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IMPACTO DO ESTRESSE OXIDATIVO EM DIFERENTES EVENTOS ENVOLVIDOS NO TRANSPLANTE PULMONAR EM RATOS

Tese de Doutorado

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Porto Alegre, 2005.

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Há duas formas para se viver na vida.
Uma é acreditar que não existe milagre.
A outra é acreditar que todas as coisas são um milagre.
Albert Einstein (1879-1955)

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LISTA DE SÍMBOLOS E ABREVIATURAS

ABAP	2-2´- azo-bis(2-aminopropano)
ATP	adenosina tri-fosfato
ANOVA	Análise de variância
AP-1	Ativador de proteína-1
CAT	Catalase
CL	Quimiluminescência
CO_2	dióxido de carbono
cps	contagem por segundo
Cu	cobre
^0C	grau celsius
cm :	centímetro
DNA	ácido desoxirribonucléico
EC	Euro-Collins
EPM	Erro padrão da média
EAO	Espécies ativas de oxigênio
Fe	ferro
g	gramas
g	gravidade
GC:	glicocorticóide
GPX	glutationa
GSH	glutationa reduzida
Hb	hemoglobina
HCl	Ácido clorídrico
H_2O_2	peróxido de hidrogênio
$HO^{\cdot}$	*r*adical hidroxila
IL	interleucina
i.p.	intraperitoneal
I-R	isquemia-reperfusão
i.v.	intravenoso

KCl	cloreto de potássio
L	Litro
$L^{\cdot}$	radical lipídico
$LO^{\cdot}$	radical alcoxila
$LOO^{\cdot}$	radical peroxil
LOOH	hidroperóxido lipídico
LPD	Low-potassium dextran
LPO	lipoperoxidação
MDA	malondialdeído
Mg	miligrama
mL	mililitro
mmol	milimol
µmol/L	micromol/litro
min:	minuto
µL	microlitro
L	litro
NaCl	cloreto de sódio
nm	nanômetro
NAD^{+}	nicotinamida – adenina dinucleotídeo
NF-κB	Fator Nuclear- KappaB
nmoles	nanomoles
NO	óxido nítrico
NOS	óxido nítrico sintase
O_2	oxigênio molecular
$^1O_2$	oxigênio *singlet*
$O_2^{\cdot -}$	radical superóxido
PaO_2	pressão parcial de oxigênio arterial
pH:	potencial hidrogeniônico da solução
prot	proteína
QL	Quimiluninescência
RL	Radical Livre

ROS	Reactive Oxygen Species
rpm:	rotações por minuto
SOD	superóxido dismutase
TNF	Fator de Necrose Tumoral
TRAP	Potencial Antioxidante Total (*Total RadicalTrapping Antioxidant Potential*)
Trolox	Vitamina E hidrossolúvel
UW	University of Winconsin

RESUMO

TORRES, R.T. **Impacto do Estresse Oxidativo em diferentes eventos envolvidos no Transplante Pulmonar em Ratos.** Tese (Doutorado em Medicina) - Programa de Pós-Graduação em Medicina: Pneumologia, UFRGS, Porto Alegre, 2005.

O transplante é o único tratamento definitivo para doença pulmonar terminal. A injúria pulmonar induzida por isquemia-reperfusão (I-R) permanece como importante causa de mortalidade após o transplante. A injúria por I-R está relacionada com a falência do enxerto pulmonar, sendo associada ao aumento das espécies ativas de oxigênio (EAO). A preservação do enxerto tem como objetivo diminuir a incidência da falência primária do mesmo. Soluções preservadoras têm sido estudadas, sendo que a utilizada em nosso meio é a LPD (low-potassium dextran). Estudos mostram que o uso prolongado de corticóide pode causar aumento das EAO no endotélio vascular, mas quando administrado “in bolus” em doadores após declaração de morte cerebral, melhora o desempenho do pulmão doador. Nesta tese avaliamos o estresse oxidativo em situações relacionadas ao transplante pulmonar, utilizando tecido pulmonar ou sangue. A peroxidação lipídica foi determinada com a técnica de quimiluminescência (CL) e as defesas antioxidantes com as técnicas de TRAP (total radical trapping antioxidant potential) e medidas de atividade das enzimas Catalase (CAT) e Superóxido Dismutase (SOD). A tese foi dividida em três trabalhos (A, B e C).

A. O estresse oxidativo periférico foi avaliado em um modelo de transplante pulmonar em ratos. Em nove transplantes o enxerto pulmonar foi conservado com solução preservadora LPD, e submetido a 90 minutos de isquemia fria. Amostras de sangue arterial periférico do receptor foram coletadas em diferentes tempos do transplante. Ocorreu aumento significativo da CL no final do período de isquemia (imediatamente antes da reperfusão) e no período de reperfusão tardia em relação aos períodos basal (antes da toracotomia) e inicial da reperfusão. Sem diferença nos demais testes. Esses resultados podem ser

indicativos de resposta adaptativa e/ou efeito protetor da solução preservadora LPD contra a lipoperoxidação.

B. Avaliação do impacto da administração i.v. da solução de preservação LPD no estresse oxidativo periférico. Amostras de sangue arterial foram coletadas em diferentes tempos, em duas situações: com ou sem isquemia pulmonar por meio de dois desenhos experimentais: **Experimento 1**: Dois grupos de ratos: LPD e SAL. Receberam 0.5 mL i.v. de solução preservadora LPD ou salina (SAL) respectivamente. Foi observado aumento significativo na TRAP do grupo LPD em relação ao grupo SAL, sem alteração na CL. **Experimento 2**: Quatro grupos de ratos: controle (CON), isquemia (ISQ), salina (SAL) e LPD (solução preservadora). Exceto os do grupo CON, todos os animais dos demais grupos foram submetidos à toracotomia com clampeamento do hilo pulmonar esquerdo por 30 min, acompanhado por reperfusão de 30 min. Salina ou solução preservadora LPD foram administradas, 0.5 mL i.v., imediatamente antes da remoção do clampeamento, nos grupos SAL e LPD. Os resultados mostraram aumento significativo da CL nos grupos SAL e ISQ em relação aos grupos CON e LPD. Por outro lado, na TRAP houve diferença significativa entre tempos, grupos e interação tempo-grupo com aumento significativo no grupo LPD comparado com os demais grupos. Esses resultados sugerem que a isquemia pode ser desencadeadora de estresse oxidativo, efeito esse inibido pela utilização de LPD. A LPD mostrou-se potencializadora das defesas antioxidantes, levando-nos a sugerir a possibilidade de que seja utilizada em situações de potencial estresse oxidativo tanto in vitro quanto in vivo. Sem alteração nos demais testes.

C. Avaliação do estresse oxidativo em tecido pulmonar de ratos submetidos a diferentes regimes de tratamento com metilprednisolona (MP). Seis grupos de ratos, três tratados e os respectivos controles, foram submetidos a diferentes tratamentos com MP - dose única de 50 mg/kg, i.p. (agudo), e dose oral de 6 mg/kg durante 15 (sub-crônico) ou 30 dias (crônico). Ao final dos tratamentos, os animais foram mortos e os pulmões retirados foram homogeneizados para medir CL e TRAP. Os resultados mostraram aumento significativo de CL (animais submetidos a tratamento crônico), e na medida de TRAP (animais

submetidos ao tratamento agudo). Os resultados sugerem que o tratamento agudo com MP não induz dano oxidativo pulmonar e melhora o sistema de defesa antioxidante, enquanto o tratamento crônico pode induzir o dano oxidativo, podendo indicar envolvimento dos radicais livres nos efeitos farmacológicos (terapêuticos ou adversos) dos corticóides.

Palavras-chaves: Transplante pulmonar, estresse oxidativo, metilprednisolona, solução preservadora – LPD.

INTRODUÇÃO

1. CONSIDERAÇÕES GERAIS

O transplante é o único tratamento definitivo para doença pulmonar terminal. Apesar de avanços dos métodos de preservação, das técnicas cirúrgicas e dos cuidados perioperatórios, a injúria pulmonar induzida por isquemia-reperfusão (I-R) permanece como importante causa de morbidade e mortalidade precoces após o transplante pulmonar (Perrot et al, 2003). A hipótese de injúria publicada por Land, inicialmente em 1994 e modificada muitas vezes entre 1996 e 2002, baseia-se na hipótese de que as espécies ativas de oxigênio (EAO) são mediadoras da injúria de reperfusão ao enxerto iniciando e induzindo resposta imune adaptativa (rejeição aguda) por meio de ativação de células apresentadoras de antígeno. Além disso, EAO contribuem para o desenvolvimento de arteriosclerose, nos vasos do doador (rejeição crônica) por meio de lesão endotelial pela proliferação de células musculares lisas (Land, 2005).

Vários termos são utilizados para descrever esta síndrome, mas injúria por I-R é o mais comum. A falência primária do enxerto tem sido considerada como uma das mais severas formas de injúria, que leva à morte ou à ventilação mecânica prolongada (de Perrot et al., 2003) e é o resultado final de uma série de eventos que ocorrem desde a morte cerebral até a reperfusão pulmonar após o transplante (Ware *et al*., 2002). Essa injúria é histologicamente caracterizada por edema pulmonar e extravasamento de neutrófilos (*apud* Belperio et al., 2005).

Disfunção precoce de enxertos pulmonares por I-R continua sendo a mais importante causa de mortalidade após transplante pulmonar, bem como um fator de risco significativo para o desenvolvimento da síndrome de bronquiolite obliterante (Belperio et al., 2005). Interações de leucócitos ativados com células endoteliais do enxerto participam do desenvolvimento de injúria por I-R

(Ardehali et al, 2003). Os mecanismos recrutadores de leucócitos nos pulmões durante a injúria por I-R pós-transplante pulmonar não estão totalmente elucidados.

Estudos sugerem um papel central das espécies ativas do oxigênio (EAO) na injúria por I-R (Conte and Baumgartner, 2000), que está associada com marcado aumento das EAO e de outros radicais livres, podendo ter papel crucial na sucessão de eventos que levam à falência pulmonar (Hefner and Repine, 1989).

EAO são produtos do metabolismo normal da célula. As maiores fontes desses radicais são a cadeia de transporte de elétrons da mitocôndria, o retículo endoplasmático e a nicotinamida-adenina dinucleotideo (NADH/NADPH) oxidase associada à membrana. Em baixas concentrações, atuam como mediadores fisiológicos de resposta celular e reguladores da expressão gênica (Sanner et al., 2002). Em altas concentrações, podem gerar uma cascata bioquímica, produzindo lipoperoxidação lipídica (quando a molécula atacada é um lipídeo), oxidação de proteínas, dano de DNA, morte celular, e contribuir para a ocorrência de situações patológicas (Liu et al., 1996; Liu et al., 1999). O estresse oxidativo é definido pelo aumento da concentração das espécies ativas de oxigênio ou por uma diminuição dos níveis de antioxidantes. É caracterizado pela formação de EAO, tais como ânion superóxido, peróxido de hidrogênio e radical hidroxil (McCord, 1985), e tem sido identificado como um importante fator patológico em doenças cardiovasculares (Singal *et al*., 2000), pulmonares e doenças auto-imunes, doenças metabólicas, câncer e envelhecimento (Alho *et al*., 1998; Pettenuzzo *et al*., 2003; Latini *et al*., 2003; Heffner *et al*., 1989).

Nos pulmões, as EAO são relacionadas à inicialização de processos inflamatórios por meio da ativação de fatores de transcrição, tais como o fator nuclear - *kappaB* (NF-κB) e o ativador de proteína-1 (AP-1), levando ao remodelamento de cromatina e à expressão gênica de mediadores pró-inflamatórios (Rahman *et al*., 2004). A geração de espécies ativas de oxigênio intracelulares tem sido encontrada em muitas células pulmonares, incluindo

células endoteliais, epiteliais alveolares do tipo II, células claras, epiteliais ciliadas das vias aéreas e macrófagos alveolares (Al Mehdi, 1997). O sucesso do transplante requer preservação dos enxertos até que possam ser implantados e reperfundidos. (Kelly, 2000).

Algum grau de injúria é inevitável durante o período de preservação do órgão (Conte and Baumgartner, 2000). Com o objetivo de reduzir a incidência de falência primária do enxerto, a composição da solução preservadora tem sido estudada tanto clínica quanto experimentalmente (Kelly, 2000). Duas categorias de soluções cristalóides, intracelulares e extracelulares, surgiram baseadas na sua composição eletrolítica. As intracelulares são caracterizadas por conterem moderada a alta concentração de potássio (>100 mmol/L), com pouco ou nenhum cálcio e baixas concentrações de sódio. Têm por objetivo reduzir o gradiente eletroquímico através da membrana celular e prevenir o edema celular. *University of Winconsin* (UW) e *Euro-Collins* (EC) são exemplos de soluções intracelulares (Conte and Baumgartner, 2000).

As extracelulares, por sua vez, tais como as soluções de *Stanford, St Thomas Hospital* e LPD (low-potassium dextran), são caracterizadas por alta concentração de sódio e baixa a moderada de potássio, objetivando evitar o dano celular e o aumento da resistência vascular (vasoconstrição) associada a soluções hipercalêmicas. Agentes farmacológicos são adicionados, sendo cada solução eletrolítica uma composição única (Conte and Baumgartner, 2000). Estudos têm demonstrado que o uso da solução preservadora extracelular (LPD), com baixa concentração de potássio, tem diminuído a incidência de falência primária do enxerto pós-transplante (Fischer *et al*., 2001; Struber *et al*., 2001).

Os glicocorticóides (GC), conhecidos agentes antiinflamatórios, são drogas utilizadas no tratamento de pacientes com várias doenças, incluindo as auto-imunes, alérgicas e linfoproliferativas (Takahiko et al., 2003), e podem ter alguns de seus efeitos mediados por interferência de EAO (Sanner et al., 2002). O excesso de GC pode desencadear uma série de sinais e sintomas, como obesidade do tronco, face em lua cheia, hirsutismo, catarata, estrias

cutâneas, osteoporose, diabetes mellitus, imunossupressão e doenças cardiovasculares. O uso prolongado pode desencadear superprodução de EAO pelas células endoteliais, o que pode causar distúrbio do óxido nítrico disponível no endotélio vascular, acarretando complicações vasculares (Takahiko et al., 2003). Estudos mostram que a administração *in bolus* de glicocorticóides em doadores após declaração de morte cerebral pode melhorar a tensão de oxigênio (PaO_2), o desempenho do pulmão doador e ter efeitos protetores no endotélio vascular pulmonar (Follette *et al*., 1998).

2. CONSIDERAÇÕES ESPECÍFICAS

2.1 Preservação pulmonar

A perfusão do enxerto com soluções de preservação gerou uma série de publicações científicas sobre a preservação pulmonar nos últimos anos. Inúmeras soluções de preservação têm sido propostas, entretanto apenas algumas se firmaram para uso clínico corrente. Com base em estudos experimentais de preservação de outros órgãos, mais tarde foram incorporadas substâncias conhecidas como “aditivos antiinjúria”, bem como métodos alternativos de perfusão pulmonar do enxerto com solução de preservação no momento da extração, sempre com o intuito de reduzir os efeitos nocivos causados pela isquemia-reperfusão.

Entretanto, apesar dos avanços obtidos, a preservação pulmonar permanece como objeto de estudo e pesquisa, uma vez que os métodos de que se dispõe ainda não permitem períodos prolongados de isquemia com segurança como nos demais órgãos sólidos. Isso se deve à complexidade fisiológica do pulmão e à sua extrema suscetibilidade à injúria isquêmica, cujo produto final será a alteração da permeabilidade da membrana alvéolo-capilar, com a transudação do conteúdo vascular para o interstício, com conseqüente edema pulmonar e prejuízo das trocas gasosas. A necessidade de encontrarem-se novas opções de preservação do enxerto é urgente, já que o suprimento cada vez menor de doadores para a demanda de receptores exerce pressão permanente sobre os

centros de transplante, forçando-os a rever constantemente os critérios de seleção e a procurar alternativas para a doação de órgãos.

2.2 Soluções de preservação

A escolha da solução para perfusão pulmonar ainda é uma questão em aberto, dividindo opiniões. Entretanto, há um consenso na literatura no que tange à solução considerada “ideal” para preservação pulmonar, cujos quesitos fundamentais são:

a) ser isosmótica para não alterar a permeabilidade da membrana celular;

b) possuir poder tamponante (*buffer*) capaz de neutralizar a acidose tecidual causada pela lactacidemia e pelo acúmulo de outros metabólitos que ocorrem durante o período de isquemia;

c) conter pelo menos um doador energético (por exemplo, glicose), que auxiliará na manutenção das funções celulares vitais durante o armazenamento do órgão até seu implante no receptor;

d) ser capaz de fluir livremente pela circulação pulmonar, distribuindo-se de forma uniforme pela vasculatura de grandes e pequenos calibres;

f) possuir componentes eletrolíticos que possam ser trocados na membrana celular durante o período isquêmico.

