UNIVERSIDADE FEDERAL DE UBERLÂNDIA
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MECANISMOS MOLECURARES ENVOLVIDOS NA MODULAÇÃO DO SGLT1 PULMONAR EM MODELO MURINO DE SEPSE EXPERIMENTAL

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Área de concentração: Ciências da Saúde

Orientador: Robinson Sabino da Silva
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À minha mãe, Geralda Mendes Cardoso,

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"Porque ainda que a figueira não floresça, nem haja fruto na vide; ainda que decepcione o produto da oliveira, e os campos não produzam mantimento; ainda que as ovelhas da malhada sejam arrebatadas, e nos currais não haja gado;
Todavia eu me alegrarei no Senhor; exultarei no Deus da minha salvação.
O Senhor Deus é a minha força, e fará os meus pés como os das cervas, e me fará andar sobre as minhas alturas."

Habacuque 3:17-19

RESUMO

Introdução: A infecção respiratória pode ser exacerbada pela alta concentração de glicose no líquido da superfície das vias aéreas (ASL). **Objetivo:** O objetivo deste estudo foi investigar o papel da atividade do SGLT1 na concentração de glicose presente no ASL e pulmão de ratos com sepse. **Material e métodos:** A sepse foi induzida em ratos Wistar machos por ligação e perfuração cecal (CLP) 24 horas antes da coleta de amostras. Os animais Sham e CLP foram tratados intranasalmente com solução salina, salbutamol (para aumentar a atividade de SGLT1) ou florizina (para diminuir a atividade de SGLT1). Após 2 horas os animais foram anestesiados para análise dos parâmetros ventilatórios (volume corrente, frequência respiratória e volume-minuto) e coleta de lavado broncoalveolar (BAL) e pulmão. A concentração de proteínas foi medida no BAL. Os danos histopatológicos, imunohistoquímicos e estresse oxidativo foram analisados no pulmão. Os resultados foram expressos como média ± SEM e comparados por ANOVA/Newman-Keuls (p< 0.05). **Resultados:** Atelectasia e inflamação brônquica não estavam presentes no pulmão dos animais Sham mas estavam presentes nos animais CLP-sal. O tratamento com salbutamol reduziu os escores de inflamação brônquica e promoveu hiperinsuflação em ratos CLP. A florizina promoveu um aumento nos escores de atelectasia, inflamação brônquica e danos nas vias aéreas associados à aumento na frequência respiratória em animais CLP. A sepse, salbutamol e florizina não alteraram os níveis de estresse oxidativo, no entanto, a atividade da catalase no pulmão aumentou em CLP-sal em comparação com o grupo Sham. A florizina bloqueou o aumento de catalase em ratos CLP. O salbutamol translocou o SGLT1 para a membrana plasmática de pneumócitos em animais CLP. **Conclusão:** Em conjunto, a sepse promove atelectasia e inflamação pulmonar associada ao aumento da enzima antioxidante catalase. Além disso, nossos dados indicam que a inibição da função de SGLT1 com florizina aumentou a gravidade da atelectasia e da inflamação brônquica associada com maior taxa respiratória e inibição de catalase no pulmão de ratos CLP, sugerindo que SGLT1 poderia participar na modulação de efeitos pulmonares nos estágios iniciais de sepse.

Palavras chave: SGLT1. Salbutamol. Florizina. Infecção respiratória. Sepse. Pulmão.

ABSTRACT

Introduction: Respiratory infection can be exacerbated by high glucose concentration in the airway surface liquid (ASL). **Objective:** the objective of this study was to investigated the role of SGLT activity on ASL glucose concentration and lung of rats with sepsis. **Material and methods**: Sepsis was induced in rat Wistar male by cecal ligation and puncture surgery (CLP) 24 hours before samples collection. Sham and CLP rats were intranasally treated with saline, salbutamol (to increase SGLT1 activity) or phlorizin (to decrease SGLT1 activity). After 2 hours, animals were anesthetized to analyze ventilation parameters (tidal volume, respiratory rate and minute volume) and collect bronchoalveolar lavage (BAL) and lung. Protein concentration were measured in BAL. Histopathological damages, immunohistochemistry and oxidative stress levels were analyzed in lung. The results were expressed as mean ± SEM and compared with ANOVA/Newman-Keuls test ($p < 0.05$). **Results:** Atelectasis and bronchial inflammation were not present in lung of control rats and were present in CLP-sal rats. Salbutamol treatment reduced bronchial inflammation scores and promoted hyperinsuflation in CLP rats. However, phlorizin,increasesd atelectasis, bronchial inflammation and airway damage scores associated with respiratory rate in CLP rats. Sepsis, salbutamol and phlorizin do not change oxidative stress levels, however,the activity of catalase in lung increased in CLP-sal compared with Sham rats. Phlorizin blocked the increase of catalase in CLP rats. The salbutamol also provided the SGLT1 translocation to the plasma membrane of pneumocytes in CLP animals. **Conclusion:** Taken together, the sepsis promotes atelectasis and lung inflammation associated with increased in antioxidant enzyme catalase. Besides, our data indicate that inhibition of SGLT function with phlorizin increased the severity of atelectasis and bronchial inflammation associated with higher in respiratory rate and inhibition of catalase in lung of CLP rats, suggesting that SGLT could participate in the modulation of earlier stage of septic effects in lung.

