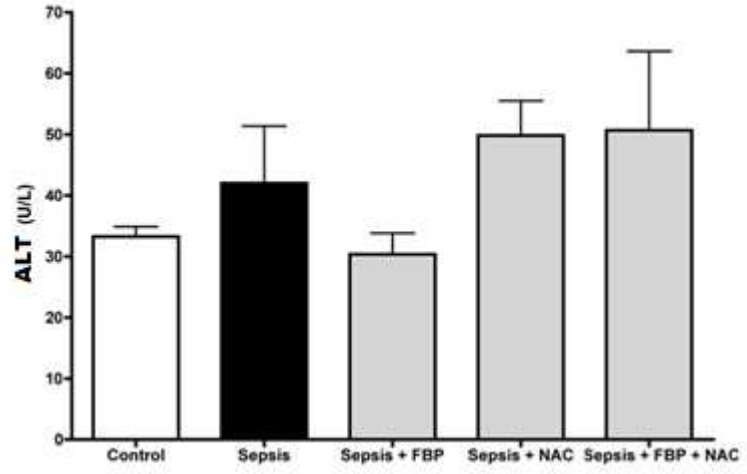

Figure 9: ALT serum levels. The results were evaluated by an automated device. expressed in mean ± SEM and performed in triplicate. No significant differences were demonstrated.

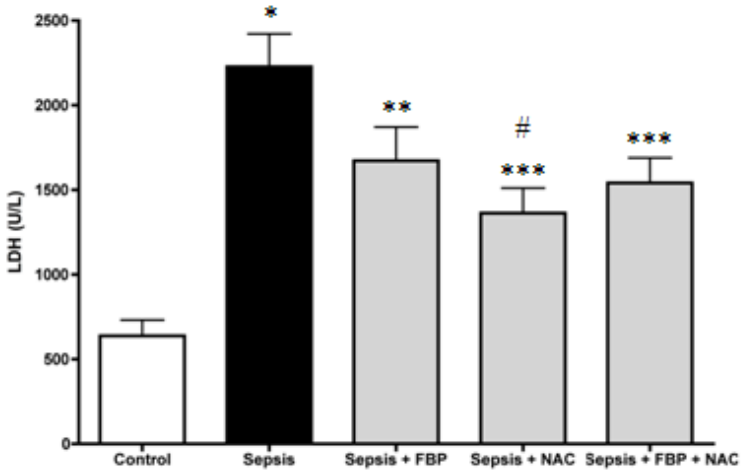

Figure 10: LDH serum levels. The results were evaluated by an automated device. expressed in mean ± SEM and performed in triplicate. * Indicates significant difference compared to the control ($P<0.001$). ** Indicates significant difference compared to the control ($P<0.01$). *** Indicates significant difference compared to the control ($P<0.05$). # Indicates significant difference compared to the sepsis group ($P<0.01$).

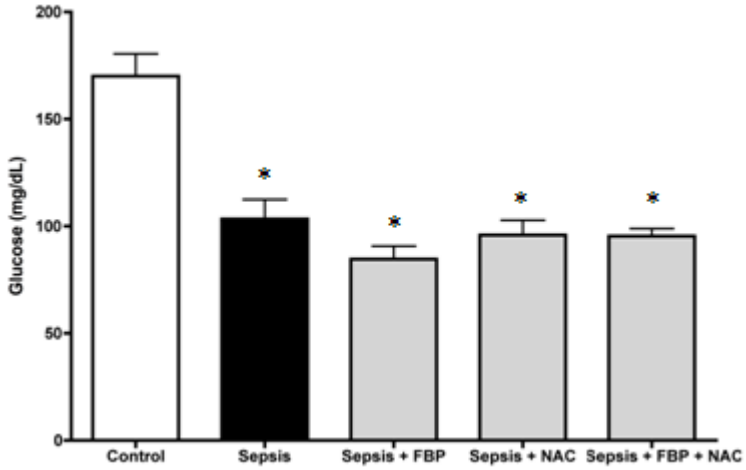

Figure 11: Glucose serum levels. The results were evaluated by an automated device, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control (P<0.001).