As dificuldades inerentes ao uso de soluções intracelulares para perfusão pulmonar (por exemplo, vasoconstrição), provocaram a necessidade de desenvolvimento de soluções específicas para o pulmão. Fujimura et al (1985) demonstraram a superioridade de uma solução extracelular para a preservação de pulmões caninos transplantados após 24 horas de isquemia hipotérmica. Dois anos mais tarde, o mesmo autor relatou o sucesso com preservação pulmonar hipotérmica de até 48 horas em um modelo canino de transplante pulmonar unilateral, utilizando soluções extracelulares contendo tampões

fosfatados (HPO_4^-) e 2% de dextran com baixo peso molecular (Fujimura *et al*., 1985).

A experiência preliminar do grupo da Universidade de Toronto demonstrou, em um modelo experimental de perfusão *ex vivo* em pulmões de coelhos, que a concentração de potássio da solução de *Euro-Collins* pode ser a principal responsável pelas dificuldades e pela má qualidade da perfusão e preservação dos pulmões. Com base nessas informações, o laboratório experimental da Divisão de Cirurgia Torácica da Universidade de Toronto desenvolveu uma solução contendo baixo teor de potássio e dextran-40 (LPD=*low potassium dextran*), baseada na solução proposta por Fujimura. Em um modelo experimental em coelhos, Yamazaki *et al* (1990) demonstraram a superioridade do LPD sobre a solução de *Euro-Collins* em pulmões de coelhos preservados por 18 horas a 10 °C.

Cerca de dois anos antes, um novo modelo experimental de transplante unilateral esquerdo havia sido desenvolvido em cães na mesma instituição por Jones et al*., 1988*. Este veio a tornar-se um dos modelos mais utilizados em animais de médio porte para os estudos de preservação pulmonar em transplante, uma vez que permitia a avaliação em separado do pulmão nativo e transplantado, oclusão temporária e seletiva das artérias pulmonares por manguitos infláveis**.** Utilizando este modelo experimental, Keshavjee et al. (1989) descreveram o desempenho funcional superior em pulmões perfundidos com solução LPD e submetidos a isquemia hipotérmica (4 °C) por 12 horas, quando comparados a pulmões preservados com solução de Euro-Collins. Com este estudo, os autores esclareceram alguns pontos fundamentais que ainda norteiam os princípios da preservação pulmonar na atualidade.

Inicialmente, observou-se que as soluções extracelulares eram capazes de preservar pulmões de maneira consistente e por períodos mais longos, quando comparadas a outras soluções. A presença do tampão fosfatado reduz a acidose tecidual, que ocorre naturalmente durante os períodos de isquemia. A adição de dextran-40 funciona como substância oncóticamente ativa, reduzindo o edema intersticial pela manutenção da água no compartimento intravascular.

Outras propriedades benéficas do dextran-40 incluem o aumento da deformabilidade e a redução da agregação dos eritrócitos, facilitando, por conseguinte, o seu trânsito pela microcirculação (Eisenberg, 1969). Keshavjee et al*.,* (1992) também testaram a adição de dextran-40 a soluções com alto e baixo teor de potássio, respectivamente, comparando-as a uma solução de baixo potássio sem dextran-40. Este estudo demonstrou uma evidente superioridade das soluções contendo dextran-40, sendo a de baixo teor de potássio (LPD) a que se relacionou ao melhor desempenho funcional pulmonar após o transplante. Os autores criaram um sistema de graduação das alterações histopatológicas dos pulmões, concluindo também pela superioridade das soluções que continham dextran-40 neste particular. No mesmo modelo, demonstrou-se um índice de viabilidade brônquica superior ao das anastomoses nos animais que haviam recebido soluções de perfusão contendo dextran-40. Esses achados consolidaram a utilização desse tipo de solução para preservação pulmonar em uso clínico, atualmente comercializado sob o nome de Perfadex® (*Biophausia*-Suécia).

As composições das soluções mais utilizadas em preservação pulmonar estão sumarizadas na tabela a seguir:

Composição original das soluções mais utilizadas em preservação pulmonar

Sol./Componente	E-C*	E-C 40**	LPD**	EP4**	UW*	LP-UW**	Celsior**
Na^+ (mmol/L)	10	85	168	142	20	140	100
K^+ (mmol/L)	113	40	4	26,2	140	20	15
Cl^- (mmol/L)	15	15	103	-	-	-	41
Mg^{++} (mmol/L)	2	-	2	0,5	5	5	13
SO_4 (mmol/L)	2	-	-	-	5	5	-
HCO_3 (mmol/L)	10	-	-	-	-	-	-
PO_4 (mmol/L)	57,5	57	37	-	25	25	-
Hidróxietil-amido (g/L)	-	-	-	-	50	50	-
Dextran 40® (g/L)	-	-	20	20	-	-	-
Glicose (g/L)	3,5	3,5	0,91	10	-	-	-
Lactobionato (mmol/L)	-	-	-	-	100	100	80
Alopurinol (mmol/L)	-	-	-	-	1	1	-
Rafinose (mmol/L)	-	-	-	-	30	30	-
Glutationa (mmol/L)	-	-	-	-	3	3	3
Glutamato (mmol/L)	-	-	-	-	-	-	20
Adenosina (mmol/L)	-	-		-	5	5	-
Histidina (g/L)	-	-	-	-	-	-	20
Manitol (g/L)	-	-	-	-	-	-	60
Solumedrol (mg)	-	-	-	500	-	-	-
K_2HPO_4 (g/L)	-	-	-	2,6	-	-	-
Na_2HPO_4 (g/L)	-	-	-	6,4	-	-	-
$Na_2H_2PO_4$ (g/L)	-	-	-	0,6	-	-	-
NaCl (g/L)	-	-	-	5,8	-	-	-
Heparina (unidades)	-	-	-	2000	-	-	-
Osmolaridade (mOsm/L)	359	-	292	364	320	320	-

(*) Intracelular
(**) Extracelular

2.3 Radicais livres

A pressão evolucionária causada pela liberação de oxigênio no ambiente nos primórdios da formação de uma atmosfera provavelmente semelhante à que vivemos hoje, há cerca de dois bilhões de anos, exigiu o desenvolvimento de mecanismos para se ocupar com os tóxicos radicais livres, produtos do metabolismo oxidativo (Halliwell and Gutteridge, 1989; Del Maestro, 1980).

Com a descoberta do oxigênio como componente químico do ar por Priestley (1774), foi descrita quase que simultaneamente, a sua toxicidade. Lavoisier, já em 1783, provou que altas pressões de oxigênio tinham efeito tóxico sobre os pulmões de animais. No entanto, foi Paul Bert (1871) quem estabeleceu as bases da toxicidade do oxigênio hiperbárico. Bert concluiu que todas as formas de matéria viva sofriam efeitos tóxicos sob essas circunstâncias experimentais. Os estudos de toxicidade do oxigênio dependem do organismo em estudo, de sua idade, estado fisiológico e dieta (Halliwell, 1992). A sobrevivência de organismos aeróbicos em um ambiente com oxigênio envolve um complicado equilíbrio entre a geração de substâncias extremamente reativas, os radicais livres e a habilidade desses organismos em controlar tais substâncias (Del Maestro, 1980).

2.3.1 Definição de Radical Livre

As espécies ativas de oxigênio (EAO) também são conhecidas como “radicais livres” (RL). No entanto, essa denominação não inclui somente os radicais de oxigênio, mas vários outros, como o átomo de hidrogênio, os íons de metais de transição, o óxido nítrico, o dióxido de nitrogênio, radicais centrados no carbono etc. Portanto, *“*radical livre (RL) é qualquer espécie química (átomo, molécula) capaz de existência independente, que possua um ou mais elétrons desemparelhados em qualquer orbital, normalmente no orbital mais externo*”* (Del Maestro, 1980; Halliwel and Gutteridge, 1984; Southorn, 1988) (Figura 1).

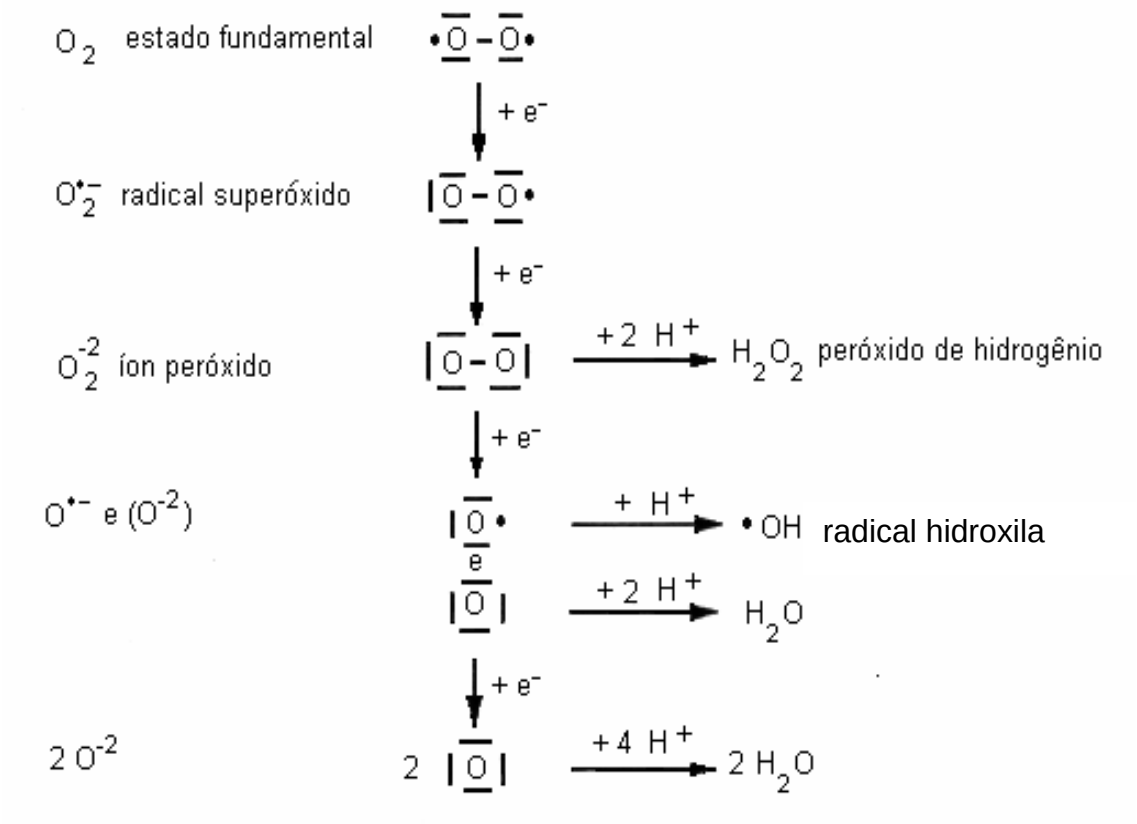


Figura 1 - Redução monoeletrônica do oxigênio formando EAO (Bast *et al*., 1991).

2.3.2. Lipoperoxidação e Efeitos Biológicos

Quando um RL reage com um composto não-radical, outro RL pode ser formado, induzindo, assim, reações em cadeia, como é o caso da lipoperoxidação (LPO). Dessa forma, podem ser produzidos efeitos biológicos distantes do sítio de geração do primeiro RL formado. As reações em cadeia seguem uma série de etapas durante as quais se consome uma espécie intermediária. Os reativos convertem-se em produtos e os intermediários são regenerados, permitindo que o ciclo recomece. As etapas do processo de LPO são: iniciação, propagação e terminação.

A iniciação é o primeiro passo das reações em cadeia. É necessário que o RL ataque uma molécula orgânica, abstraindo um átomo de hidrogênio de um grupamento químico. Na LPO, o RL é geralmente o radical hidroxila, e o

grupamento químico é um metileno pertencente a um ácido graxo poli-insaturado da membrana (Meerson et al., 1982).

A retirada de um átomo de hidrogênio do grupamento metileno leva à formação de um radical centrado no carbono (-•CH-), o qual tende a se estabilizar por um rearranjo molecular, formando um dieno conjugado (Figura 2). Este, por sua vez, ao se combinar com o oxigênio, produz o radical peroxil, também chamado "radical peróxi".

No estágio de propagação, os radicais peroxil são capazes de abstrair hidrogênio de outra molécula lipídica, ou seja, de um ácido graxo adjacente. O radical peroxil pode, também, se combinar com o átomo de hidrogênio que abstraiu, produzindo um lipídio hidroperóxido. Os lipídios hidroperóxidos decompõem-se numa reação catalisada por complexos de ferro e cobre, produzindo aldeídos, como o malondialdeído (MDA), hidrocarbonetos voláteis, como o gás pentano, e outros produtos que podem ser detectados experimentalmente (Figura 2) (Halliwell and Gutteridge, 1989). Foi sugerido que as reações de degradação ocorridas durante a LPO podem originar oxigênio *singlet,* acelerando esse processo (Halliwell and Gutteridge, 1989).

Na etapa de terminação, dois radicais peroxila reagiriam entre si, formando um tetróxido instável que se decompõe, dando origem ao oxigênio *singlet* ($^1O_2$) e a carbonilas excitadas, as quais retornam ao seu estado fundamental emitindo quantas de luz visível (Russel, 1957). Essas carbonilas podem ser medidas pelo processo de quimiluminescência (QL), que se constitui num importante método de quantificação de LPO (Castro *et al*., 1990).

Além da perda da fluidez da membrana, há desarranjo dos receptores e potenciação da lise celular. O dano dos RL a enzimas que contêm enxofre e a outras proteínas culmina em sua inativação, em ligações cruzadas e em desnaturação (Figura 3A, B) (Machlin and Bendich, 1987).

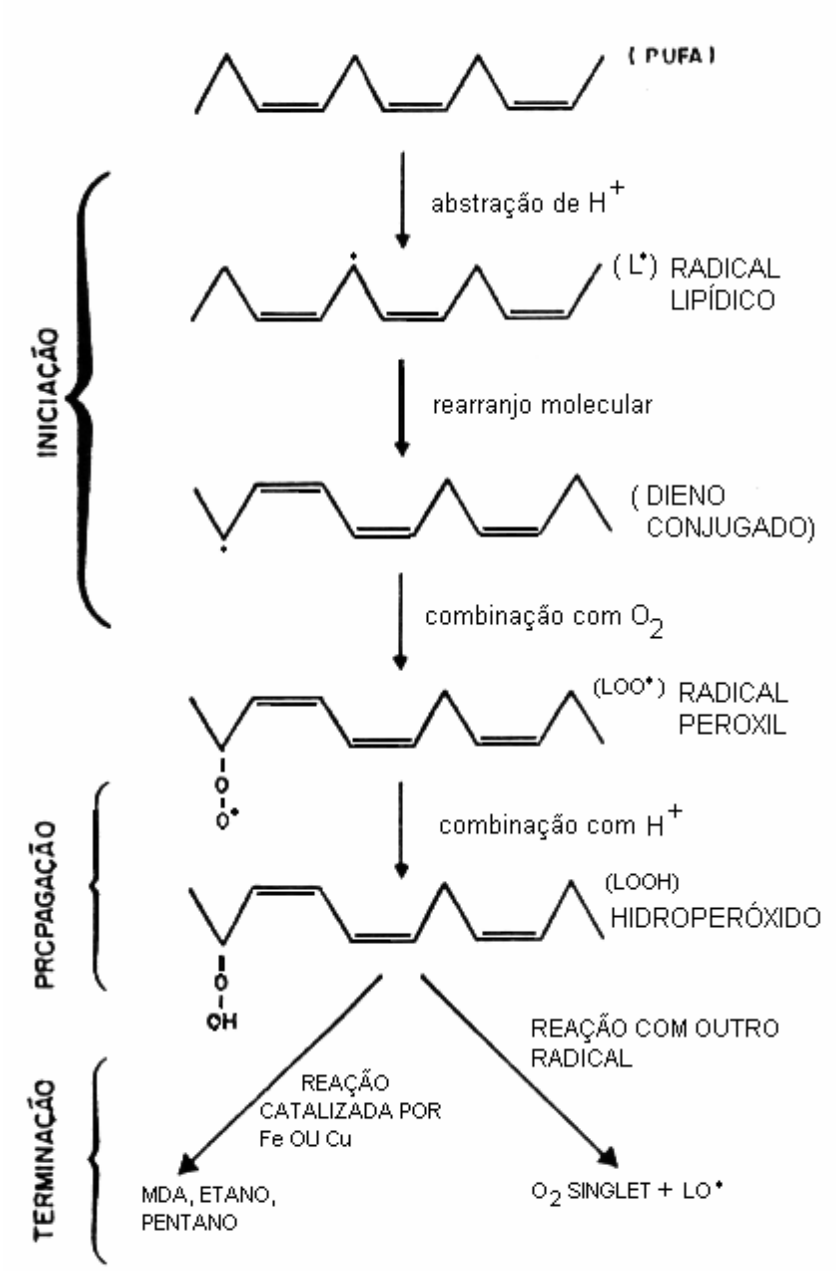
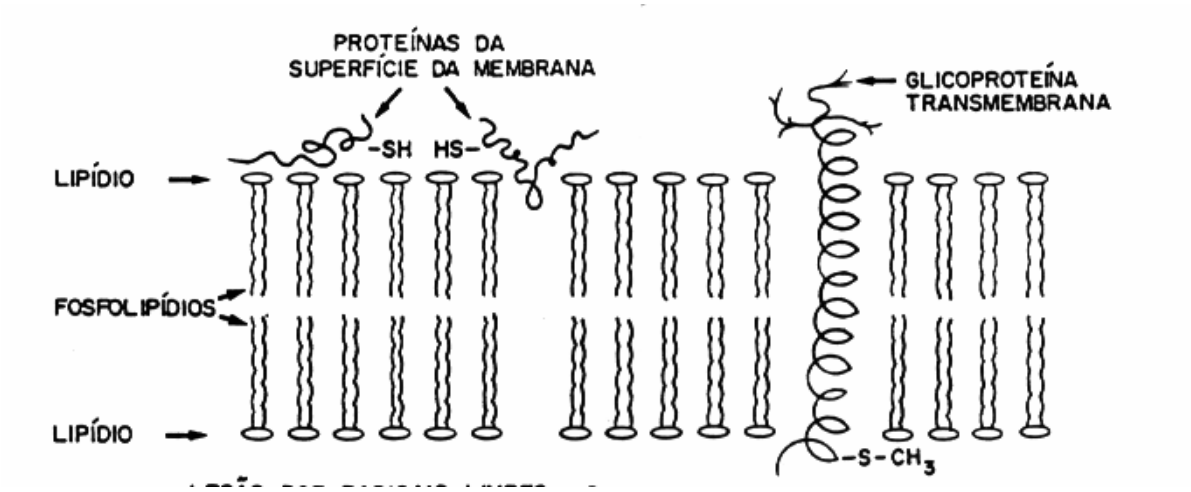