 Keywords: SGLT1. Salbutamol. Phlorizin. Respiratory infection. Sepsis. Lung.

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LISTA DE ABREVEATURAS E SIGLAS

AMPc	Monofostato Cíclico de Adenosina
ASL	Líquido de superfície das vias aéreas
BAL	Lavado broncoalveolar
β-AR	Receptor β-adrenérgico
CLP	Ligação e perfuração cecal
CLP-sal	Ratos Wistar sépticos tratados com solução salina
CLP-salb	Ratos Wistar sépticos tratados com salbutamol
CLP-phlo	Ratos Wistar sépticos tratados com florizina
FR	Frequência Respiratória
GLUTs	Família de transportadores de glicose por difusão facilitada
LPA	Lesão Pulmonar Aguda
mm	milímetro
MRSA	Staphylococcus aureus resistente à meticilina
PKA	Proteína quinase A
SDRA	Síndrome do desconforto respiratório agudo
SGLT1	Cotransportador de Na^+/glicose/H_2O tipo 1
SGLTs	Família de cotransportadores de glicose acoplado ao sódio
Sham	Ratos Wistar controle
SIRS	Síndrome da Resposta Inflamatória Sistêmica
SOFA	Avaliação sequencial de Falência Orgânica
TBARS	Substâncias Reativas ao Ácido Tiobarbitúrico

UTI	Unidade de Terapia Intensiva
VC	Volume Corrente
VM	Volume Minuto

Sumário

1- Introdução

A sepse é caracterizada como uma síndrome clínica resultante de uma resposta inflamatória sistêmica, relacionada a um foco de infecção, podendo apresentar severidade variada. Em quadros mais graves os pacientes podem progredir para o choque séptico e a falência múltipla dos órgãos (HOTCHKISS; KARL, 2003; LEVY et al., 2003). Em decorrência da heterogeneidade dos pacientes em sepse há um grande investimento para que se desenvolva meios de diagnósticos precoce e tratamentos eficientes (ANGUS et al., 2001).

Dados do *Brazilian Sepsis Epidemiological Study* mostram que no Brasil os pacientes possuem um maior tempo de internação, além de apresentarem gravidade aumentada em comparação à outras regiões no mundo. Pacientes sépticos possuem uma alta taxa de mortalidade, além de gerarem custo muito elevado com seu tratamento nos setores público e privado (SALES-JÚNIOR et al., 2006).

A sepse pode promover lesão pulmonar aguda e síndrome do desconforto respiratório agudo (SDRA). Na lesão pulmonar aguda são encontrados infiltrado inflamatório bilateralmente e hipoxemia arterial e a síndrome do desconforto respiratório agudo é caracterizada por uma inflamação aguda mediada por neutrófilo acompanhada comumente de aumento de citocinas inflamatórias e estresse oxidativo (YEH et al., 2005).

O epitélio das vias pulmonares possui uma membrana luminal que é recoberta pelo líquido de superfície das vias aéreas (ASL) (WIDDICOMBE; WIDDICOMBE, 1995). Os pacientes com concentrações aumentadas de glicose no ASL possuem um maior risco de infecção brônquica por bactérias patogênicas (PHILIPS et al., 2005).

Em humanos, a glicose é a principal fonte de energia desempenhando papel fundamental no metabolismo celular (SCHEEPERS; JOOST; SCHURMANN, 2004). Devido a sua característica de polaridade, a glicose não pode atravessar a bicamada lipídica da membrana plasmática das células. Desta forma, o transporte da glicose é realizado por meio de proteínas carreadoras localizadas na membrana plasmática. Estas proteínas carreadoras, chamadas de transportadores de glicose, podem ser divididas em duas famílias: transportadores de glicose por difusão facilitada (GLUTs) e cotransportadores de Na^+/glicose (SGLTs) (BELL et al., 1990; CARRUTHERS, 1990).

Os SGLTs fazem parte da família de proteínas transportadoras de soluto acoplado ao Na$^+$ (KONG; YET; LEVER, 1993). Entre os SGLTs, o cotransportador de Na$^+$/glicose/H$_2$O tipo 1 (SGLT1) é o principal subtipo presente no pulmão. Neste território, o SGLT1 já foi descrito em superfície de pneumócitos tipo I (BODEGA et al., 2010) e tipo II (BOYD, 1990). A proteína SGLT1 é codificada pelo gene SLC2A1 e funcionalmente transporta 2 íons Na$^+$, uma molécula de glicose e 264 moléculas de H$_2$O em cada ciclo (ZEUTHEN, 2000; WRIGHT; HIRAYAMA; LOO, 2011). A direção do transporte de glicose pelo SGLT1 pela membrana plasmática ocorre devido à força motriz produzida pelo gradiente de Na$^+$ que é mantida pela bomba Na$^+$/K$^+$. Desta forma, promove o transporte do Na+, da glicose e da água sempre do meio extracelular para o meio intracelular (WRIGHT; HIRAYAMA; LOO, 2007). O SGLT1 é composto por 14 segmentos transmembrana com a face N-terminal voltada para o interstício e a face C-terminal ancorada no interior da membrana plasmática (WRIGHT; TURK, 2004).