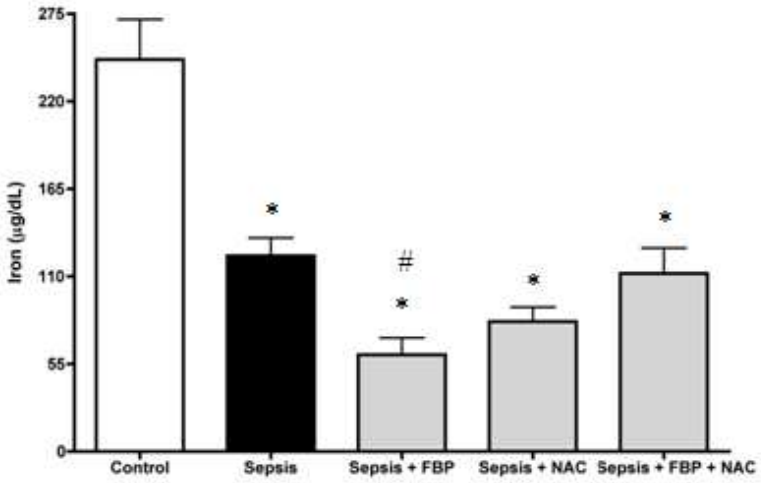

Figure 12: Iron serum levels. The results were evaluated by an automated device, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control (P<0.001). # Indicates significant differences compared to the sepsis group (P<0.05).

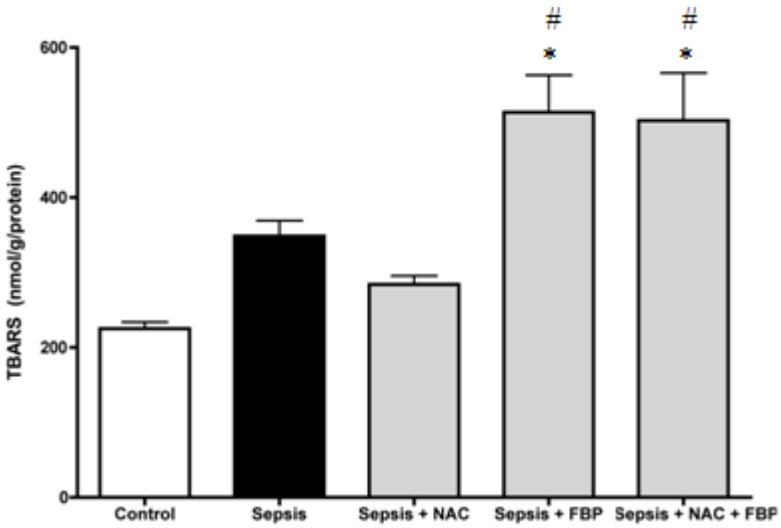

Figure 13: Renal thiobarbituric acid reactive substances. The results were evaluated by spectrophotometry, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control group (P<0.01). # Indicates significant differences compared to the sepsis group (P<0.01).