Figura 2: Reações em cadeia da lipoperoxidação (Del Maestro, 1980). PUFA: ácidos graxos poliinsaturados.

A



B

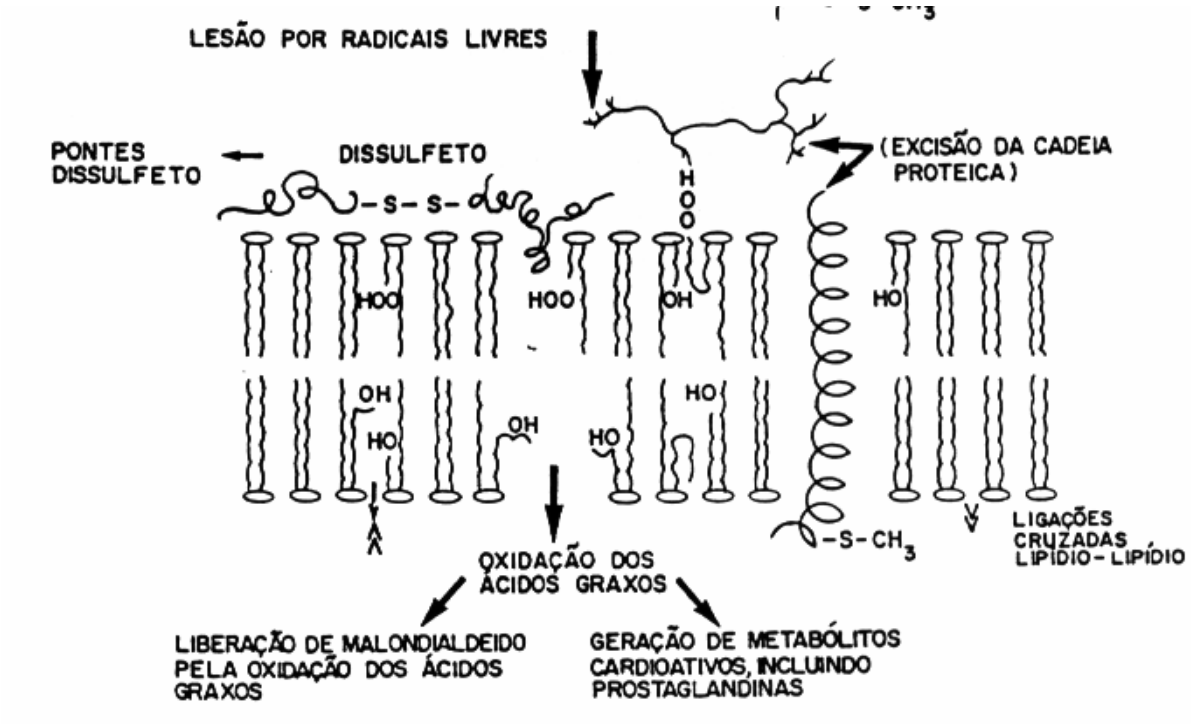


Figura 3: A - Membrana normal; B - Efeito da LPO sobre as membranas celulares.

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2.3.3. Defesas Antioxidantes

As EAO, quando formadas, ao reagirem com biomoléculas, causam diferentes tipos de danos biológicos que podem levar à morte celular. Os organismos aeróbios desenvolveram diferentes tipos de defesas antioxidantes - enzimáticas e não-enzimáticas, prevenindo a formação de EAO, bem como mecanismos para reparar os danos causados.

Antioxidante é qualquer substância que, quando presente em baixas concentrações, se comparadas às de um substrato oxidável, retarda ou inibe significativamente a oxidação deste substrato - enzimático ou não-enzimático (Halliwell and Gutteridge, 1989).

A defesa do organismo contra as EAO vai desde:

a) *prevenção da formação das EAO:* Os sistemas que previnem a formação de EAO são considerados biomoléculas ligantes de metais (Fe e Cu) chamados “quelantes”. A presença de proteínas quelantes é de vital importância aos seres vivos, porque protegem as células dos processos oxidativos catalisados por íons metálicos. As enzimas que controlam os níveis de EAO são glutationa (GPx), superóxido dismutase (SOD) e, catalase (CAT);

b) *interceptação dos radicais formados:* A interceptação é a desativação das EAO, as quais são destruídas de forma a impedir a oxidação posterior de outras moléculas. São chamadas *scavengers* de RL as substâncias que se combinam com um RL, levando à formação de um não-radical ou de um radical menos lesivo, como tocoferóis e carotenóides. Substâncias que funcionam como *quenchers* de oxigênio singlet são as que absorvem a energia de excitação e a liberam em forma de calor ou movimento;

c) *reparo das células danificadas (Figura 4):* Acredita-se que enzimas, como a fosfolipase, removam o ácido graxo peroxidado da membrana lipídica, substituindo-o por ácido graxo normal.

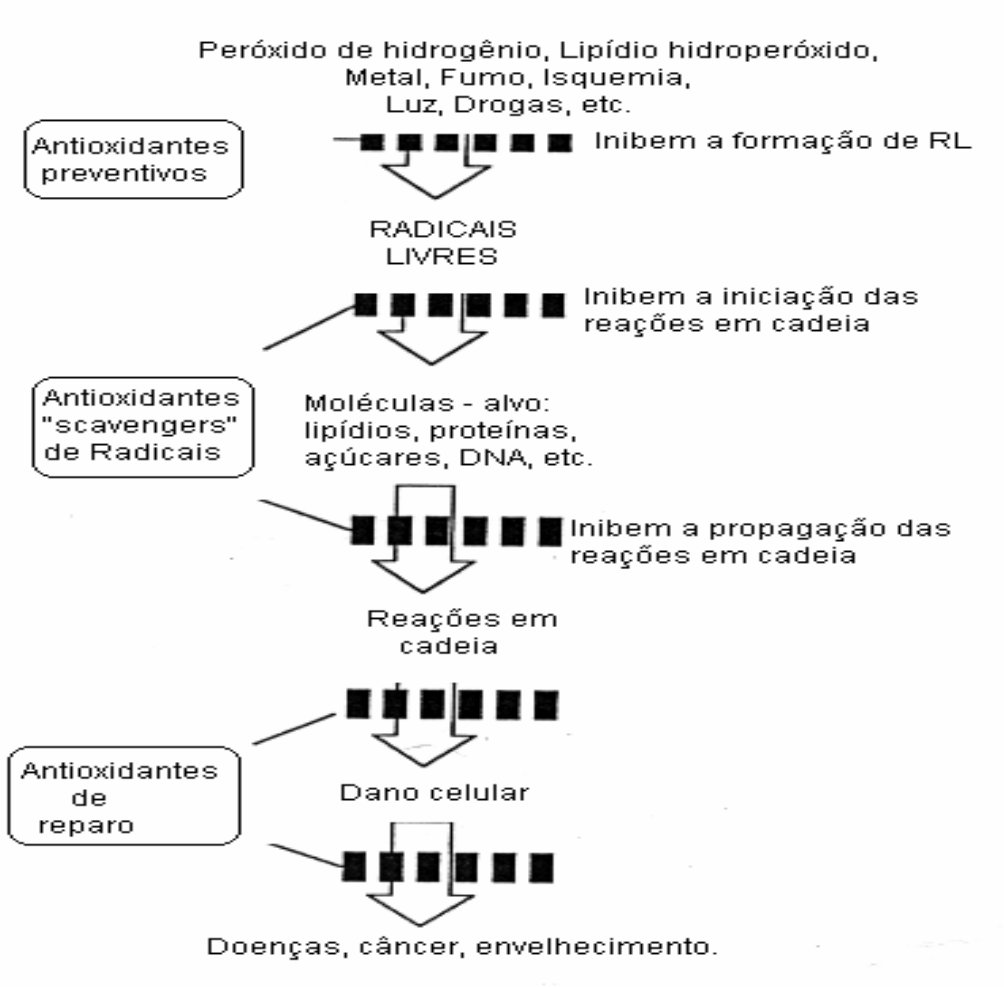

Figura 4: Sistemas de defesa antioxidante contra dano oxidativo induzido por RL (Niki, 1993).

A determinação do grau de estresse oxidativo no sangue por meio de métodos padronizados pode ser útil para definir o seu papel em diferentes patologias. No plasma estão presentes moléculas e macromoléculas cuja capacidade é transformar espécies ativas (radicais) em inativas. Esse tipo de defesa inclui vários compostos. Dessa forma, é interessante determinar a capacidade antioxidante total em fluidos biológicos. Um dos métodos mais importantes para avaliar a capacidade antioxidante de um fluido biológico é o TRAP (*total radical trapping potencial*). Os eritrócitos são especialmente sensíveis à peroxidação por terem alta concentração de ácidos graxos poliinsaturados, por estarem expostos a altas concentrações de oxigênio e possuírem metais de transição,

como o ferro, os quais atuam como catalisadores de reações que geram EAO. As membranas dos eritrócitos podem ser afetadas pelos radicais livres e, dessa forma sofrer dano oxidativo, que pode ser avaliado com técnicas de quimioluminescência (CL) (Travacio and Llesuy, 1995). Ensaios de procedimentos bioquímicos no plasma e células sangüíneas são considerados marcadores periféricos minimamente invasivos (Repetto *et al*., 1999).

3. OBJETIVOS

3.1 Objetivo Geral

Avaliação do estresse oxidativo em ratos submetidos a diferentes situações relacionadas ao transplante pulmonar.

3.2 Objetivos Específicos

3.2.1 Avaliar o estresse oxidativo periférico em um modelo de transplante pulmonar em ratos;

3.2.2 Avaliar o estresse oxidativo periférico em ratos que receberam administração endovenosa da solução preservadora LPD em experimentos nos quais eram ou não submetidos à isquemia pulmonar;

3.2.4 Avaliar o estresse oxidativo em tecido pulmonar de ratos submetidos a tratamentos agudo, subcrônico e crônico de metilprednisolona.

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ARTIGO I

PERIPHERAL MARKERS OF OXIDATIVE STRESS IN A RAT LUNG TRANSPLANTATION MODEL

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Abstract

Ischemia-reperfusion (IR) injury is a serious problem in organ transplantation. Reactive oxygen species (ROS) are considered important mediators of this event. The overproduction of ROS plays a crucial role in the cascade of events leading to graft failure. This study focused on the evaluation of the peripheral markers of oxidative stress in a rat lung transplantation model. Nine lung transplants were performed. The lung grafts were maintained with preservation solution low potassium dextran (LPD) and submitted to 90 minutes of cold ischemia. Peripheral arterial blood samples were collected from the receptor in 4 different times to evaluate the peripheral markers of oxidative stress: prior to thoracotomy (time 0 = baseline); immediately before reperfusion following lung transplantation after 1 hour of hilar clamping (time 1); immediately (time 2) and 15 minutes after reperfusion of the transplanted lung (time 3). Lipid peroxidation (LPO) was evaluated by means of chemiluminescence (CL), and the antioxidant defenses were evaluated by the total radical trapping antioxidant potential (TRAP) and antioxidant enzyme activities of superoxide dismutase (SOD) and catalase (CAT). There were significant differences among the analyzed times ($P=0.03$) in the chemiluminescence (CL), with a significant decrease in CL levels in the time 2, when compared to the times 1 and 3 ($P<0.05$)**.** On the other hand, TRAP, CAT, and SOD measurements demonstrated no differences in the different times. We conclude that the results could be indicative of an adaptative response and/or a protective effect of LPD against LPO through natural scavenging mechanisms.

Key Words: lung transplant, lipid peroxidation, ischemia-reperfusion, low potassium dextran

Introduction

Thoracic organ transplantation is the only definitive treatment for end-stage lung disease. Despite the refinements in the lung preservation, surgical techniques and perioperative care, ischemia- reperfusion (I-R) induced lung injury remains an important cause of early morbidity and mortality after lung transplantation. A number of terms have been used to describe this phenomenon, but ischemia-reperfusion injury is the most commonly used. The primary graft failure has been attributed to the most severe form of injury that frequently leads to death or prolonged mechanical ventilation beyond 72 hours (de Perrot et al, 2003).

Primary graft failure is the end-result of a series of events occurring from the time of brain death to the time of lung reperfusion after transplantation. However, other factors may have influence on the donor lungs before harvesting and may contribute and intensify the severity of ischemia and reperfusion injury (Ware et al., 2002).

The injury hypothesis, holds that the reactive oxygen species-mediated reperfusion injury to allografts iniciates and induces the alloimmune response and contributes to alloatherogenesis (Land, 2005). Pulmonary ischemia-reperfusion (I-R) injury is associated with a marked increase in reactive oxygen species (ROS) and other free radicals. This overproduction of reactive oxidants may overload natural scavenging mechanisms, thus playing a crucial role in the cascade of events leading to lung failure (Heffner and Repine, 1989).

ROS are products of normal metabolic processes in cells. The major sources of these radicals are leakages from the electron transport chains of mitochondria and endoplasmic reticulum. Putatively, another important source of ROS is a membrane-associated nicotinamide-adenine dinucleotide (NADH/NADPH) oxidase. At low concentrations, ROS act as physiological mediators of cellular responses and regulators of gene expression (Sanner et al, 2002). Increased production of ROS can generate a biochemical cascade, producing lipid peroxidation, protein oxidation, DNA damage and cell death, and contribute to the occurrence of pathological conditions (Liu et al, 1996, Liu et al, 1999).

Oxidative stress is characterized by the formation of ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radical (McCord, 1985). The generation of intra-cellular oxygen species has been found in most lung cells, including the endothelial cells, type II alveolar epithelial cells, clara cells, ciliated airway epithelial cells and alveolar macrophages (Al Mehdi, 1997).

The determination of the degree of oxidative stress in blood through standardized methods can be useful to define the role of oxidative stress in different pathologies. Biochemical procedures assayed in plasma and blood cells are considered minimally invasive peripheral markers (Repetto et al., 1999).

The objective of the present study was to evaluate some peripheral markers of oxidative stress in a rat model of lung transplantation, through the determination of catalase (CAT), and superoxide dismutase (SOD) activities, lipid peroxidation by chemiluminescence (CL) in erythrocytes, and total radical-trapping potential (TRAP) in plasma.