Levando em consideração que o SGLT1 é translocado por meio da proteína quinase A (PKA), que é ativada pela interação de agonistas β-adrenérgicos com seus receptores, o presente estudo avaliou o papel do salbutamol (agonista β2-adrenérgico) e da florizina (inibidor de SGLTs) nos parâmetros ventilatórios, danos histopatológicos e estresse oxidativo de ratos portadores de sepse.

2- Fundamentação teórica

2.1- Epitélio pulmonar

Uma das principais funções do pulmão é permitir as trocas gasosas. As estruturas pulmonares se dividem em zona condutora e zona respiratória, sendo a primeira as responsáveis por permitir o movimento de entrada e saída de ar entre a atmosfera com o pulmão e a segunda é o local de troca gasosa entre o pulmão e o sangue (SALDIVA, 1990).

Figura 1: Organização do sistema pulmonar
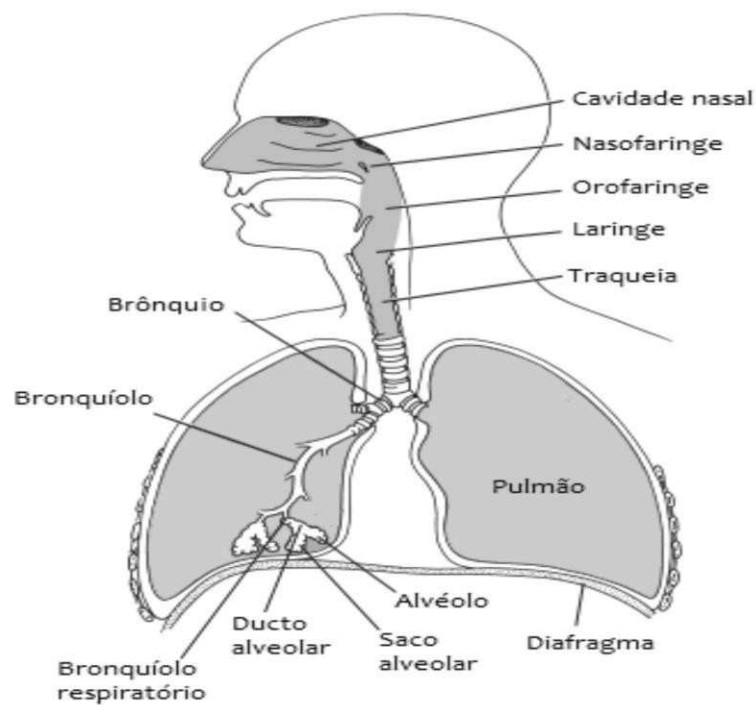
Fonte: Adaptado de ROSS; PAWLINA, 2012.

A zona condutora é composta pela fossa nasal, nasofaringe, laringe, traqueia, brônquios, bronquíolos e bronquíolos terminais e a zona respiratória é composta por bronquíolos respiratórios, ductos alveolares, sacos alveolares e alvéolos (Fig.1) (LECHNER; MATUSCHAK; BRINK, 2013). A estrutura histológica do epitélio pulmonar é composta por uma camada celular contínua (QU et al., 2005; ROSS; PAWLINA, 2012). Nas vias aéreas de maior calibre esse epitélio é pseudoestratificado cilíndrico ciliado associado com células caliciformes, e torna-se colunar ou cuboide nas vias aéreas de menor calibre. Deste modo, os

tipos celulares que compões este epitélio são: colunar ciliado, em escova, basal, caliciforme e granular (Fig. 2) (CRYSTAL et al., 2008; JUNQUEIRA; CARNEIRO, 2011).

Figura 2: Estrutura Histológica do epitélio pulmonar.
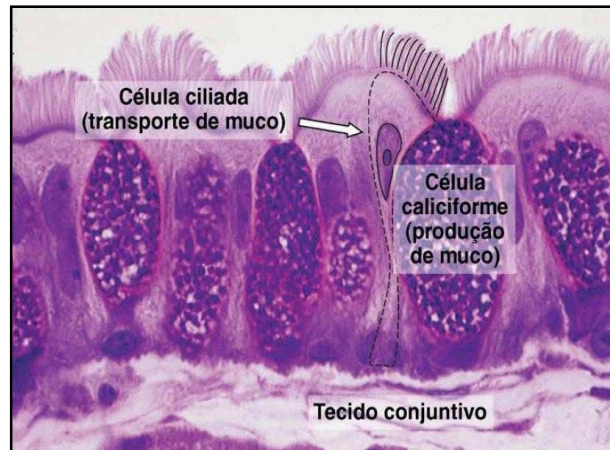
Fonte: Modelo adaptado de JUNQUEIRA; CARNEIRO, 2011.