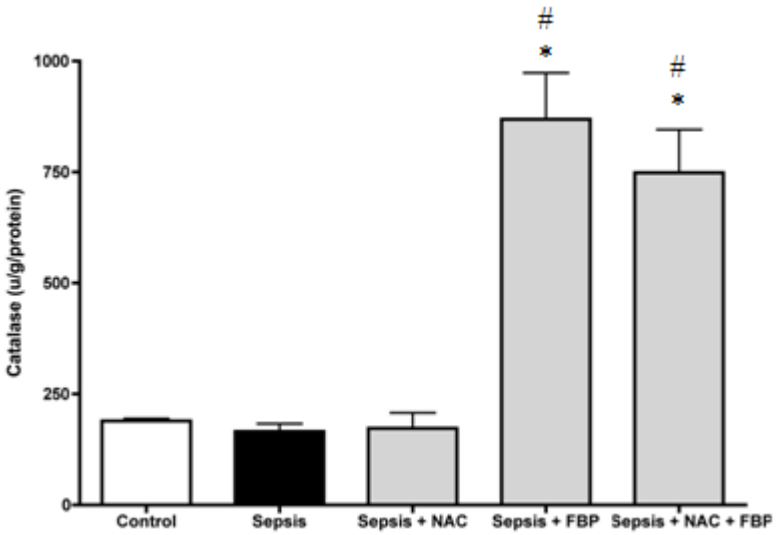

Figure 14: Renal catalase levels. The results were evaluated by spectrophotometry, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control group (P<0.001). # Indicates significant differences compared to the control sepsis group (P<0.001).

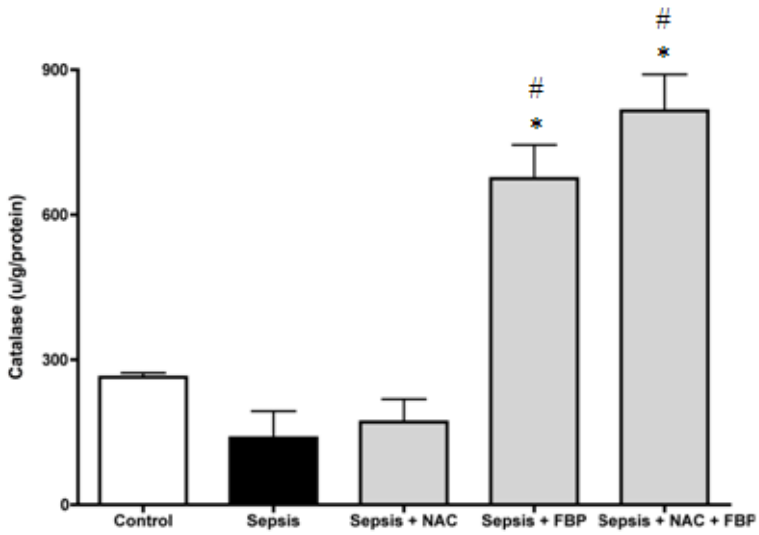

Figure 15: Liver catalase levels. The results were evaluated by spectrophotometry, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control and control group (P<0.001). # Indicates significant differences compared to the control and sepsis group (P<0.001).

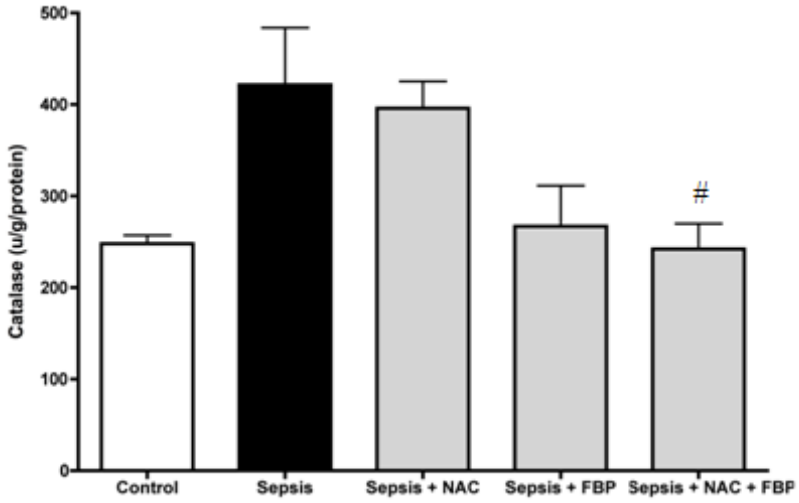

Figure 16: Pulmonary catalase levels. The results were evaluated by spectrophotometry, expressed in mean ± SEM and performed in triplicate. # Indicates significant difference compared to the sepsis group (P<0.05).