Methods

Eighteen adult male Wistar rats (90 days old, 250-300 g) were used. The animals were housed in groups of five in cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained in a controlled environment (light/dark cycle of 12 h, temperature of 22 $\pm$ 2^{o} C) before the experimental period and were given free access to food (standard lab rat chow) and water. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals"(http://www.nap.edu/catalog/5140.html).

Transplant model

Donor rats were anesthetized with intraperitoneal sodium penthotal (0.35 mL/100 g body weight), submitted to orotracheal intubation and mechanical ventilation (Harvard 683-Rodent Ventilator- Harvard Apparatus; FiO2=1.0, tidal volume=1 mL/100 g body weight, respiratory rate=65 bpm). Median sternotomy

was carried out, heparin was administered through the inferior vena cava (50 U/100 g body weight), and the heart-lung block was prepared by the dissection of the inferior vena cava right above the diaphragm. The main pulmonary artery was cannulated through the right ventricle outflow tract, and antegrade perfusion was performed with LPD, 5 mL/100 g body weight, at 4^{o}C, with an infusion pressure of 20 cm of H_2O obtained by elevation of the perfusion bag. The outflow was drained through the severed left atrial appendix while the lungs were inflated. The heart-lung block was then dissected out and extracted. The graft was prepared as follows. Under a surgical microscope (10x power, Carl Zeiss-GmB), the left lung was dissected out from the heart-lung block, three cuffs previously cut from #14 teflon Abocath® venous catheters were put into the pulmonary artery, left atrium and left main bronchus, respectively, and secured in place by 8-0 Prolene® ties. The prepared graft was placed in a plastic bag with cold saline for storage for one hour in a cold chamber (4^{o}C).

The receptor animals were anesthetized and intubated using the same procedure described for the donor rats. Under the same surgical microscope (10x power), a left postero-lateral thoracotomy was performed. The inferior pulmonary ligament was released, the pulmonary artery and veins of the recipient were dissected and clamped. The left main bronchus was dissected and ligated proximal to the carina (6-0 Vicryl®) and the left pneumonectomy was carried out. The prepared left lung graft was brought, and the cuffs were inserted into the pulmonary artery, left atrium and left main bronchus and secured in placed by encircling 8-0 Prolene® ties. Upon completion of the anastomoses, the clamps were removed and the left lung was reperfused. The thoracotomy was left open and blood samples were obtained serially from the right femoral artery for the evaluation of oxidative stress, TRAP, enzymes, protein and hemoglobin. No attempts were made to assess the gas exchange during reperfusion.

Blood samples and preparation

Blood was drawn (0.5 mL /sample) from a peripheral artery (right femoral artery) from the receptor prior to thoracotomy (time=0, baseline); immediately before

reperfusion following lung transplantation after 1 hour of hilar clamping (time 1); immediately (time 2) and 15 minutes after the graft reperfusion (time 3). Heparinized arterial blood samples were centrifuged at 1000g for 15minutes (Sorval RC 5b-rotor SM 24, Du Pont Instruments, USA). Plasma was separated and stored at -70^{o}C for further TRAP determination. The erythrocytes were washed three times in saline solution and centrifuged at 1000g for 3 minutes at room temperature. The supernatant was discarded by aspiration. The washed erythrocytes were used for the lipid peroxidation assay (Repetto et al., 1999). The erythrocytes were diluted 1/10 in 1 mmol/L of acetic acid and 4mmol/L magnesium sulphate, and stored at -70^{o}C until analysis for enzyme assays CAT and SOD.

Oxidative damage assessment

Chemiluminescence (CL) was measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB – Produkter AB, Sweden) to assess oxidative stress. Blood samples (10μL) were placed in low-potassium vials, and the background chemiluminescence was measured using 4 mL of 30 mmol/L phosphate buffer and 120 mmol/L of KCl (pH=7.4). Measurements were started by the addition of 400 mmo/L tert-butyl hydroperoxide (final concentration 3mmol/L). Data were expressed as counts per second per milligram of hemoglobin (cps/mg Hb) (Repetto et al., 1999).

Antioxidant defenses

Determination of Total Radical-Trapping Potential (TRAP).The total antioxidant capacity present in the plasma was measured by luminescence, using 2,2'-azo-bis(2-amidinopropane) (ABAP, a source of alkyl peroxyl free radicals) and luminol. The background chemiluminescence was measured using 50 μmol/L ABAP, 40 μmol/L luminol, and 50 mmol/L phosphate buffer (pH=7.4). This emission was almost completely quenched by the addition of Trolox (hidrosoluble vitamin E), rendering induction times that were linearly related to the free radical scavenger concentration added. A calibration curve was

obtained by using different Trolox concentrations between 0.5 and 1 µmol/L. The addition of plasma samples instead of Trolox elicited an induction time related to the initial amount of sample added (Lissi et al., 1995). Luminescence was measured in a scintillation counter, in the out-of-coincidence mode, and the results were expressed in µM Trolox.

Determination of Catalase (CAT)

Catalase activity (CAT) was determined by the evaluation of the decrement of 240-nm absorption of hydrogen peroxide (H_2O_2) in a reaction medium containing 50mmol/L phosphate buffer (pH=7.2), and 10mmol/L hydrogen peroxide (H_2O_2) (Aebi, 1984). Activity was expressed as ρmoles of H_2O_2 reduced per minute per milligram of protein.

Determination of Superoxide Dismutase (SOD)

The measurement of SOD activity, expressed as units per milligram of protein, was based on the inhibition of the superoxide radical reaction with pyrogallol (Marklund, 1985). The SOD activity was determined by measuring the velocity of oxidized pyrogallol formation and the reaction medium contained Tris buffer (50mmol/L, pH=8.20), pyrogallol (24mmol/L), and catalase (30mmol/L). Absorbance changes were observed at 420 nm for 2 min.

Protein determination

Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard (Lowry et al., 1951).

Hemoglobin determination

The conversion of hemoglobin to cyanomethaemoglobin by Drabkin reagent was measured against a standard curve. The values were expressed as mg per mL (mg/mL) (Drabkin and Austin, 1935).

Statistical analysis

Data were expressed as median (interquartile range). Data were analyzed by the Friedman test, followed by Multiple Comparisons' Friedman test when indicated. Values of $P<0.05$ were considered significant. The results were analyzed as the percentage of the baseline measurement of the rat (time 0= 100%).

Results

Erythrocyte's chemiluminescence was analyzed to evaluate the peripheral oxidative damage to membrane lipids. This technique evaluates the oxidative stress and is currently used as a measurement of lipid peroxidation. The method is very sensitive and capable of detecting small amounts of peroxidative products. There were significant differences among the different times ($P=0.030$) in the chemiluminescence (CL). The Multiple Comparison Friedman's test demonstrated a significant decrease in CL levels ($P<0.05$) at the time 2 (immediately after reperfusion), when compared to the time 1 and time 3 (15 minutes after graft reperfusion). Results demonstrated that lipid peroxidation (CL) was increased in time 1 (26.68%) and in the time 3 (22.90%) when compared to the baseline. Conversely, no significant differences were found between times 1 and 3. Similarly, no differences were detected between time 2 and baseline measurements (Figure 1A).

TRAP was performed in order to verify if the antioxidants were altered. In the plasma, as well as in other human tissues, there are substances with antioxidant capacity, including reduced glutathione (GSH), uric acid, ascorbic acid, ceruplasmin, transferrin, bilirrubin, carotenoids, etc. The relative concentration of these substances determines the total plasma antioxidant capacity. There were no significant differences in TRAP among the time points analyzed (Figure 1B). Some enzymatic defenses were also quantified in this protocol. SOD and CAT enzymes were chosen because they represent the first step in the enzymatic detoxification of ROS, being considered as primary defenses. Almost 3% to 10% of the oxygen utilized by the tissues is converted into ROS, such superoxide radicals which impair the function of cells and

tissues. Superoxide dismutase (SOD) catalyzes the conversion of single electron reduced species of molecular oxygen to hydrogen peroxide and oxygen. Catalase (CAT) catalyzes the conversion of hydrogen peroxide to water and oxygen. No significant difference was found in the activity of these enzymes (Fig 2A; Fig 2B).

These results are in table 1.

Discussion

This study showed that, in this rat lung transplantation model, there was a significant decrease in the erythrocytes lipid peroxidation during the initial reperfusion, when compared to the late ischemic and end-reperfusion period. On the other hand, no differences were observed in all the other parameters analyzed. The possible mechanisms that could explain such increase in LPO immediately prior to reperfusion is the possibility of remote tissue ischemia-reperfusion injury. Ischemia-reperfusion injury is a systemic phenomenon. Esme et al (2006) demonstrated experimentally that other organs and tissues, such as liver and myocardium, also suffer the effects of such phenomena when lungs are submitted to I-R injury, and recommended that these organs should also be protected during lung ischemia. Pulmonary ischemia-reperfusion induces liver and heart injury characterized by activated neutrophil sequestration and release of significant amounts of reactive oxygen species (Esme et al., 2006). Tissue ischemia is associated with an overwhelming generation of oxygen radicals (Napoli et al 2005). Surgery is a recognized cause of SIRS (systemic inflammatory response syndrome). Neutrophil activation is a key histopathological feature of SIRS, and neutrophil clearance through programmed cell death or apoptosis, is an essential step in its resolution. It is recognized that ROS such as those generated by activated neutrophils during cardiac surgery, may have a regulatory role influencing neutrophil lifespan and thus inflammation. We consider recent evidence for the regulation of neutrophil apoptosis by ROS (Melley et al., 2005).

The possible mechanism, by which there is a significant decrease in CL levels during initial reperfusion when compared to late ischemic period and end reperfusion period, is the use of low-potassium dextran (LPD) as a lung preservation solution. The composition of lung preservation solutions has been studied both clinically and experimentally (Kelly, 2000). Previous studies have shown the beneficial effects of low-potassium dextran (LPD), an extracellular solution designed specially for lung preservation. It has been shown that the high potassium concentrations of some preservation solutions, besides inhibiting potassium currents and depolarizing the pulmonary artery smooth muscle cells, also signalize to ROS production. Nicotinamide-adenine dinucleotide (NADH/NADPH) oxidase activation, through membrane depolarization, is a potential pathway for this effect (Fischer et al., 2001; Struber et al., 2001). Although the signaling or pathway of ROS production has not yet been determined, the use of a low potassium concentration in lung preservation solution seems to decrease the incidence of primary graft failure through reduction in ROS production from the pulmonary vasculature (Kelly et al., 2003). The beneficial effect of preservation with LPD has been attributed to the combination of both a low potassium concentration and the presence of dextran (Keshavjee et al., 1992). The low potassium concentration may be less detrimental to both functional and structural integrity of endothelial cells, which may lead to a decreased production of oxidants (Kennedy et al., 1989; Henderson et al., 1988; Kitagawa and Johnston, 1985) and release of lower levels of pulmonary vasoconstrictors (Yamazaki et al, 1990; Sasaki et al., 1995; Kimblad et al., 1991; Bando et al., 1998). Furthermore, dextran itself improves erythrocyte deformability, prevents aggregation, and has an antithrombotic effect induced by coating endothelial surfaces and platelets (Keshavjee et al., 1992). The final result is an improvement in pulmonary microcirculation and preservation of the endothelial-epithelial barrier, which may ultimately prevent the no-reflux phenomenon and reduce the degree of water and protein leakeage at the time of reperfusion (Schneuwly et al., 1999). In vitro studies have demonstrated that LPD solution can exert a suppressive effect on polymorphonuclear chemotaxis (Sakamaki et al., 1999), is less cytotoxic for

Type II pneumocytes (Maccherini et al., 1991; Carbognani et al., 1997) and maintains a better activity for alveolar epithelial Na+/K+-ATPase function during the cold storage period, when compared to other solutions, such as Euro-Collins or University of Wisconsin (Suzuki et al., 2000). These effects may result in less lipid peroxidation and better surfactant function both at the end of the ischemic time and after reperfusion (Sakamaki et al., 1997; Struber et al., 2000). We therefore hypothesize that the presence of LPD solution in the pulmonary vascular bed, when flushed out of the lung and into the systemic circulation, may have some degree of antioxidant capacity, temporarily decreasing the amount of lipid peroxidation. This hypothesis agrees with the results of previous studies (Kelly et al., 2003; Kennedy et al., 1989; Henderson et al., 1988; Kitagawa and Johnston, 1985).

The possible mechanisms that could explain an increase in LPO in the late ischemic period is that usually, ischemia-reperfusion corresponds to anoxia-reoxygenation in organ transplantation. However, the lung should be considered separately, because it contains oxygen within the alveoli during ischemic time of preservation. The alveolar oxygen helps to maintain aerobic metabolism and prevents hypoxia (Date et al., 1993; Fischer et al., 1991; Eckenhoff et al., 1992). Hence, in the lung the oxidative stress resulting from ischemia should be distinguished from the oxidative stress resulting from hypoxia. Hypoxia and ultimately anoxia result in a sharp decrease in intracellular adenosine triphosphate (ATP) and a corresponding increase in the ATP degradation product hypoxanthine, which generates superoxide when oxygen is reintroduced with reperfusion and/or ventilation. These phenomena can occur in the lung when alveolar oxygen tension drops below 7mmHg during ischemia (Fischer and Dodia, 1981), and can be blocked by the inhibitors of xanthine oxidase, such as allopurinol (Zhao et al., 1997; Kennedy et al., 1989). Ischemia is characterized by the absence of blood flow into the lung, which can cause lipid peroxidation and oxidant injury despite the presence of oxygen (Fischer et al., 1991; Zhao G et al., 1997). This mechanism of oxidative stress is somewhat different from that occurring during anoxia-reoxygenation because it is not associated with ATP depletion and it can occur during the storage period

(Fischer et al., 1991; Eckenhoff et al., 1992; Zhao et al., 1997). Furthermore, inhibitors of xanthine oxidase cannot block it (Zhao et al., 1997; Al Mehdi et al., 1998). One could speculate that the absence of the mechanical component of flow during lung ischemia can stimulate membrane depolarization of endothelial cells, with the activation of nicotinamide-adenine dinucleotide (NADH/NADPH) oxidase. This phenomenon can generate superoxide anion, nuclear factor κb, and calcium/calmodulin-dependent nitric oxide synthase (NOS) (Al Mehdi et al., 1998a; Al Mehdi et al., 1998b). Other cells such as macrophages and/or marginated neutrophils, which are known to have higher NADPH oxidase activity, could also contribute to the lung oxidant burden that takes place during the ischemic storage (Henderson et al., 1988; Kitagawa & Johnston, 1985). After revascularization the mediators from the ischemic tissue enter the systemic circulation and can affect other organs. It has been reported that the systemic effects of IR injury are caused by activated neutrophils, the complement system, and proinflammatory and vasoactive mediators such as eicosanoids, nitric oxide, cytokines, and oxygen-free radicals (Esme et al., 2006).

Since the results of our experiments have not shown significant differences over time in TRAP, CAT and SOD, one may speculate whether such findings are artifacts, or whether this represents some yet unknown properties of LPD solution. Additionally, one can infer that LPD is capable of carrying some intrinsic potential for increasing the endogenous antioxidant potential.

We conclude that the decrease in LPO immediately after reperfusion in this animal model can be indicative of an adaptive response or a protective effect of LPD against LPO through an induction of natural scavenging mechanisms. Future studies are necessary to evaluate the antioxidant capacity of LPD itself, as well as to clarify whether LPD has intrinsic antioxidant properties when administered systemically.

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LEGENDS OF FIGURES

Figure 1. Erythrocytes CL* (**A**) and plasma TRAP (**B**) level. CL is expressed as median (interquartile range) of counts per second per milligram of hemoglobin (cps/mg Hb) (n=9). TRAP is expressed as median (interquartile range) of equivalents in μM Trolox. (n=9). The results were analyzed as the percentage of the baseline measurement of the rat (time 0= 100%).

◆(**A**) *Significant differences (Friedman test, followed by Multiple Comparisons' Friedman, P= 0.03). Significant decrease in CL levels at the time 2 when compared to times 1 (26.68%) and in the time 3 (22.90%) relative to the baseline.

◆(**B**) No significant differences among the times (Friedman test, P >0.05).

Figure 2.Effect of rat lung transplant on Catalase (**A**) and SOD activities (**B**) in erythrocytes. Catalase activity is expressed as median (interquartile range) of ρmol per milligram of protein (n=9). SOD activity is expressed as median (interquartile range) of U /mg protein (n=9). The results were analyzed as the percentage of the baseline measurement of the rat (time 0= 100%).

◆(**A**) and (**B**). No significant differences among the times (Friedman test, P>0.05).