O sistema respiratório possui bronquíolos e alvéolos que estão diretamente relacionadas ao seu funcionamento (Fig.3). Os bronquíolos possuem um calibre menor com diâmetro médio de 1mm e são as ramificações de menor calibre da árvore brônquica. No bronquíolo o epitélio é inicialmente cilíndrico simples ciliado, passando a cúbico simples, podendo ou não ser ciliado, com sua porção final não apresentando cartilagem, glândulas ou nódulos linfáticos (JUNQUEIRA; CARNEIRO, 2011; ROSS; PAWLINA, 2012).

Figura 3- Epitélio respiratório.

Fonte: http://www.unifesp.br/morfo/histologia/ensino/pulmao/histologia.htm
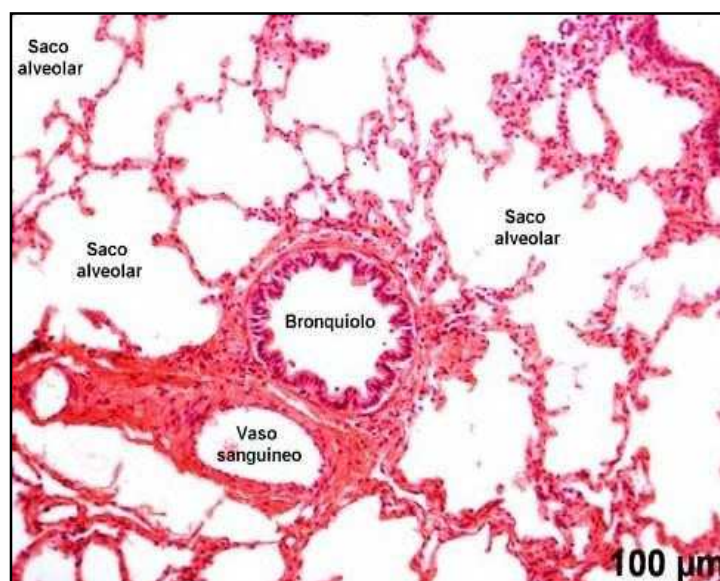
2.1.1- Líquido de superfície das vias aéreas

A membrana do epitélio pulmonar possui um revestimento denominado líquido de superfície das vias aéreas (ASL) (WIDDICOMBE; WIDDICOMBE, 1995). O ASL é uma solução aquosa composta por cerca de 95% de água; 1% de sais inorgânicos; 0,5-1% de lipídeos; 0,5-1% de proteínas livres e 1-2% de glicoproteínas. O ASL apresenta duas camadas, sendo a primeira e mais densa denominada muco ou fase gel, composta principalmente por mucinas, e a segunda menos densa e mais profunda, denominada de líquido pericilar ou fase sol (HOUTMEYERS et al., 1999; ROGERS, 2007).

Desse modo, a umidificação do ar inalado, lubrificação do epitélio respiratório, inativação de agentes agressores exógenos e condução desses agentes para fora das vias aéreas por meio do transporte mucociliar são funções do ASL (ROGERS, 2007). A funcionalidade do transporte mucociliar é totalmente dependente a fatores ligados aos cílios, composição do muco e interação adequada entre cílios e muco (HOUTMEYERS et al., 1999).

2.1.2-Volumes pulmonares

Os volumes pulmonares podem ser estáticos ou dinâmicos. Os volumes estáticos são compartimentos pulmonares, resultantes da complementação das manobras respiratórias, e os volumes dinâmicos são aqueles decorrentes de manobras respiratórias forçadas. O volume corrente, volume inspiratório e expiratório de reserva, capacidade inspiratória e capacidade vital são volumes de determinação direta, que podem ser medidos por espirometria. O volume residual não pode ser avaliado da mesma forma, necessitando de exames específicos como a pletismografia. Levando em consideração que o comportamento mecânico do pulmão é fundamentado em suas propriedades elásticas e em seu volume, a mensuração de tais volumes oferece informações que são essenciais para a caracterização fisiopatológica decorrente de anormalidades dos processos pulmonar-ventilatórios (BARRETO, 2002). Os volumes pulmonares são convencionalmente divididos em três volumes primários denominados como:

Volume Corrente (VC): É a quantidade de ar inspirada ou expirada espontaneamente em cada ciclo respiratório, ou seja, a cada respiração normal, também pode ser chamado de volume tidal.

Frequência Respiratória (FR): Número de movimentos respiratórios por minuto.

Volume Minuto (VM): Quantidade total de ar novo que entra nas vias respiratórias a cada minuto e equivale a VCxFR.