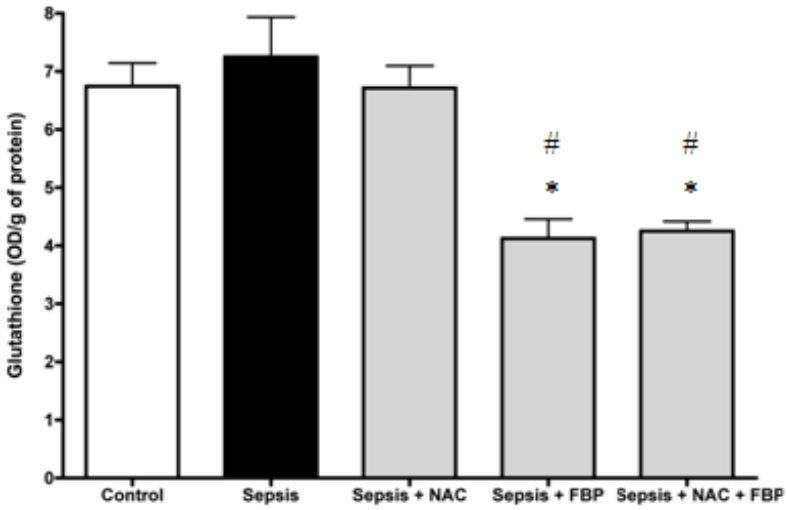

Figure 17: Liver glutathione levels. The results were evaluated by spectrophotometry, expressed in mean ± SEM and performed in triplicate. * Indicates significant differences compared to the control ($P<0.01$). # Indicates significant differences compared to the sepsis group ($P<0.001$ vs. Sepsis).

4. CAPÍTULO 4

4.1 CONSIDERAÇÕES FINAIS

A sepse tem representado um grave problema epidemiológico para os sistemas de saúde em todo o mundo, sendo caracterizada como uma síndrome complexa secundária ao desequilíbrio entre respostas pró e anti-inflamatórias.[54] A resposta do organismo se dá através da ativação de componentes celulares e humorais. Esta ativação por sua vez, pode causar deficiência orgânica mitocondrial. O dano mitocondrial e o déficit de energia parecem ser o principal estimulo para a modificação de proteínas mitocondriais.[55] Estas alterações podem diminuir a síntese do heme e a biossíntese dos clusters Fe-S.[56] A formação desses clusters é essencial para prevenção do acumulo de ferro e do estresse oxidativo.[57]

Evidências na literatura têm demonstrado que espécies reativas de oxigênio (EROs) desempenham um papel importante no desenvolvimento de falências múltiplos órgãos e choque séptico.[58] Quando ocorre a liberação de EROs dentro da matriz extracelular, a lesão celular pode envolver mecanismos como a degradação de alguns componentes da matriz (ácido hialurônico e o colágeno), a destruição das membranas celulares pela peroxidação lipídica, o rompimento das membranas das organelas, a interferência em importantes sistemas enzimáticos, e promoção da entrada excessiva de oxigênio na célula.[59]

Tratamentos que reduzem a geração ou previnem os efeitos das EROs, tem mostrado efeitos benéficos através de uma variedade de modelos de choque séptico.[60,61] A NAC tem sido usado na intervenção terapêutica como um antioxidante. Porém, seu uso isolado pode ter algumas limitações devido a seu a efeito pró-oxidante, provavelmente pela sua interação com o ferro.[62] A NAC também,

tem sido relacionada com à intensificação dos efeitos biológicos por combinação do NO, formando o S-nitrosotiol, este ocasiona a inativação complexo I mitocondrial, que por sua vez é um importante regulador de EROs, além de estar envolvido na permeabilidade mitocondrial.[63]

Primeiramente, investigamos *in vitro* o possível efeito da NAC isolada ou associada à FBP, já com conhecido efeito imunomodulador,[43] sobre a proliferação de Linfócitos T, com o intuito de se obter drogas alternativas para o tratamento do choque séptico. Além disso, tentamos explicar o possível mecanismo imunomodulatório, através da mensuração dos níveis citocinas pró-inflamatórias, como a IL-1β, IL-6 e MCP-1, tendo em vista que as mesmas estão envolvidas no choque séptico.