Table 1: CL* TRAP, CAT and SOD values are expressed as Median and interquartile range of 9 animals. The results were analyzed as the percentage of the baseline measurement of the rat (time 0= 100%).

* Significantly different relative to the baseline measurements of rat (Friedman test, followed by Multiple Comparisons' Friedman, P=0.03).Table 1: CL, TRAP, CAT and SOD values are expressed as Median and interquartile range

		Time 1	**Time 2***	**Time 3**
CL*	Median	128.19	99.38	123.95
	25%	103.28	84.28	104.67
	75%	130.17	107.27	138.20
TRAP	Median	91.41	56.48	106.99
	25%	84.24	42.81	92.95
	75%	100.00	117.13	109.76
CATALASE	Median	76.00	78.41	82.67
	25%	76.00	63.30	62.50
	75%	77.53	94.58	104.59
SOD	Median	98.62	92.00	89.82
	25%	91.83	63.41	89.18
	75%	100.00	144.82	100.00

FIGURE 1A

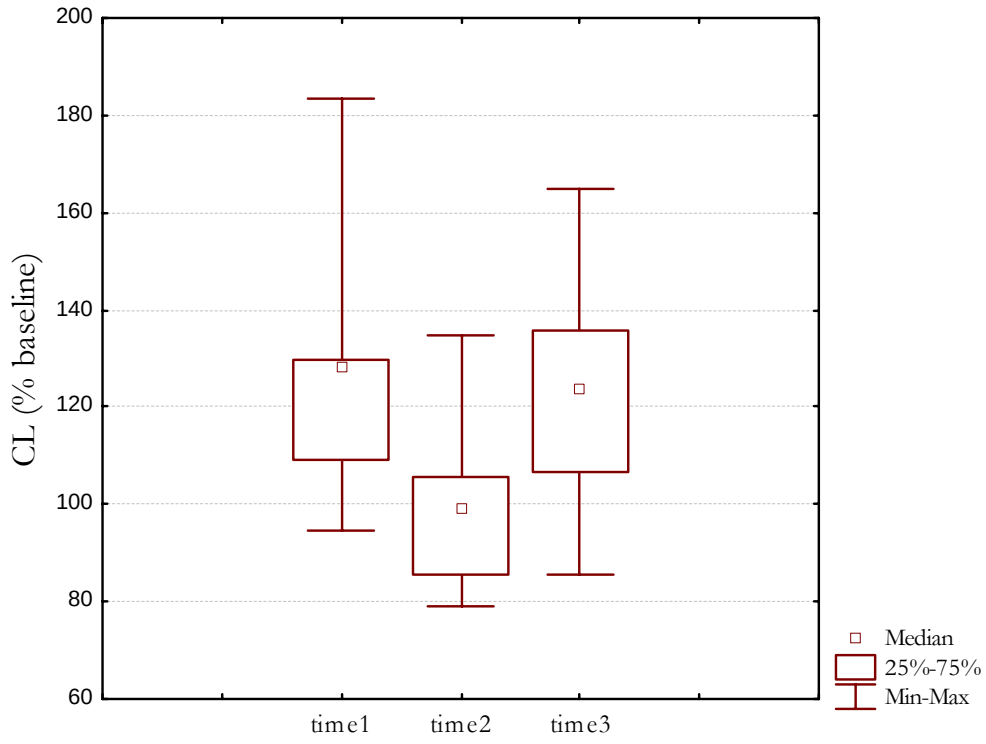


FIGURE 1B

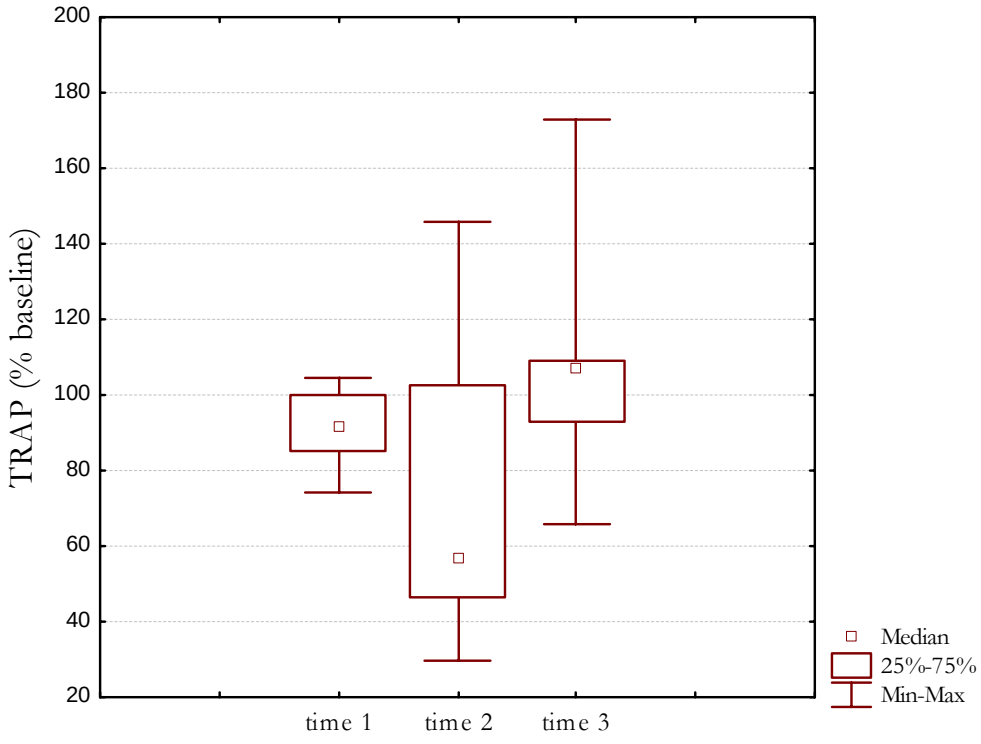


FIGURE 2A

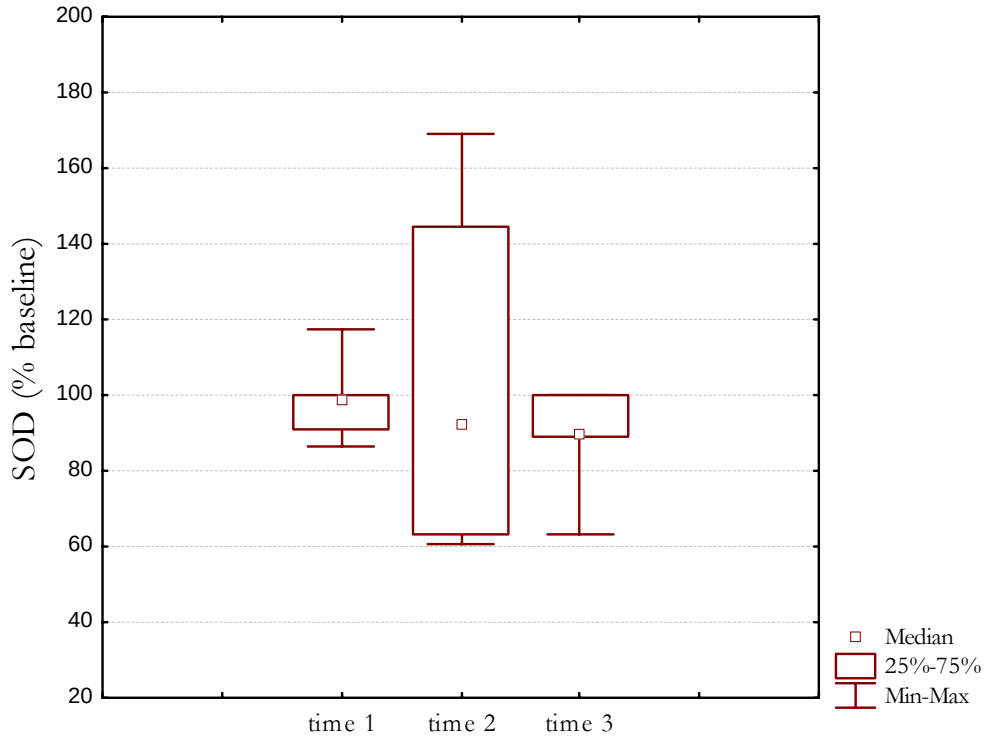
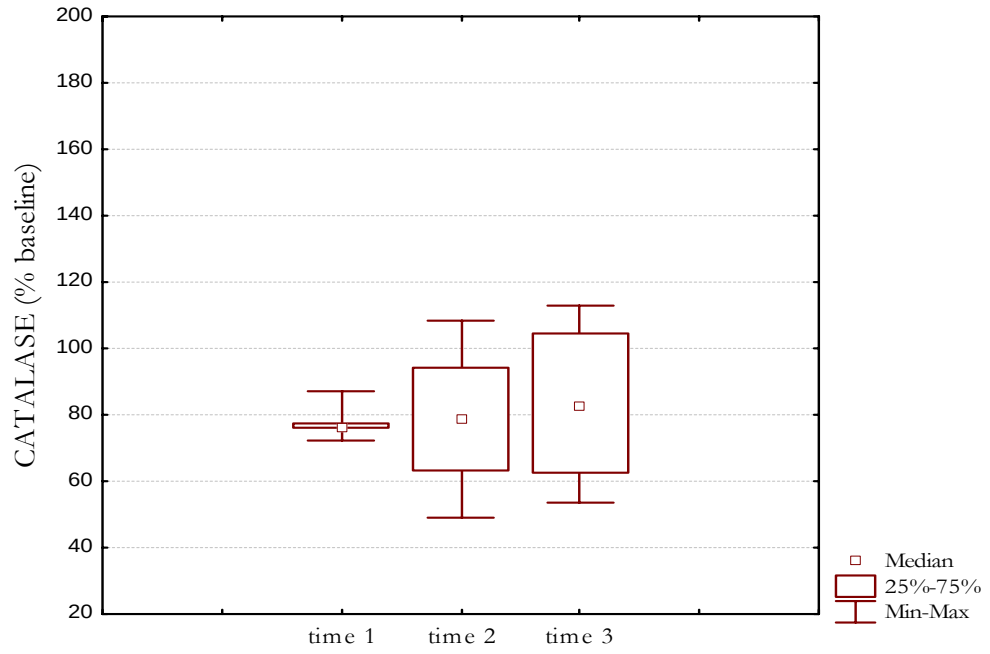


FIGURE 2B



ARTIGO II

EFFECT OF SYSTEMICALLY ADMINISTERED LOW POTASSIUM DEXTRAN SOLUTION ON OXIDATIVE STRESS IN A RAT MODEL OF LUNG ISCHEMIA

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Abstract

Lung ischemia-reperfusion injury induces a systemic inflammatory response and production of reactive oxygen species. Optimal allograft preservation is paramount for successful lung transplantation. *Objective*: Evaluate the effect of the systemic administration of the lung preservation solution low-potassium dextran (LPD) on the peripheral oxidative stress, in an animal model of lung ischemia-reperfusion in rats. *Methods*: In experiment 1, adult Wistar rats were anesthetized, randomized in 2 groups (n=5 each), and given 0.5mL i.v. of LPD or saline (SAL). Arterial blood samples were obtained before, immediately, 15 and 30 minutes after the administration of the solutions; In experiment 2, animals were divided in 4 groups (n=5 each): control (CON), ischemia (ISQ), saline(SAL) and LPD. Except for the CON animals, all groups were submitted to left hilar clamping for 30minutes, followed by reperfusion for 30minutes. The arterial blood samples were obtained before thoracotomy (baseline), immediately after release of a 30 minutes crossclamping, and at 15 and 30minutes of reperfusion. Saline or LPD were administered intravenously iv (0.5mL) to SAL and LPD groups, respectively, immediately before clamp removal. Lipid peroxidation was measured by chemiluminescence (CL) and antioxidant defenses by total radical trapping potential (TRAP) in both experiments to estimate oxidative stress. On experiment 2, catalase (CAT) and superoxide dismutase (SOD) enzyme activities were added. *Results***:** In experiment 1, there were no differences in CL between groups; the LPD group showed an increase in TRAP relative to SAL (P=0.037). In experiment 2, there was an increase in CL in both SAL and ISQ groups compared to CON (P=0.004) and LPD (P=0.018); TRAP showed significant differences over time, among groups (P=0.006; P<0.0001); LPD group showed increased TRAP compared to other groups. There were no differences in CAT and SOD activities between groups. *Conclusion*: Both experiments suggest that LPD has increased antioxidant activity in plasma with and without lung ischemia-reperfusion and has reduced erythrocyte LPO in lung ischemia.

Key words: lung transplantation, free radicals, ischemia- reperfusion

Introduction

Optimal allograft preservation is very important for successful lung transplantation (Kelly,2000). Pulmonary ischemia during donor organ retrieval and transplantation is associated with ischemia-reperfusion injury (I-R), resulting in endothelial cell damage and surfactant dysfunction (Sommer et al., 2004). Some degree of tissue injury is inevitable, regardless of the quality of the organ preservation process. This injury can be exacerbated by multiple factors that can act before harvesting, during preservation, and remain active after implantation (Conte and Baumgartner, 2000). Early lung allograft dysfunction is closely related to ischemia-reperfusion injury,that remains the most common cause of early mortality after lung transplantation, as well as a significant risk factor for the development of bronchiolitis obliterans syndrome (Belperio et al., 2005). Interaction of activated leukocytes with injured graft endothelial cells participates in the development of ischemia-reperfusion injury (Ardehali et al., 2003). This injury is characterized histopathologically by lung edema and neutrophil leukocyte extravasation. The mechanisms recruiting leukocytes into the lung during post-lung transplantation ischemia-reperfusion injury have not been completely understood (Belperio et al., 2005). Studies have suggested that reactive oxygen species (ROS) play an important role in primary graft failure (Conte and Baumgartner, 2000). Oxidative stress is characterized by the formation of ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radical (McCord, 1985). The generation of intra-cellular oxygen species has been found in the majority of lung cells, including the endothelial cells, type II alveolar epithelial cells, clara cells, ciliated airway epithelial cells and alveolar macrophages (Al Mehdi, 1997). Studies on lung preservation focus on local effects following the administration of the preservation solution directly into the pulmonary circulation, using either antegrade or retrograde routes (Keshavjee 1992; Chen 1996). To date, there have been no studies addressing the effects of the lung preservation solution administered systemically on oxidative stress. The objective of this study is to assess the potential effects LPD on oxidative stress when it is administered into the peripheral blood circulation. Such

potential effects were then evaluated in the presence or absence of lung ischemia.

Methods

Adult male Wistar rats (90 days-old, 250-300 g body weight) were used. They were housed in groups of five in cages made of Plexiglas (65 x 25 x 15 cm), with the floor covered with sawdust. They were maintained in a controlled environment (light/dark cycle of 12 h, temperature of 22 $\pm$ 2^{o} C) before the experimental period and were given free access to food (standard lab rat chow) and water. Rats had free access to food (standard lab rat chow) and water. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (http://www.nap.edu/catalog/5140.html).

Experiment 1

Ten animals were anesthetized with intraperitoneal sodium penthotal (0.35ml/100g body weight) injection. Afterwards, they were submitted to orotracheal intubation and mechanical ventilation (Harvard 683-Rodent Ventilator- Harvard Apparatus; FiO2=1.0, tidal volume=1ml/100g body weight, respiratory rate=65 bpm). Indwelling catheters were introduced into the femoral artery and vein after dissection. The animals were then randomly divided into two groups: SAL and LPD (n=5/group): one received iv saline (SAL) (0.5mL). Animals in the LPD group were given the preservation solution - LPD (12.5ml/kg body weight), via the femoral venous catheter. Peripheral blood samples were collected (0,5ml/sample), via the femoral artery at the baseline (time 0), immediately (time1), 15 minutes (time 2) and 30 minutes (time 3) after the administration of the solutions above. Oxidative stress was evaluated using the chemiluminescence (CL) technique, and the antioxidant defenses were determined by the total radical-trapping potential (TRAP) technique, which indicates the total antioxidant potential present in the plasma. For the CL technique, erythrocytes were utilized, whereas only plasma was utilized for TRAP.

Experiment 2

Twenty animals were anesthetized, intubated and canullated, using the same procedures described in experiment 1. Animals were randomly distributed in four groups (n=5/group): control (CON), ischemia (ISCH), saline (SAL), and LPD. With the exception of the CON animals, all other groups were submitted to left hilar clamping for 30 minutes followed by reperfusion for 30 minutes. The arterial blood samples (0,5mL/sample) were obtained at time 0 (baseline=before thoracotomy), time 1 (immediately after release of a 30 minutes crossclamping), time 2 (15 minutes after reperfusion) and time 3 (30 minutes after reperfusion). In the CON group, samples were collected at the same times, although without thoracotomy or clamping. Saline (0,5ml) or LPD (12.5ml/kg body weight) were administered intravenously at room temperature to animals in SAL and LPD groups respectively, immediately before clamp removal. The lipid peroxidation was assessed through CL, and the antioxidant defenses through determination of the TRAP and the enzyme activities (catalase-CAT and superoxide dismutase-SOD). For the catalase and superoxide dismutase activities and the CL, erythrocytes were utilized, whereas plasma used for TRAP.