2.2- Transportador de Glicose

Considerando que a glicose é a fonte principal de energia para organismos eucarióticos, seu transporte se torna foco de diversos estudos. Devido às características de uma molécula polar e insolúvel, a glicose depende de proteínas transportadoras para atravessar a membrana plasmática e atingir o citoplasma. Entre essas proteínas encontra-se o cotransportador de Na^+/glicose/água SGLT1 (Fig. 4) que promove o transporte de 2 íons de Na^+, uma molécula de glicose e 264 moléculas de água a cada ciclo (ZEUTHEN, 2000; WRIGHT; HIRAYAMA; LOO, 2007; THORENS; MUECKLER, 2010; WRIGHT; HIRAYAMA; LOO, 2011). O SGLT1 apresenta 14 segmentos transmembrana com a face N-terminal voltada para o interstício e face C-terminal ancorada no interior da membrana plasmática (WRIGHT; TURK, 2004). O processo de captação de glicose do ASL para interior do pneumócito pode ocorrer por meio do SGLT1, que já teve sua expressão confirmada na superfície de pneumócitos do tipo I, que constitui cerca de 95-97% da superfície alveolar, e do tipo II, distribuído em cerca de 3-5% do epitélio alveolar (SAUMON; MARTET; LOISEAU, 1996).

Figura 4: Estrutura secundária do cotransportador SGLT1.
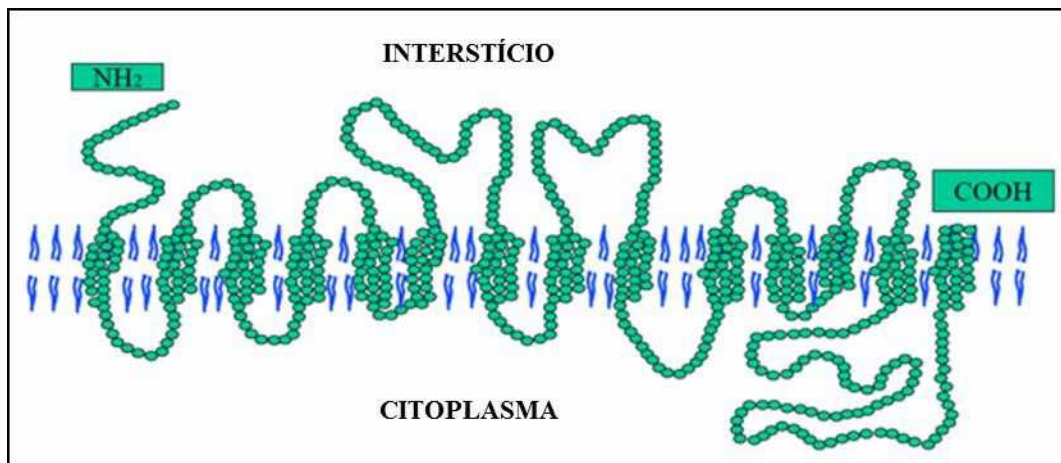
Fonte: Modelo adaptado de WRIGHT; TURK, 2004.

2.2.1- Modulação do SGLT1

Em situações fisiológicas o SGLT1 é modulado por meio da proteína quinase A (PKA) em órgãos tais como intestino (ZIMMERMAN et al., 2012), glândulas salivares (SABINO-SILVA et al., 2010) e pulmão (OLIVEIRA, 2016). Isso ocorre pela ativação da proteína G

acoplada ao receptor β-adrenérgico que irá desencadear a produção de segundo mensageiro (AMP cíclico) pela enzima adenilatociclase. O AMP cíclico promoverá a ativação da PKA e a translocação do SGLT1 para a membrana plasmática celular, aumentando desse modo a absorção de Na^+, glicose e água (de PROST. SAUMON, 2007). Nosso grupo de pesquisa demonstrou que o bloqueio do SGLT1 por meio de florizina, aumenta o volume e a concentração de glicose do ASL. Neste mesmo trabalho demonstramos que isto facilita a proliferação de *Staphylococcus aureus* resistente à meticilina (MRSA) e *Pseudomonas aeruginosa* neste território (OLIVEIRA, 2016). No entanto, as regulações gênicas e funcionais do SGLT1 pulmonar em situações de sepse permanecem desconhecidas.

2.2.2- Florizina

Dentre as drogas que possuem capacidade de inibição do transporte de glicose, usa-se a florizina, um glicosídeo derivado de plantas que atua como inibidor específico, através do mecanismo de competição, da atividade dos SGLTs, como ferramenta para investigação da regulação do transporte de glicose no organismo (RAJA; TYAGI; KINNE, 2003). Ela é um membro da família dos dihidrocalcones extraídos em altas concentrações na macieira e sua estrutura química (Fig. 5) é constituída por uma molécula de glicose e dois anéis aromáticos unidos por um radical alquila (EHRENKRANZ et al., 2005). É sabido que o mecanismo de inibição do transporte de glicose por meio da florizina é saturável e eletrogênico (EHRENKRANZ et al., 2005).