Nossos resultados demonstram que a NAC isolada nas concentrações de 10 e 15 mM e na concentração de 15 mM associada à FBP na concentração de 1,25 mM, possuem um importante efeito imunomodulatório. Entretanto Hadzic *et al.*,[64] através da inibição da proliferação linfocitária induzida por bloqueio da glutationa (GSH), relataram que a NAC aumenta a proliferação dos linfócitos T, propondo que os grupamentos tiois seriam um regulador da proliferação de linfócitos T. Já a concentração de NAC 1 mM associada à FBP reduz significativamente a viabilidade celular, sugerindo que esta associação pode ser tóxica para as células. O aumento nas concentrações de NAC isolada ou associada à FBP está associado com uma proporcional redução na proliferação de linfócitos T.

Existem diversos relatos na literatura, demonstrando a correlação existente entre o aumento da produção de citocinas pró-inflamatórias e o índice de mortalidade na sepse, tanto em humanos como em modelos experimentais. Dentre estas citocinas destacam-se a IL-1 e a IL-6. Portanto, as terapias clínicas atuais

estão avaliando a segurança e eficácia do uso de anti-citocinas isoladas ou em conjunto.[65]

A IL-1 é formada por duas moléculas diferentes; IL-1α e IL-1β. A IL-1 produz aumento na concentração de fatores estimuladores de colônia, de IL-6, MCP-1, aumento das proteínas de fase aguda hepática, reabsorção óssea, inibição da lipoproteína-lipase e indução da síntese do colágeno.[66,67] O mecanismo de inibição natural dessa citocina envolve o bloqueio da ligação no receptor por antagonistas de receptores de citocinas, como o IL-1Ra, antagonista do receptor de IL-1. A IL-1Ra é uma proteína da família das interleucinas originalmente descrita como uma molécula secretada por monócitos e macrófagos, que modula uma variedade de respostas imunes e inflamatórias relacionadas a IL-1.[68,69] A participação da IL-1, na fisiopatologia da sepse, foi bem demonstrada pela observação deste antagonista (IL-1Ra) na qual, reduz a letalidade causada pela administração de endotoxina[70] ou *E. Coli*.[71] No nosso estudo, verificamos um aumento nos níveis de IL-1β, tanto com NAC isolada quanto associada à FBP com ou sem a estimulação com PHA, sugerindo que o efeito protetor da NAC e/ou sua associação com a FBP, seja devido ao seu poder imunomodulador e não ao seu efeito antiinflamatório. Porém, relatos na literatura têm mostrado uma diminuição dos níveis de IL-1β quando tratados com a NAC.[72,73]

A IL-6 faz parte dos processos da imunidade inata e adquirida, produzida em resposta ao TNF, a IL-1, e a algumas células T ativadas. Na resposta inata a IL-6 estimula a síntese de proteínas hepáticas, como a proteína amilóide A e o fibrinogênio, que constituem parte da resposta de fase aguda da inflamação sistêmica. Na imunidade adquirida, a IL-6 estimula o desenvolvimento dos linfócitos B produtores de anticorpos. Isto pode ser observado nos plasmócitos neoplásicos dos mielomas e nas células neoplásicas produtoras de anticorpos monoclonais,

denominadas hibridomas.[74,75] Apesar de não estar clara a relevância de seus efeitos na sepse, essa citocina é a que apresenta melhor correlação com a mortalidade em modelos experimentais e em pacientes com sepse. Isto é, quanto maior os níveis plasmáticos de IL-6, maior a probabilidade de o paciente chegar a óbito.[76,77] Neste estudo, verificamos uma redução nos níveis de IL-6 quando comparados ao grupo controle, tanto no grupo séptico tratado com a NAC isolada quanto associada a FBP, sem a estimulação com PHA. Este resultado também demonstra que a ação imunomodulatório da NAC sobre as células tratadas com PHA não envolve a produção de IL-6.