Blood sample preparation

Blood samples from the peripheral artery were collected for oxidative stress evaluation. Heparinized arterial blood samples were centrifuged at 1000 g for 15 min (Sorval RC 5b-rotor SM 24, DuPont Instruments, USA). Plasma was separated and stored at -70^{o}C for further TRAP determination. The erythrocytes were washed three times in saline solution and centrifuged at 1000g for 3 minutes at room temperature. The supernatant was discarded by aspiration. The washed erythrocytes were used for the lipid peroxidation assay (Repetto et al., 1999). The erythrocytes were diluted 1/10 in 1 mmol/L of acetic acid and 4 mmol/L magnesium sulphate and then stored at -70^{o}C, until analysis for enzyme assays (catalase-CAT and superoxide dismutase-SOD).

Oxidative damage assessment

Chemiluminescence (CL) was measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB – Produkter AB, Sweden) to assess oxidative stress. Blood samples (10 μL) were placed in low-potassium vials, and the background chemiluminescence was measured using 4 mL of 30 mmol/L phosphate buffer and 120 mmol/L of KCl (pH=7.4). Measurements were started by the addition of 400 mmo/L tert-butyl hydroperoxide (final concentration 3mmol/L). Data were expressed as counts per second per milligram of the blood samples' hemoglobin (cps/mg Hb) (Repetto et al., 1999).

Antioxidant defenses

Determination of Total Radical-Trapping Potential (TRAP)

The total antioxidant potential present in the plasma was measured by luminescence, using 2,2'-azo-bis(2-amidinopropane) (ABAP, a source of alkyl peroxyl free radicals) and luminol. The background chemiluminescence was measured using 50 μmol/L ABAP, 40 μmol/L luminol, and 50 mmol/L phosphate buffer (pH=7.4). This emission was almost completely quenched by the addition of Trolox (hydrosoluble vitamin E), rendering induction times that were linearly related to the free radical scavenger concentration added. A calibration curve was obtained by using different Trolox concentrations between 0.5 and 1μmol/L. The addition of plasma samples instead of Trolox elicited an induction time related to the initial amount of sample added (Lissi et al., 1995). Luminescence was measured in a scintillation counter, in the out-of-coincidence mode, and the results were expressed in μmol of Trolox.

Determination of Catalase (CAT)

Catalase activity (CAT) was determined by the evaluation of the decrement of 240-nm absorption of hydrogen peroxide (H_2O_2) in a reaction medium containing 50mmol/L phosphate buffer (pH=7.2), and 10mmol/L hydrogen peroxide (H_2O_2) (Aebi, 1984). Activity was expressed as ρmoles of H_2O_2 reduced per minute per milligram of protein.

Determination of Superoxide Dismutase (SOD)

The measurement of SOD activity, expressed as units per milligram of protein, was based on the inhibition of the superoxide radical reaction with pyrogallol (Marklund, 1985). The SOD activity was determined by measuring the velocity of oxidized pyrogallol formation and the reaction medium contained Tris buffer (50mmol/L, pH=8.20), pyrogallol (24mmol/L), and catalase (30mmol/L). Absorbance changes were observed at 420 nm for 2 min.

Protein determination

Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard (Lowry et al., 1951).

Hemoglobin determination

The conversion of hemoglobin to cyanomethaemoglobin by Drabkin reagent was measured against a standard curve. The values were expressed as mg per mL (mg/mL) (Drabkin and Austin, 1935)**.**

Statistical analysis

Results are expressed as mean $\pm$ SEM. The differences among baseline groups were analyzed using student t test or one-way ANOVA. The comparison of the effects along the different periods of time was done by repeated measures ANOVA, followed by Tukey´s multiple comparisons test when needed. Values of $p<0.05$ were considered significant. The results were analyzed as the percentage of the baseline for the animal (time 0=100%).

Results

Experiment 1

This experiment tested the effect of the preservation solution on peripheral markers of oxidative stress, in vivo, by means of CL and TRAP. CL is currently regarded as a measurement of the level of oxidative free-radicals formed the process of lipid peroxidation. Erythrocyte chemiluminescence was analyzed to evaluate peripheral oxidative damage to membrane lipids. This method is very

sensitive, and capable of detecting small amounts of peroxidative products. A comparison between the groups that received i.v. saline or preservation solution - LPD, showed no significant difference in the CL measurement in the baseline, nor was any difference observed over time or among the groups or time-group interaction (Fig 1A).

TRAP was performed in order to verify whether the non-enzymatic antioxidants were altered. In plasma, as well as in other human tissues, there are substances with antioxidant capacities, including reduced glutathione (GSH), uric acid, ascorbic acid, ceruloplasmin, transferrin, bilirrubin, carotenoids, etc. The relative concentration of these substances determines the total plasma antioxidant capacity. The TRAP technique measures the total antioxidant capacity of the plasma. There were no significant differences in the TRAP measurements at baseline. When the effects were compared over time, a significant difference was found between the groups. Animals that received the preservation solution (LPD) showed a significant increase in TRAP when compared with the CON animals group (27% increase; P=0.037). However, there were no differences over time, as well as no time-group interactions (Fig. 1B). Experiment 1 values are depicted in Table 1.

Experiment 2

Analyzing the baseline, there were no significant differences among the groups for the CL, TRAP, CAT and SOD measurements. Comparing the measurements over the different periods of time, significant differences were observed among the four groups in CL (P=0.004). A comparison between the groups ISCH and SAL showed no significant differences but a significant increase in CL, when compared with the CON animals (17% increase; P =0.004) and with the LPD animals (14% increase; P=0.018). No significant difference was found in animals in the CON and LPD groups. There were also no differences over time and no significant time-group interaction (Fig 2A).

In the TRAP analysis, significant differences were observed among the periods of time (P=0.006), within the groups (P<0.0001), and with a significant time-group interaction (P<0.0001) (Figure 2B). The LPD group demonstrated an

increased TRAP in comparison with the other groups (20% increase compared to the control group; 27% compared to the ischemia group and 11% compared to the saline group).

SOD and CAT enzymes were chosen as they represent the first step in the enzymatic detoxification of ROS, considered as primary defenses. Almost 3 to 10% of the oxygen utilized by tissues is converted to a ROS, such a superoxide radicals, which impair the function of cells and tissues. Superoxide dismutase (SOD) catalyzes the conversion of single electron reduced species of molecular oxygen to hydrogen peroxide and oxygen. Catalase (CAT) catalyzes the conversion of hydrogen peroxide to water and oxygen. No significant difference was found in the activity of these enzymes (Fig 2C and 2D). The results of experiment 2 are shown in Table 2.

Discussion

The results of the present study showed that the presence of LPD preservation solution increased the total antioxidant potential in the plasma, both in the presence and absence of the lung ischemic event. It was also observed that there was a decrease in erythrocyte LPO in the presence of lung ischemia. In experiments 1 and 2, we found no differences between LPD and Control groups in the lipid peroxidation, as evaluated by CL. In experiment 2 there was an increase in LPO in the animals submitted to lung ischemia, and it was not modified after the parenteral administration of saline. On the other hand, it was modified by the administration of LPD solution, which caused the LPO to reduce down to levels similar to those found in the Control animals. When TRAP was evaluated in experiments 1 and 2, a consistent and significant increase in its values was observed in the LPD group, when compared to the other groups. Such a finding may be indicative of the anti-oxidant properties of LPD, even when used at room temperature in the peripheral circulation at low doses which, in our study was approximately 25% of the dose administered into the pulmonary circulation during flush. This dose was established by trial and error in the pilot study (unpublished data), in which higher doses caused the animals to die. Conversely, the antioxidant enzymatic activities of CAT and SOD were

similar when both groups and periods of time of measurement were compared. The use of intracellular solutions (high potassium concentration), such as University of Winconsin (UW) and Euro-Collins (EC), may contribute to cellular injury during lung procedure. Studies have demonstrated increased pulmonary vascular resistance in lungs preserved with EC (Kawahara et al., 1991). Vasodilators such as calcium-channel blockers, prostaglandins and prostacyclin have been used in conjunction with EC to improve graft tolerance to ischemia-reperfusion injury, by reducing pulmonary vasoconstriction in experimental models (Sasaki et al., 1999a; Sasaki et al., 1999b; Hachida and Morton, 1988). The pulmonary vasculature is known to respond to high serum potassium concentration with membrane depolarization and subsequent vigorous vasoconstriction (Unruh et al., 1990)**.** Exposing the pulmonary artery smooth muscle cells to preservation solutions with a high potassium concentration causes a sustained membrane depolarization that reflects a decrease in outward changes in potassium exchange. Such an exchange is affected by both the steep concentration gradient and the inhibition of voltage-gated potassium channels (Kelly et al., 2003). Previous studies have demonstrated the benefits of LPD (extracellular low potassium solution) in decreasing the incidence of primary graft failure after transplantation in the clinical setting (Fischer et al., 2001; Struber et al., 2001). The physiological factors have pointed to less tissue edema, reduced pulmonary vascular resistance and better oxygenation compared to historical controls using lungs preserved with EC or UW solutions. In fact, experimentally, there is evidence of ROS production in the transplanted lung. Furthermore, lower lipid peroxidation has been demonstrated in lungs preserved with LPD. However, the mechanism by which an extracellular low potassium solution reduces lung ischemia-reperfusion injury is not fully understood (Kelly et al., 2003). In contrast, with the high potassium solutions, the LPD does not cause membrane depolarization, therefore the cells flushed with LPD remain at, or near, resting membrane potential. Since the membrane depolarization does not occur, there is no stimulus for vasoconstriction, which explains the relatively normal pulmonary vascular resistance noted in physiologic studies. This indicates stability of the resting potential of the

pulmonary artery smooth muscle cells in the presence of a low potassium concentration preservation solution. This solution may provide a more appropriate ionic milieu that minimizes cell injury or activation, as indicated by the reduction of vasoconstriction or ROS production (Kelly et al., 2003). The role of the potassium concentration in lung preservation solutions is important, since potassium channels have many functions in the regulation of pulmonary vascular tone. Although there are many modulators of pulmonary circulation, the regulation of hypoxic vasoconstriction by voltage-gated potassium channels suggests that the potassium concentration of lung preservation solution may dynamically alter pulmonary vasculature tone. Hypoxic pulmonary vasoconstriction occurs when hypoxia inhibits voltage-gated potassium channels in pulmonary artery smooth muscle cells, leading to the depolarization and entry of calcium through the L-type calcium channels (Weir and Archer, 1995). It is possible that a similar mechanism may activate reactive oxygen species (ROS) production from the pulmonary vasculature (Kelly et al., 2003). High potassium preservation solutions used in lung transplantation increase the production of ROS from the pulmonary arteries. Therefore, high potassium concentrations may inhibit the potassium current and depolarize the pulmonary artery smooth muscle cell and also act as a signal for the production of ROS. Nicotinamide-adenine dinucleotide (NADH/NADPH) oxidase activation through membrane depolarization is a potential pathway of ROS production (Fischer et al., 2001; Struber et al., 2001). Although the signaling or the pathway of ROS production is not yet completely understood, the use of a low potassium concentration in lung preservation solution seems to decrease the incidence of primary graft failure through the reduction of ROS production in the pulmonary vasculature (Kelly et al., 2003). Based on the findings of CL and TRAP measurements in the current experiments, we suggest that LPD itself may have an intrinsic antioxidant potential mediated either by one of its components or by a conjunction of the benefits of an extracellular solution. On the other hand, LPD may be capable of inducing increased endogenous antioxidant potential by as yet unknown mechanisms.

In conclusion, the present study supports the hypothesis that ischemia-reperfusion injury mediated by ROS might be attenuated by the use of LPD. Such properties of LPD may play a role in the apparent ability of this solution in reducing graft failure. The potential benefits to the lung graft itself were not addressed in our study. Future studies are therefore required to compare the antioxidant potential of LPD with other lung preservation solutions in order to verify whether these features are specific for LPD or not.

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LEGENDS OF FIGURES

Figure 1. Effect of systemic administration of saline or LPD on CL (1A) and TRAP* (1B) levels. CL values are expressed as mean ± SEM of cps/mg Hb (n=5/group). TRAP is expressed as mean ± SEM of equivalents in µM trolox (n=5/group). The results were analyzed as the percentage of the baseline measurement in the animal.

(1A) No significant difference between the groups (P>0.05). No significant differences among the different periods of time (P>0.05), nor in the time-group interaction (P>0.05).

(1B) *Significant difference between the saline and LPD groups (P=0.037). No significant differences among the different periods of time (P>0.05), nor in the time-group interaction (P>0.05).

Figure 2. Effects of the systemic administration of saline or LPD solutions upon CL, TRAP, SOD and CAT activities, on the lung ischemia-reperfusion injury model. CL* values (2A) are expressed as mean ± SEM of cps/mg Hb (n=5 /group). TRAP** values (2B) are expressed as mean ± SEM of equivalents µM trolox trolox (n=5/ group). SOD activity is expressed as mean ± SEM of U SOD/mg protein (n=5/group) (2C). Catalase activity is expressed as mean ± SEM of nmol per milligram of protein (n=5/group) (2D). The results were analyzed as the percentage of the baseline measurement in the animal.

(2A)* The groups ISCH and SAL showed significant difference when compared with the CON animals (17% increase; P =0.004) and with the LPD animals (14% increase; P=0.018). No significant difference was found in animals in the CON group in relation to the LPD group, and in the group ISCH in relation to the group SAL. There were also no differences over time and no significant time-group interaction (Fig 2A).

(2B**)** **Significant difference among times (P=0.006), groups (P<0.0001), and the time-group interaction (P<0.0001). # Significantly increase LPD group in relation the others groups.

(2C and 2D) No significant differences among times, groups, nor for the time-group interaction (P>0.05).