Figura 5: Estrutura molecular e tridimensional da Florizina
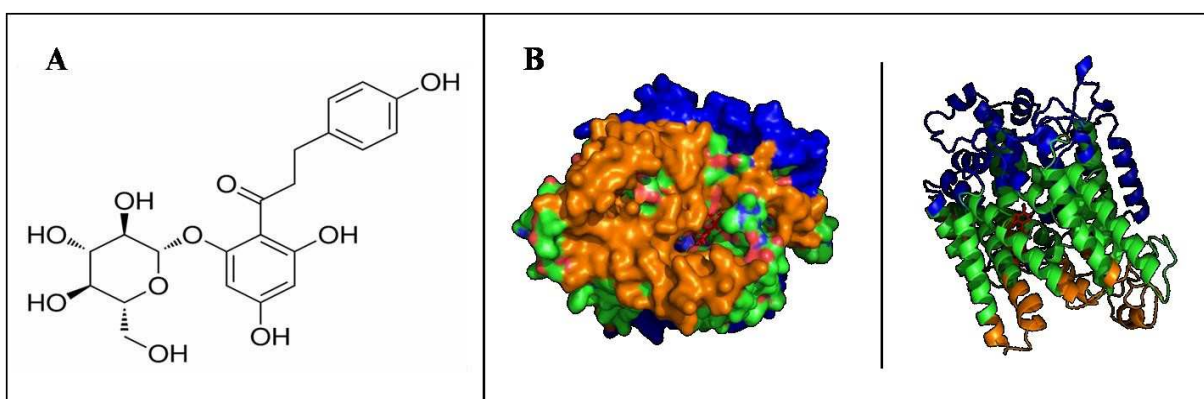
**A.** Fórmula Molecular: $C_{21}H_{26}O_{11}$. Fórmula química: glicose, 1-2-(beta-D-oxiglicopiranosil)-4,6-diidroxifenil-3-(4 hidroxifenil)-1-propanona. Peso molecular: 454.41 g/mol. Fonte: Sigma-Aldrich. **B.** Descrição: em vermelho: florizina e em azul, verde e laranja: *loops* extracelulares, transmembranares e citoplasmáticos do SGLT1, respectivamente. Fonte: modelo adaptado de MELO, et al., 2016.

2.2.3- Salbutamol

No pulmão ocorre a expressão de receptores do tipo β- adrenérgicos (β-AR) sendo que 90% deles encontram-se nos alvéolos. Sabe-se que os subtipos β_1 e β_2 são distribuídos de forma uniforme nas paredes alveolares, porém, os receptores tipo β_2 tem predominância de 70% de sua superfície (CARTAIRS; NIMMO; BARNES, 1985).

Os agonistas β_2 são broncodilatadores seletivos que possuem uma curta duração e efeito dose-dependente (VIEIRA et al., 2000; GONZÁLES-MUNOZ et al., 2009). Eles atuam sobre receptores específicos levando a uma elevação da concentração de AMPc intracelular que ativam canais de potássio e a abertura de tais canais hiperpolariza a célula, inibindo o influxo de cálcio e, em consequência, promovendo uma broncodilatação (AMNTÉA et al., 2002). O salbutamol (Fig.6) é um fármaco pertencente a esta classe de broncodilatadores vastamente utilizado na terapêutica para asma brônquica e possui tempo de meia-vida plasmática de 4-6 horas, promove um relaxamento muscular das vias aéreas (BAI; MAK; BARNES, 1992; SAKUMA et al., 1997; DONOVAN et al., 2014), inibe a liberação de mediadores de mastócitos, monócitos e pode aumentar a eliminação de muco (RANG et al., 2004).

Figura 6: Estrutura química do salbutamol
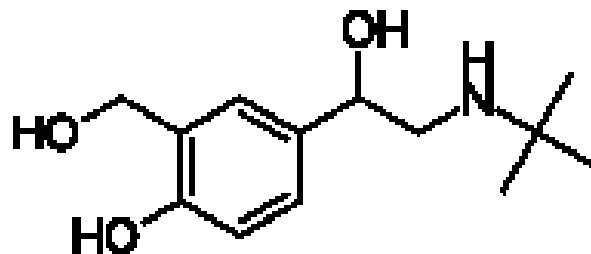
Fonte: modelo adaptado de RANG et al., 2004.

2.3- Sepse

A sepse apresenta uma acentuada resposta sistêmica com uma diversidade de distúrbios imunológicos e fisiológicos (MOHR et al., 2012) e, por esta razão, a terapia continua sendo um desafio para a clínica médica. Além disso, a evolução da Síndrome da Resposta Inflamatória Sistêmica (SIRS) para sepse, choque séptico e falência múltipla de órgãos e os mecanismos que levam a isto necessitam de maior elucidação para que se entenda sobre a progressividade e alterações nos diversos sistemas envolvidos (HOESEL; GAO; WARD, 2006).

Por definição a sepse é uma síndrome clínica resultante de uma resposta inflamatória sistêmica, relacionada a um foco de infecção, podendo apresentar severidade variada e, de acordo com o III Consenso Internacional de Sepse e Choque séptico realizado em 2016, a sepse é definida como uma alteração pontual no índice de Avaliação sequencial de Falência Orgânica (SOFA) (Tabela 1) sendo que SOFA ≥ 2 reflete risco de morte por volta de 10%.

Tabela 1: Índice de Avaliação Sequencial de Falência Orgânica.