O MCP-1 tem a capacidade de atrair monócitos da circulação para se transformarem em macrófagos no tecido adiposo. Estes macrófagos são fontes de citocinas com atividades inflamatórias. Pré-adipócitos e adipócitos produzem MCP-1 (além de outros produtos) em resposta a estímulos como óxido nítrico (NO) e TNF-α, IL-1β, IL-4 e Interferon-γ (IFN-γ).[78] Em nosso trabalho, apesar do aumento da IL-1β, verificou-se uma redução nos níveis de MCP-1 quando usado a NAC associada à FBP sem a estimulação com PHA, sugerindo que este efeito provavelmente seja devido a FBP, já que a NAC isolada não demonstrou diferença significativa. Entretanto, Maruno *et al.,*[79] relataram uma diminuição nos níveis de MCP-1, quando tratados com NAC, após indução do fator de crescimento endotelial vascular, mostrando que a NAC isoladamente pode ter uma ação sobre a produção desta citocina.

Nossos resultados sugerem que tanto a NAC isolada como sua associação com a FBP pode inibir a proliferação celular, agindo como potentes agentes imunomoduladores, sugerindo o uso destas contra processos inflamatórios, incluindo a sepse. Quando avaliamos a produção de IL-1β, IL-6 e MCP-1, concluímos que as mesmas não explicam a ação imunomoduladora em culturas celulares de linfócitos T

em presença de PHA, pois não apresentaram diminuição da sua produção no grupo tratado com NAC e FBP. Entretanto, a NAC e a sua associação com a FBP, quando testadas em culturas celulares de linfócitos T sem a presença de PHA, reduziram a produção dos níveis de IL-6 e MCP-1, sugerindo que as mesmas de alguma forma, modulam a síntese destas interleucinas, e que esta ação pode estar envolvida com seu efeito imunomodulador. Portanto, resolvemos verificar o efeito terapêutico dessas duas drogas em sinergismo, no tratamento da sepse experimental (*in vivo*) com o intuito de minimizar a morbidade e a mortalidade decorrente do processo séptico.

Os resultados encontrados neste trabalho revelaram que os animais sépticos tratados com a NAC aumentaram o tempo de sobrevida em 50% a partir de 15 horas após indução séptica, sendo que 16,68% sobreviveram. No grupo séptico não tratado o percentual de mortalidade atingiu 100%, 15 horas após a indução séptica. A mortalidade dos animais sépticos tratados com a FBP foi reduzida para 66,66% em 15 horas, porém não houve sobreviventes, como descrito no trabalho de Nunes *et al.,*[80] que obteve um percentual de sobrevivência de 50%. Esta discrepância pode ser decorrente das cepas bacterianas usadas nos dois trabalhos, tendo em vista que a Nunes *et al.,* utilizaram uma cepa selvagem. Já, quando tratados com a associação da NAC com a FBP, a mortalidade foi de 100% em 15 horas.

As hemoculturas foram 100% positivas para *Escherichia coli* em todos os grupos, mostrando que houve indução séptica. Às análises hematológicas dos grupos sépticos apresentaram uma diminuição dos níveis leucocitários, com um aumento de aproximadamente 10% em relação ao número de células bastonadas no diferencial hematológico, quando comparados com grupo controle, caracterizando uma infecção aguda (desvio à esquerda). Estes resultados confirmam a indução séptica, conforme estabelecido pela *International Sepsis Definitions Conference*,[6]

além de sugerir que a sobrevida dos animais não é decorrente da diminuição da infecção bacteriana, nem do aumento das defesas linfocitárias. Outro achado hematológico importante se deve ao fato da FBP preservar o número total das plaquetas quando comparado ao grupo séptico, o que reforça a tese da FBP agir como importante inibidor da agregação plaquetária, conforme demonstrado no trabalho de Oliveira *et al.,*[81] Esta inibição da agregação plaquetária pode ser muito importante, já que esta pode melhorar a perfusão tecidual, que está prejudicada no choque séptico pela formação de trombos.[81]