Experiment 1

Table 1: CL and TRAP expressed as mean ± SEM

Group (n=5)	Time	CL	TRAP*
Saline	1	103.30 ± 7.27	62.02 ± 10.41
	2	100.20 ± 19.59	48.66 ± 10.85
	3	134.14 ± 35.80	50.70 ± 12.87
Perfadex®	1	97.26 ± 2.11	80.02 ± 6.39
	2	100.83 ± 10.07	83.23 ± 6.51
	3	115.57 ± 16.91	94.32 ± 13.83

Experiment 2

Table 2: CL, TRAP, CAT and SOD expressed as mean ± SEM

Groups (n=5)	Time	CL*	TRAP**	CAT	SOD
Control	1	90.37 ± 4.51	95.89 ± 7.77	132.97 ± 7.25	121.84 ± 18.80
	2	83.06 ± 9.69	83.69 ± 5.29	84.36 ± 3.87	96.52 ± 18.47
	3	100.74 ± 2.57	85.55 ± 3.69	78.07 ± 5.68	83.51 ± 16.07
Saline*	1	113.31 ± 4.52	111.50 ± 6.01	95.11 ± 4.51	97.77 ± 1.41
	2	114.65 ± 5.38	90.41 ± 1.73	89.17 ± 3.10	119.38 ± 7.91
	3	119.44 ± 7.07	103.59 ±10.95	76.88 ± 9.31	94.95 ± 5.60
Ischemia*	1	113.80 ± 5.75	94.84 ± 4.26	113.80 ± 12.75	109.94 ± 13.43
	2	102.33 ± 3.75	79.54 ± 9.36	80.26 ± 4.64	121.07 ± 14.49
	3	100.94 ± 6.80	58.35 ± 1.81	87.45 ± 7.18	93.50 ± 11.06
LPD**	1	95.44 + 4.57	106.56 ± 7.40	176.95 ± 37.43	85.10 ± 5.59
	2	95.85 ± 5.45	93.98 ± 3.95	168.39 ± 36.43	93.63 ± 7.81
	3	95.56 ± 5.20	153.25 ±10.13	135.43 ± 26.14	98.56 ± 9.44

FIGURE 1A

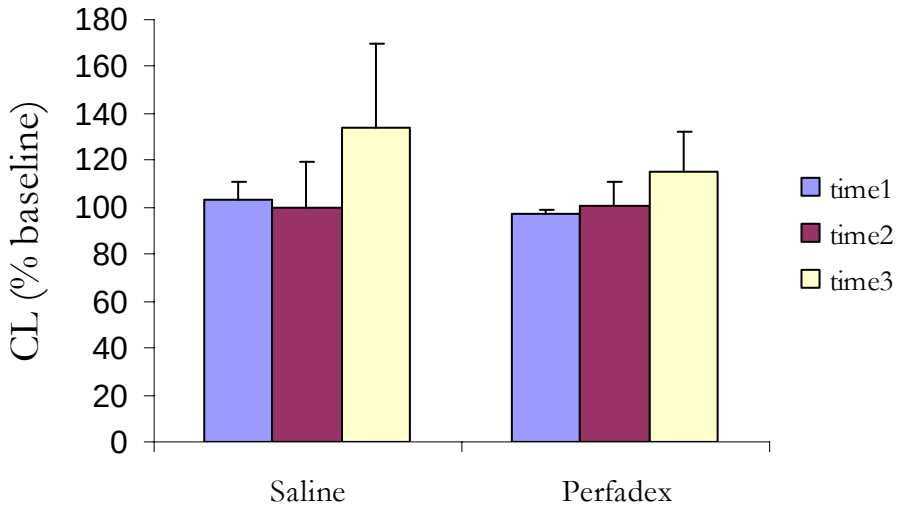


FIGURE 1B

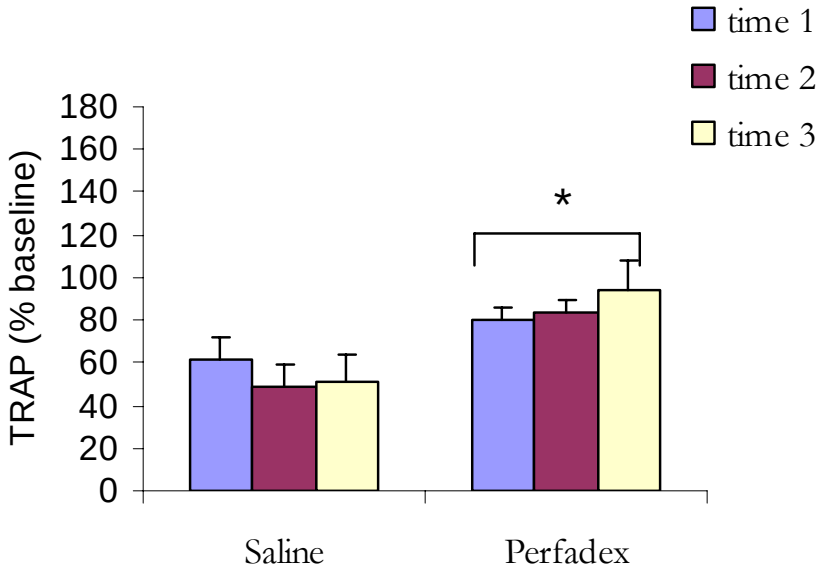


FIGURE 2A

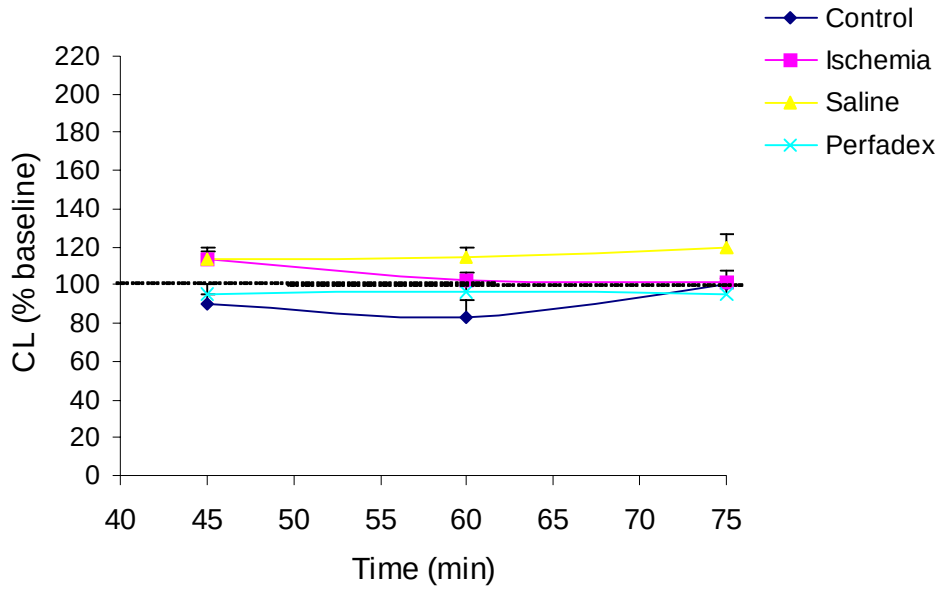


FIGURE 2B

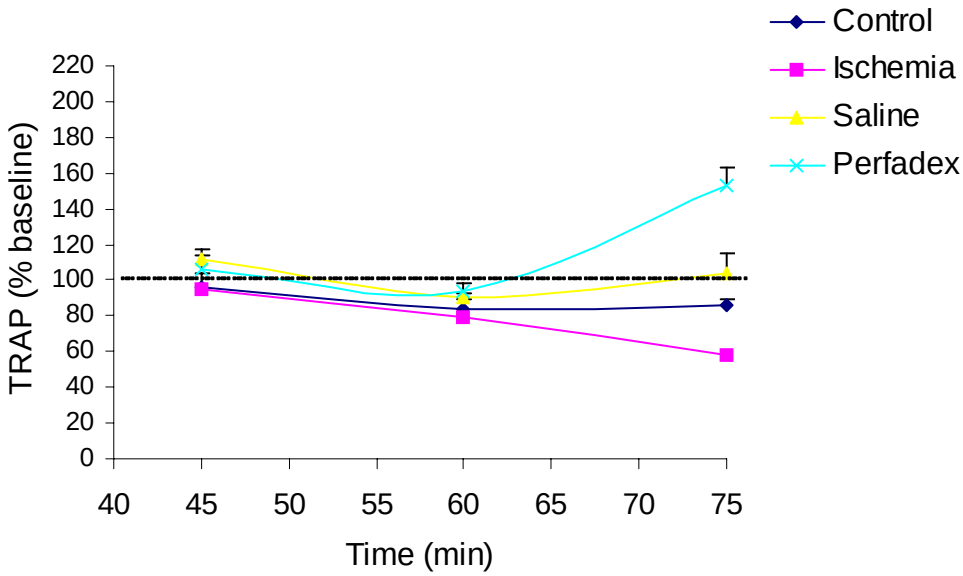


FIGURE 2C

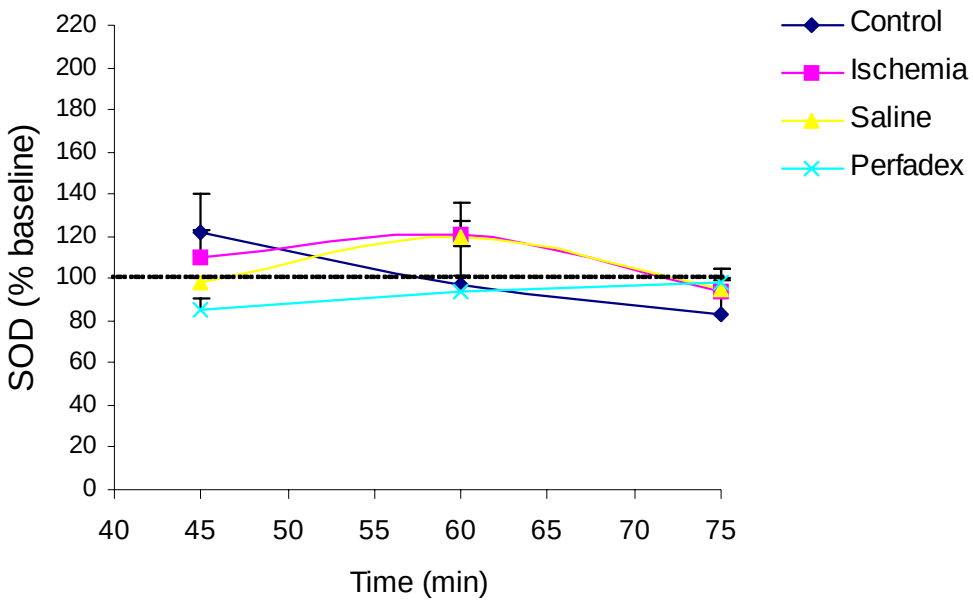
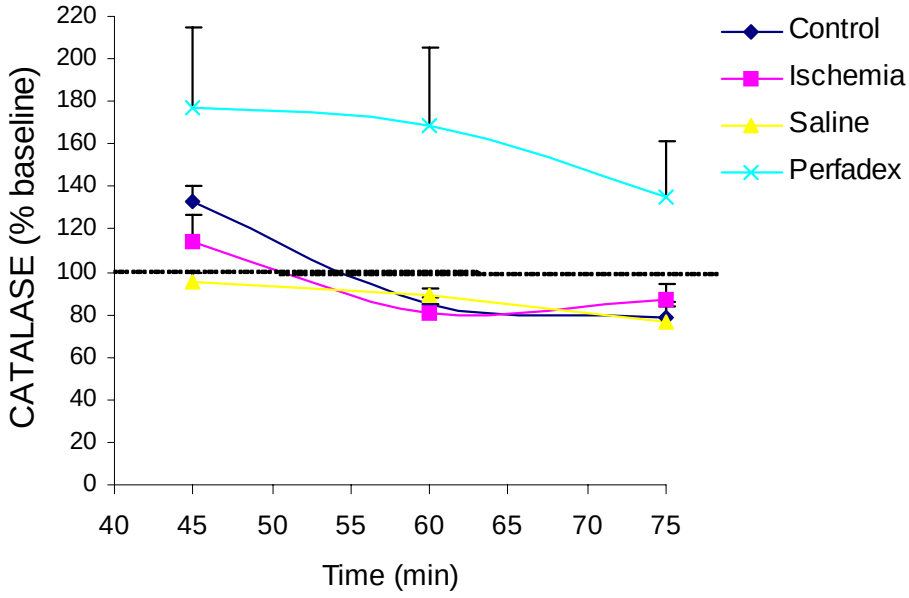


FIGURE 2D



ARTIGO III

PULMONARY OXIDATIVE STRESS UNDER DIFFERENT TREATMENT REGIMENS WITH METHYLPREDNISOLONE

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ABSTRACT

Studies have shown that prolonged use of glucocorticoids may cause overproduction of reactive oxygen species (ROS) in the vascular endothelium. Conversely, when used in bolus in all donors after brain death declaration, they have protective effects on the vascular endothelium, increasing lung donor recovery. The aim of the present study was to verify the level of lipid peroxidation (LPO) and antioxidant defenses in lungs of rats submitted to different treatment regimens with glucocorticoid. Adult male Wistar rats were submitted to the administration of methylprednisolone as a single dose of 50 mg/kg, i.p. (acute), and as an oral dose of 6 mg/kg for 15 days (sub-chronic) or 30 days (chronic). At the end of the treatments, oxidative damage, measured by chemiluminescence(CL), and antioxidant defenses, measured by total radical-trapping antioxidant potential (TRAP), were evaluated in rat lungs. Results demonstrated that lipid peroxidation (CL) was increased by chronic treatment (37,5%), and TRAP was increased (20,4%) by the acute exposure to a high dose of methylprednisolone. Findings suggest that acute treatment with methylprednisolone does not induce pulmonary oxidative damage and improve pulmonary defense system, whereas chronic treatment may induce oxidative damage, which could be related to the adverse effects of this drug.

Key words: glucocorticoid, chemiluminescence, TRAP, free radicals, lungs, lipid peroxidation.

Introduction

Glucocorticoids (GC) have been widely used for the treatment of patients with various disorders, including autoimmune, allergic, and lymphoproliferative diseases. However, chronic treatment GC can cause a variety of symptoms and signs, including truncal obesity, moon face, cutaneous striae, hirsutism, cataract, osteoporosis, myopathy, diabetes mellitus, immunossuppression, and cardiovascular disorders. This excess may also elicit the overproduction of reactive oxygen species (ROS) from endothelial cells (Iuchi et al.,2003); in turn, disturbing nitric oxide (NO) availability in the vascular endothelium, leading to vascular complications (Takahiko et al., 2003). Glucocorticoids are well known as anti-inflammatory agents, and some of their effects may be mediated by interference with ROS, which are products of normal metabolic processes in cells. The major sources of these species are leakages from the electron transport chain of mitochondria and endoplasmic reticulum. Putatively, another important source of ROS is a membrane-associated nicotinamide-adenine dinucleotide (NADH/NADPH) oxidase. At low concentrations, ROS act as physiological mediators of cellular responses and regulators of gene expression (Sanner et al., 2002). Oxidative stress has been implicated as an important pathologic factor in cardiovascular (Singal et al.,2000), pulmonary, and autoimmune diseases, inherited metabolic disorders, cancer and aging (Alho et al.,1998; Pettenuzzo et al., 2003; Latini et al.,2003; Heffner and Repine,1989). It is well known that ROS generate a biochemical cascade, producing lipid peroxidation, protein oxidation, DNA damage and cell death, and that they can contribute to the occurrence of pathological conditions (Liu et al.,1996 ; Liu and Mori,1999). In the lungs, ROS have been implicated in initiating inflammatory responses through the activation of transcription factors such as nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1), causing chromatin remodeling and gene expression of pro-inflamatory mediators (Rahman et al., 2004). Lung injury induced by ischemia-reperfusion (I-R) after lung transplantation is associated with a marked increase in ROS and other free radicals, thus playing a crucial role in the cascade of events leading to lung failure (Hefner and Repine, 1989). It has also been observed that the in bolus use of

glucocorticoids in all donors after brain death declaration, can improve arterial oxygen tension (PaO_2), increase lung donor recovery, and provide protective effects on the vascular endothelium (Follette et al., 1998).

In the present study, we evaluated the effect of acute, sub-chronic and chronic treatments with methylprednisolone upon oxidative stress by means of oxidative damage (chemiluminescence-CL) and an antioxidant defenses test (total radical-trapping potential-TRAP) in rat lungs.

Material And Methods

Animal

Sixty adult male Wistar rats (60 days old; 200-250 g) were used, housed in groups of five in cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust. Animals were maintained in a controlled environment (light/dark cycle of 12 h, temperature of 22 $\pm$ 2^{o} C) before and throughout the experimental period. Rats had free access to food (standard lab rat chow) and water. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (http://www.nap.edu/catalog/5140.html).

Chemicals

It was used methylprednisolone sodium succinate (Solu-Medrol®, Pharmacia). The lyophilized powder (500 mg) dissolved in 8 ml of 0.9% NaCl. The drug solution was prepared immediately before the administration.

Acute administration

The animals were divided into two groups (n=10 each). The control group received saline, and treatment group in which the animals were treated with methylprednisolone (50 mg/kg) as a volume of 1ml/kg of the solution administered i.p.

Sub-chronic and chronic administrations

The animals were divided into two groups (n=10 each): control that receives water and treated that received methylpredinisolone (6 mg/kg, p.o.) which was prepared as described above and was added to the drinking water for 15 days - sub-chronic treatmen or for 30 days - chronic treatment. Each 500 ml of water contained 31 mg of methylprednisolone sodium succinate (0.0625 mg/ml). Considering a mean consumption of 25 ml/rat/day, each rat consumed 1.56 mg/day of methylprednisolone, equivalent to 6 mg/kg.

Biochemical analysis

The animals were killed by decapitation, 24h following drug administration (acute) or at the end of the treatment (sub-chronic and chronic schedules). Lungs were extracted and frozen by immersion in liquid nitrogen. Samples were stored at -80ºC until analysis. The lungs were weigthed and homogenized 1:5 w/v in ice-cold (1.15% KCl and 20mmol/l phenyl methyl sulphonyl fluoride, PMSF) in Ultra-Turrax. The homogenates were centrifuged at 1000 *g* for 20 min at 0-4ºC to remove the particulate fraction and the supernatant was used for chemiluminescence, TRAP and protein content assays (Llesuy et al.,1985).

Total Radical-Trapping Antioxidant Potential (TRAP): This technique was performed in order to verify if the antioxidants were altered. In human tissues, there are substances with antioxidant capacity. The relative concentration of these substances determines the total tissue antioxidant capacity.TRAP represents the total antioxidant capacity of the tissue and was determined by measuring the luminol chemiluminescence intensity induced by thermolysis of 2-2'-azobis(2-amidinopropane) dihydrochloride (ABAP). The background chemiluminescence was measured using 4000 μl of 10 mmol/L ABAP and 10 μl of 4 mmol/l luminol, both in 100 mmol/l glycine buffer, pH=8.6. The addition of 10 μl of 80 μmol/l Trolox (hydrosoluble vitamin E) or 10 μl of tissue homogenate (diluted 1:4) to the incubation medium reduces chemiluminescence. The time necessary to return to the levels observed before the addition was considered as the induction time (I.T.). The I.T. is directly proportional to the antioxidant

capacity of the tissue (Evelson et al., 2001), and it was compared to the I.T. of Trolox. Results were expressed as μM Trolox/mg protein.