Sistema	Score 0	1	2	3	4
Respiratório					
-PaO2/FiO2 mmHg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) com suporte respiratório	<100 (13.3) com suporte respiratório
Coagulação					
- Plaquetas x10³/µL	≥150	<150	<100	<50	<20
Fígado					
- Bilirrubina, mg/dL (µmol/L)	<1.2 (20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12.0 (204)
Cardiovascular	MAP≥70 mm Hg	MAP<70 mm Hg	Dopamina <5 ou dobutamina (qualquer dose)	Dopamina 5.1-15 ou epinefrina ≤0.1 ou norepinefrina ≤0.1	Dopamina >15 ou epinefrina >0.1 ou norepinefrina >0.1
SNC					
- Escala de Coma de Glasgow	15	13-14	10-12	6-9	<6
Renal					
- Creatinina mg/dL (µmol/L)	<1.2 (110)	1.2-1.9 (110-170)	2.0-3.4 (171-299)	3.5-4.9 (300-440)	>5.0 (440)
- Débito urinário mL/d				<500	<200

Fonte: Adaptado de SINGER, 2016.

Foi demonstrado que a mortalidade por sepse é diretamente proporcional à gravidade da síndrome. No mundo a taxa de mortalidade por SIRS corresponde a 7% dos pacientes, por sepse 16%, na sepse grave 20% chegando a atingir 46% no choque séptico. Esses dados demonstram a importância de um diagnóstico precoce e uma terapêutica adequada (ENDO et al., 2008). Outros estudos epidemiológicos indicam taxa de mortalidade de 35% em pacientes sépticos e esse valor atinge 60% quando a sepse está associada a doenças com estadiamento avançado ou choque séptico (COHEN, 2009). No Brasil os valores na taxa de mortalidade por sepse grave e choque séptico seguem as tendências mundiais onde o somatório de mortes em Unidades de

Terapia Intensiva (UTIs) de hospitais públicos e privados chega a 65,7% em determinadas regiões, tais valores podem ser observados na Figura 7.

Figura 7: Taxa de mortalidade por sepse grave e choque séptico em UTIs de hospitais públicos, privados e no Brasil no período de 2005-2015.
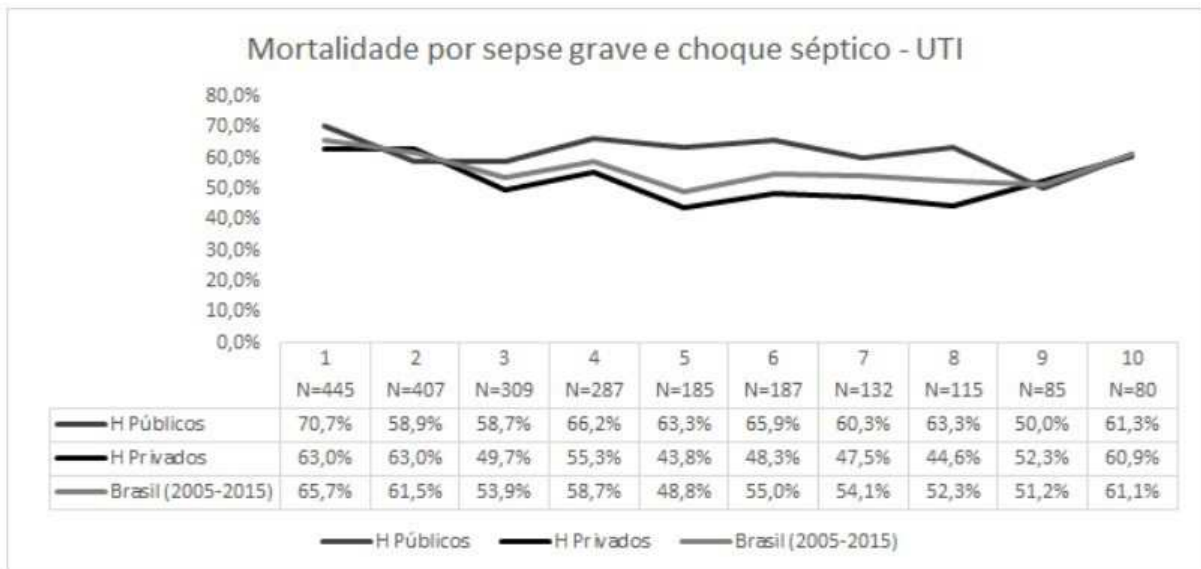
Fonte: Banco de dados ILAS 2005-2015 - ILASonline®

Em pacientes internados em UTI's com necessidade de ventilação mecânica, a alta concentração de glicose no líquido de superfície das vias aéreas aumenta o risco de infecções brônquicas causadas por bactérias patogênicas como MRSA aumente (PHILIPS et al.,2005) e, deste modo, agrave o quadro clínico do paciente.

2.3.1-Síndrome do desconforto respiratório agudo e sepse

O pulmão é um território afetado frequentemente na sepse, onde pode desenvolver lesão aguda pulmonar (BHARGAVA & WENDT, 2012). Essa lesão pulmonar é caracterizada por edema pulmonar e infiltrado de células inflamatórias como os neutrófilos que, consequentemente, promovem redução das trocas gasosas. Em formas severas, o agravamento da hipóxia pode levar à falência múltipla de órgãos (HOTCHKISS & KARL, 2003; FILGUEIRAS et al., 2012).