Na tentativa de se explicar os resultados da curva de sobrevida, foram mensurados os níveis séricos de uréia e creatinina, aonde observamos que os animais induzidos septicamente apresentaram níveis uréicos significativamente elevados em relação ao grupo controle ($P<0.001$) e que os tratamentos não revertem esta tendência. Entretanto, os níveis de creatinina não apresentaram diferença estatística, e quando calculada a relação de uréia/creatinina, observou-se relações significativamente mais elevadas, caracterizando este quadro como azotemia pré-renal, em parte explicada pela hipotensão ocasionada pela sepse.[18] Estes resultados sugerem que o aumento dos níveis séricos de uréia sejam decorrente do aumento da reabsorção renal e não propriamente dito decorrente de uma lesão renal, além de não explicar o mecanismo de ação das drogas testadas. Observou-se também, que o rim foi o único órgão que apresentou um aumento significativo nos níveis do TBARS, demonstrando que os grupos sépticos e tratados com FBP e a sua associação com NAC, ocasionam um aumento do dano tecidual renal ($P<0.01$ vs Control/Sepsis). Porém, Andrade *et al*.,[82] demonstraram que ratos tratados com NAC, melhoram a função renal após a indução de insuficiência renal aguda isquêmica. Nossos resultados sugerem que este aumento no dano seja

decorrente da FBP ou de sua associação com a NAC e não da NAC isolada, tendo em vista que a mesma não alterou os níveis de TBARS.

Quando analisados os parâmetros de lesão hepática (TGO e TGP), somente o grupo tratado com NAC, apresentou níveis plasmáticos reduzidos de TGO frente ao grupo séptico ($P<0.01$), demonstrando função hepatoprotetora da mesma. Esta capacidade protetora já foi descrita na literatura através da indução cirrótica por inalação de tetracloreto de carbono (CCl_4), onde supostamente esta proteção se deva a glutationa peroxidase, uma enzima antioxidante endógena chave no combate das EROs.[83] Para verificar se a NAC aumentaria o poder antioxidante, foram dosados os níveis de glutationa reduzida hepática, e verificamos que a NAC não aumentou os níveis teciduais de glutationa reduzida, quando comparados ao grupo séptico, descartando esta teoria. Observou-se também que a FBP, assim como sua associação com NAC, reduziram a expressão da glutationa reduzida tecidual, o que em parte poderia explicar o mal prognóstico ação sinérgica das drogas. Quando mensurados os níveis de TGP, estes não apresentaram diferença significativa em relação aos diferentes grupos experimentais. Outro achado importante é a capacidade da NAC impedir o dano tecidual, observado através da análise sérica da LDH ($P<0.01$ vs Sepsis), que em parte pode ser explicado pela capacidade da NAC, proteger os tecidos contra o dano oxidativo.

Também observamos que os níveis séricos de ferro apresentaram uma diminuição significativa em todos os grupos sépticos ($P<0.001$), quando comparados com o controle, porém o grupo induzido e tratado com FBP apresentou uma diminuição significativa dos níveis de ferro quando comparado ao grupo séptico ($P<0.05$). Nossos resultados mostram que a diminuição de ferro pela FBP poderia estar envolvida no seu mecanismo protetor, e que a NAC agiria independente do metabolismo do ferro. Entretanto é controversa a relação entre o ferro e a

imunidade. Enquanto alguns autores afirmam que as deficiências de ferro predispõem às infecções, outros sugerem que o excesso de ferro pode aumentar o risco de infecções e também sua gravidade, pois os microrganismos necessitam de ferro para o desenvolvimento das funções vitais como síntese de DNA e transporte de elétrons.[84,85] Segundo Kent *et al.*,[86] a anemia de doença crônica representa uma defesa do organismo contra a proliferação de microrganismos e de células neoplásicas e pode estar envolvida, juntamente com a febre, como estratégia complementar que o organismo emprega para se proteger da doença, sugerindo que o baixo nível de ferro plasmático inibe o crescimento bacteriano. Desta forma, as anormalidades no metabolismo de ferro podem representar um mecanismo evoluído de defesa do hospedeiro contra a invasão bacteriana (imunidade nutricional), tendo em vista que o acúmulo ou excesso de ferro é extremamente nocivo para os tecidos, uma vez que o ferro livre promove a síntese de EROs, que são tóxicas e lesam proteínas, lipídeos e DNA.[87,88]