Chemiluminescence (CL): The CL is this technique that evaluates the oxidative stress and is currently used as a measurement of lipid peroxidation. The method is very sensitive and capable of detecting small amounts of peroxidative products.CL was measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB – Produkter AB, Sweden) to assess oxidative stress. Homogenates were placed in low-potassium vials at a protein concentration of 0.5-1.0 mg of protein/ml in a reaction medium consisting of 120 mmol/l KCl, 30 mmol/l phosphate buffer (pH=7.4). Measurements were started by the addition of 3 mmol/L *tert*-butyl hydroperoxide, and data were expressed as counts per second per milligram of protein of the homogenate (cps/mg protein) (Gonzalez-Flecha et al.,1991).

Determination of protein concentration

Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Statistical analysis

The data were expressed as mean ± S.E.M. and statistically evaluated using analysis of Student´s *t* test. The values were considered significantly different when the P value was less than 0.05.

Results

The effect of acute treatment with methylprednisolone upon TRAP levels and chemiluminescence in rat lungs was first evaluated. A significant increase (20,4%) was observed in the pulmonary total radical-trapping antioxidant potential (TRAP) in the treated group (*P*=0.032) (Fig.1A). There was no significant difference in the chemiluminescence measurement between the groups (Fig.1B).

Secondly (sub-chronic treatment), a more prolonged period of administration of methylprednisolone (15 days) was evaluated; this treatment did not modify the pulmonary antioxidant defense, as assessed by pulmonary total radical-trapping antioxidant potential (TRAP) (Fig.2A), nor did it alter lipid peroxidation, as assessed by chemiluminescence ($P>0.05$) (Fig.2B). Finally, chronic (30 days) treatment with methylprednisolone showed no diference in the pulmonary total radical-trapping antioxidant potential (TRAP) (Fig. 3A) and caused a significant increase in the pulmonary oxidative damage, as assessed by chemiluminescence (CL) (37,5%) ($P< 0.0001$) (Fig.3B).

Discussion

The acute treatment with methylprednisolone demonstrated a significant increase in pulmonary total radical-trapping antioxidant potential (TRAP) in rat lungs without any change in chemiluminescence (CL). When the animals were submitted to a more prolonged administration (for 15 days) with a lower dose, this effect was not observed. Furthermore, when the treatment was maintained for 30 days, there was an increase in pulmonary oxidative damage. Previous studies have suggested that short-term administration of GC could have protective effects upon oxidative injury in different tissues and experimental models. Follette and colleagues (1998) showed that a bolus of steroids (approximately 15 mg/kg of methylprednisolone) administered to all donors after brain death can improve PaO_2 and increase lung donor recovery. The steroid bolus can potencially reduce the inflammatory reaction and compensate the deficit in hypophyseal hormones observed after brain death (de Perrot et al., 2003). Previous studies have reported that glucocorticoids also have protective effects on the vascular endothelium, such as inhibition of apoptosis mediated by lipopolysaccharide and tumor necrosis factor-α (TNFα) (Messmer et al., 1999), and inhibition of the endothelial inflammatory response (Susuki et al., 2000; Wheller; Perretti, 1997). Antinflamatory glucocorticoid drugs, such as dexamethasone, inhibit the expression of a number of cytokines, including TNFα, interleukin-6(IL-6), interleukin-8(IL-8), as well as E-selectin, intercellular

adhesion molecule-1(ICAM-1), and inducible nitric oxide synthase (iNOS) in various cell types (Barnes, 1998; McKay and Cidlowski, 1999).

Short-term administration of prednisolone and dexamethasone inhibited oxygen radical generation in platelets and recent literature indicates that steroids may inhibit oxidative phosphorylation (Sanner et al., 2002). Ozmen (2005) showed that dexamethasone phosphate may affect oxidative stress by changing antioxidant enzymatic activities. The molecular actions of methylprednisolone (MP) indicate that pretreatment with this drug may be cardioprotective against ischemic and oxidative damage, improves left ventricular function and increases coronary flow during postischemic reperfusion. The activities of heart tissue antioxidant enzymes (catalase and glutathione peroxidase) were increased during the reperfusion of MP-treated hearts (Valen et al., 2000a). The significant increase in plasma nonenzymatic antioxidants (uric acid and total antioxidant capacity) in association with the enhanced enzymatic antioxidant activity (SOD in heart) during short-term corticosterone administration indicates preventive changes to counteract oxidative injury (Lin et al., 2004). Glucocorticoid pretreatment reduces inflammation, increases cardiac heat shock protein 72 (HSP72) and protects cardiac function against ischemia-reperfusion injury (Valen et al., 2000b). A single low-dose of methylprednisolone (10mg/kg) reduces the inflammatory reaction during and after cardiopulmonary bypass (CPB), by inhibition of proinflammatory cytokine release and ROS generation after release of the aortic cross-clamp (Bourbon et al., 2004). Schmidt et al., (2002) showed an important influence of steroids on antioxidative cellular functions in neuronal and glial cells. Anderson and Means (1985) suggested that, at least in experimental animals, the effectiveness of methylprednisolone in preventing tissue necrosis and paralysis after spinal cord trauma may reside in part, in its capacity to quench peroxidative reactions in the injured tissue. These findings agree with our results. As observed in other tissues, the acute treatment with a high dose of methylprednisolone caused an increase in defense antioxidants (TRAP) in rat lungs. When the animals were exposed to a lower dose of methylprednisolone for longer periods (30 days of treatment), there was an increase in lipid peroxidation in the lungs, expressed as an

increase in CL, in contrast to the response observed with acute treatment. These data suggest that chronic glucocorticoid treatment induces lipid peroxidation, increasing the risk of stress injury in lungs.

In conclusion, the total radical-trapping antioxidant potential (TRAP) in lungs is increased by the acute exposure to a high dose of methylprednisolone, and lipid peroxidation is increased by chronic exposure to methylprednisolone. Our results, in lung tissue, agree with previous findings in the vascular endothelium, showing a protective effect of acute GC administration. These results corroborate the suggestion that acute treatment of the donor organ with GC could improve the graft's conditions for lung transplantation. Conversely, the effect of this drug on lipoperoxidation may play a role in the development of the adverse effects associated with chronic GC use.

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LEGENDS TO FIGURES

Fig. 1. Effect of acute methylprednisolone administration on TRAP(A) and chemiluminescence (B) levels in rat lungs. TRAP is expressed as mean ± SEM of equivalents in µMTrolox/mg protein (n=10/group).Chemiluminescence is expressed as mean ± SEM of counts per second per milligram protein of the homogenate (cps/mg protein) (n=10/group).

*Significant difference between the groups in TRAP levels (Student's *t* test, *P*= 0.032).

Fig. 2. Effect of sub-chronic administration (15 days) on TRAP (A) and chemiluminescence (B) levels in rat lungs. Chemiluminescence is expressed as mean ± SEM of counts per second per milligram protein of the homogenate (cps/mg protein) (n=10/group). TRAP is expressed as mean ± SEM of equivalents in µMTrolox/mg protein (n=10/group).

No significant difference between the groups in CL or TRAP levels (Student's *t* test, *P*>0.05).

Fig. 3. Effect of chronic administration on TRAP (A) and chemiluminescence (B) levels in rat lungs. The results were expressed as mean ± SEM . TRAP is expressed as equivalents in µMTrolox/mg protein (n=10/group). Chemiluminescence is expressed as counts per second per milligram protein of the homogenate (cps/mg protein) (n=10/group).

Significant difference between the groups in CL levels (Student's *t* test, *P*<0.0001).

Figure 1A

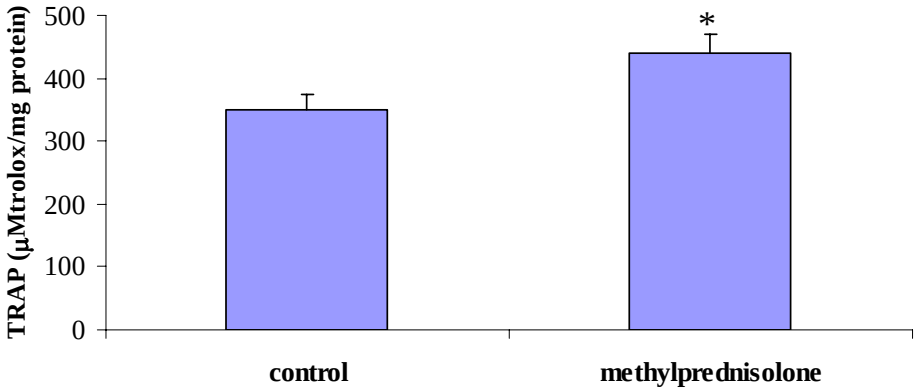


Figure 1B

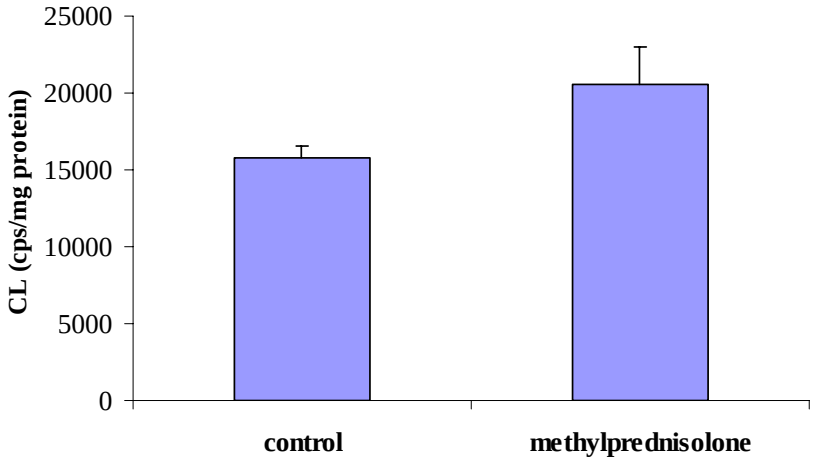


Figure 2A

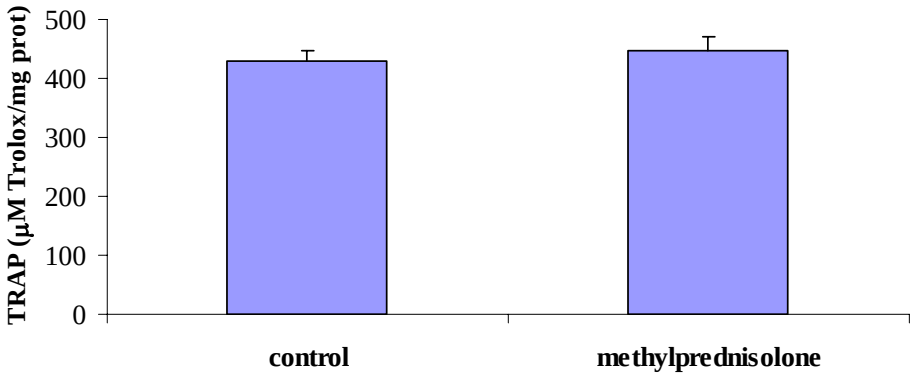


Figure 2B

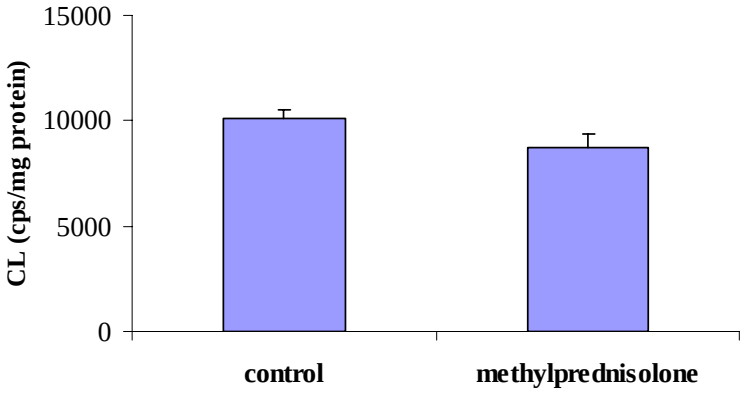


Figure 3A

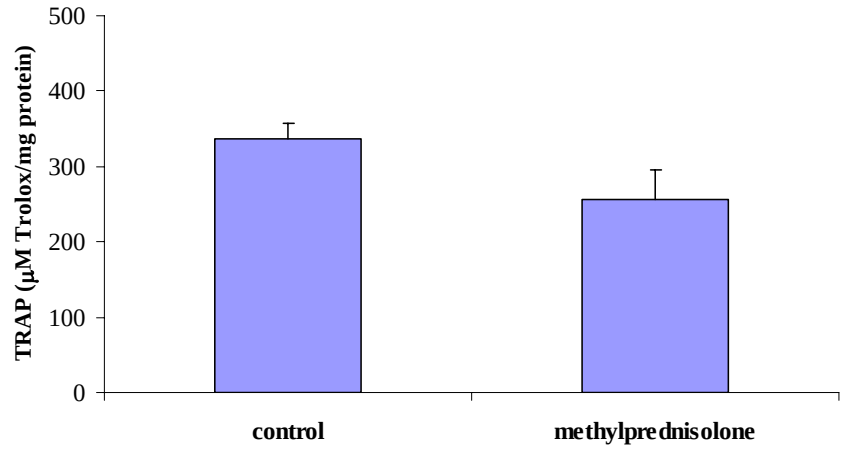
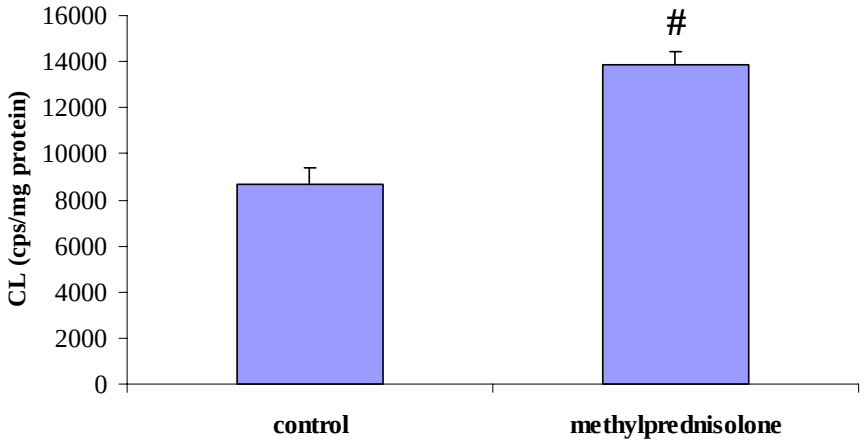


Figure 3B



CONCLUSÃO

- ✓ O uso da técnica de quimiluminescência (CL) para avaliar o dano oxidativo (peroxidação lipídica), e de técnicas de medida de defesa antioxidante, como TRAP (*total radical trapping antioxidant potential*), Catalase (CAT) e Superóxido Dismutase (SOD) avaliadas em eritrócitos, plasma ou tecido pulmonar, conforme os experimentos realizados mostraram-se adequadas, seguras e reproduzíveis.
- ✓ A isquemia pulmonar, por clampeamento do hilo, teve como efeito o aumento do estresse oxidativo periférico, medido por CL em eritrócitos.
- ✓ A administração IV da solução preservadora "*Low Potassium Dextran"* (LPD) preveniu o aumento da CL induzido pela isquemia, não havendo diferença desta medida em relação ao grupo controle (sem isquemia pulmonar).
- ✓ A administração IV da solução preservadora LPD, em situação com ou sem isquemia pulmonar, levou a um aumento significativo das defesas antioxidantes plasmáticas, medido pela técnica de TRAP.
- ✓ Catalase (CAT) e Superóxido Dismutase (SOD) nas situações avaliadas não mostraram alteração significativa.
- ✓ O uso agudo de metilprednisolona aumenta as defesas antioxidantes em tecido pulmonar; enquanto que o uso crônico aumentou o dano oxidativo tecidual (CL).
- ✓ No uso endovenoso da solução preservadora LPD, observamos presença de efeito antioxidante.

Palavras-chaves: Transplante pulmonar, estresse oxidativo, metilprednisolona, solução preservadora – LPD.

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