Apesar de a hiperglicemia ser uma característica envolvida nos processo séptico,[89] nós observamos que os grupos sépticos apresentaram uma hipoglicemia quando comparada ao controle, sendo esta em parte explicada, pela diminuição do aporte de oxigênio além da utilização da glicose como fonte de energia pelas bactérias.[90,91] Portanto, estes resultados podem ser devidos ao tempo de indução séptica (aproximadamente 15 horas), ou seja, este quadro pode ser tão agudo, que a diminuição dos níveis glicêmicos poderia ser decorrente da utilização da glicose como fonte de energia e não decorrente da resistência insulínica provocada pelo quadro inflamatório.

Na tentativa de verificar capacidade antioxidante tecidual, foram verificados os níveis das enzimas catalase (CAT) e superóxido dismutase (SOD), que por sua vez são responsáveis pelas defesas endógenas do organismo. Vários estudos têm

demonstrado a relação da SOD extracelular com diminuição do recrutamento de neutrófilos.[92,93] A SOD extracelular reduz também a expressão das moléculas de adesão. Um mecanismo pelo qual a SOD extracelular poderia modular inflamação dos neutrófilos se deve pela redução da produção de citocinas pelos macrófagos,[92] sugerindo que a SOD extracelular pode ser considerada como uma enzima anti-inflamatória, além de antioxidante. Neste estudo não se observou alteração dos níveis de SOD nos grupos experimentais, quando comparados ao grupo controle. Entretanto, Ritter *et al.*,[94] demonstraram um aumento na atividade da SOD de ratos sépticos quando tratados com NAC associado a DFX, sugerindo que o efeito protetor de NAC associada a DFX poderiam ser secundários ao seu efeito sobre a atividade da SOD, além de comprovar as limitações do tratamento com NAC devido somente a seu a efeito antioxidante.

Quando determinado os níveis CAT, tanto renal quanto hepático, os grupos sépticos tratados com FBP, assim como, os tratados com a associação de NAC com FBP, apresentaram um aumento significativo da atividade da catalase ($P<0.001$), frente ao grupo controle e séptico. Esta exacerbação dos níveis de CAT pode ser considerada como um reflexo do dano renal avaliado pelo aumento do TBARS. Já no pulmão houve uma diminuição significativa dos níveis da catalase no grupo da NAC associado à FBP quando comparado ao grupo séptico ($P<0.05$). Ritter *et al.*,[94] também demonstrou uma diminuição dos níveis de CAT pulmonar de ratos sépticos quando tratados com NAC associado a DFX, confirmando este achado.

Nossos resultados demonstram que a NAC impediu a mortalidade dos animais após a indução séptica. Estes dados comprovam a validade do uso da NAC no tratamento da sepse, sugerindo que isso seja devido a sua propriedade de ser um precursor da glutationa, pela sua capacidade hepatoprotetora e possível proteção renal. Nossos dados também demonstram que a ação sinérgica da NAC

com a FBP não melhora o quadro de sobrevida dos animais, esta em parte explicada pelo aumento do TBARS renal e pela diminuição da glutationa hepática.

5. CONCLUSÃO

Nossos resultados sugerem que tanto a NAC como a sua associação com a FBP podem inibir a proliferação celular, quando estimulados com PHA, agindo como potentes agentes imunomoduladores, sugerindo o uso destas contra processos inflamatórios, incluindo a sepse.

A NAC reduziu significativamente a mortalidade dos animais após a indução séptica, comprovando a validade do uso da NAC no tratamento da sepse, em parte explicada por sua propriedade precursora da glutationa, pela sua capacidade hepatoprotetora e possível proteção renal.

Nossos dados demonstraram que a ação sinérgica com FBP não melhora o quadro, excluindo o uso da combinação da NAC com a FBP no tratamento da sepse.

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