No estado de sepse, o volume de ASL encontra-se aumentado e sua composição alterada, ocasionando uma redução de trocas gasosas, prevalência de colônias, principalmente bacterianas bem como infiltração de células inflamatórias. Estudos apontam que quando o ASL

apresenta alta concentração de glicose, o risco de infecção respiratória é maior (BAKER et al., 2006; KORNUM et al., 2007).

2.3.2- Estresse Oxidativo

Entre as disfunções metabólicas relatadas durante a sepse incluem-se as alterações de metabolismo lipídico e a oxidação dos ácidos graxos e tais alterações resultam em consequências que são prejudiciais ao paciente (SZTEFKO et al., 2001). Alguns trabalhos relatam que em casos de sepse grave ocorre uma grande infiltração de células inflamatórias e aumento dos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) no pulmão (GONÇALVES-DE-ALBUQUERQUE et al., 2016). No entanto, outros estudos revelam níveis normais de tais substâncias na lesão pulmonar aguda secundária à sepse (CRACIUN et al., 2014; THEOBALDO et al., 2013).

Os níveis aumentados de estresse oxidativo podem estimular os sistemas peroxidase-glutationa e glutationa redutase em diferentes territórios (MARITIM et al., 2003) o que sugere que o aumento no sistema antioxidante pode bloquear a detecção do aumento de enzimas oxidativas.

3- Objetivos

3.1- Objetivo geral

Avaliar o efeito da sepse experimental e do tratamento com salbutamol e florizina na modulação do SGLT1 pulmonar em modelo murino.

3.2- Objetivos específicos

1. Avaliar frequência respiratória, volume corrente e volume minuto;
2. Avaliar parâmetros relacionados ao estresse oxidativo;
3. Identificar a localização subcelular da proteína SGLT1 no tecido pulmonar por meio da técnica de imunohistoquímica;
4. Analisar a estrutura do tecido pulmonar por meio da técnica de histologia convencional com hematoxilina-eosina;
5. Avaliar a quantidade de proteínas totais presentes no lavado broncoalveolar (BAL);
6. Avaliar a taxa de mortalidade entre os grupos.

4- Artigo

The SGLT1 co-transporter presents a key role in acute lung inflammation and disease severity in experimental sepsis

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Abstract

Respiratory infection can be exacerbated by high glucose concentration in the airway surface liquid. We investigated the role of SGLT1 activity on pulmonary function, oxidative stress levels in lung, pulmonary histopathological damages and survival rates of rats with sepsis. Sepsis was induced by cecal ligation and puncture surgery (CLP). Twenty-four hours after surgery, CLP rats were intranasally treated with saline, salbutamol or phlorizin. After 2 hours, animals were anesthetized to analyze ventilation parameters. Sepsis promotes atelectasis and bronchial inflammation associated with increase in tidal volume. Salbutamol treatment reduced bronchial inflammation and promoted hyperinsuflation in CLP rats. Sepsis led to increased expression of SGLT1 on cytoplasm of pneumocytes. Salbutamol stimulated SGLT1 translocation to plasma membrane; whereas, phlorizin promoted increase of SGLT1 in cytoplasm. Phlorizin reduced catalase activity and induced a significant decrease in the survival rate of CLP rats. Taken together, sepsis has promoted atelectasis and lung inflammation associated changes in lung function, which was associated with SGLT1 inhibition. The loss of function of SGLT1 by phlorizin, which augmented disease severity followed by increased atelectasis, bronchial inflammation, and a significant reduction of survival rate of CLP rats. We suggest that SGLT1 is an important target modulated by septic effects in lung.

Introduction

Sepsis is a serious clinical condition that represents a response to a severe infection that may lead to multiple organ damage and acute lung injury [1]. In severe sepsis a poor prognosis with high mortality rates is achieved when one or more organs are affected [2; 3]. The lung is the most vulnerable organ during sepsis. The acute lung injury (ALI) occurs in 25% to 50% of patients with sepsis [4]. Considering the high mortality rate in patients with ALI, the identification of therapeutic platforms that are innovative, safe and effective are crucial for successful sepsis treatment [5]. Composition of airway surface liquid (ASL) is a front line of lung host defense [6]. ASL glucose concentration is reduced in ASL when compared with plasma [7], and results from the balance of epithelial glucose efflux and influx [8]. Glucose diffuses in both proximal (trachea, bronchi and bronchioles) and distal (alveolar) lung from plasma into ASL via paracellular permeability [9]. In type I and type II pneumocytes, glucose is removed from ASL via transcellular through SGLT1-mediated secondary active sodium-coupled transport [10; 11; 12]. The intranasal administration of phlorizin, an inhibitor of SGLT cotransporters, increased glucose concentration in ASL under normoglycemic and hyperglycemic conditions [8]. On the other hand, the stimulation of SGLT1 activity with isoproterenol, a non-selective beta-adrenergic agonist, reduced glucose concentration in ASL, suggesting an imperative role of SGLT1 protein in bacterial proliferation [8]. However, despite the clearly demonstrated activation of cAMP-PKA pathway enhances the SGLT1-mediated glucose transport, the capacity of salbutamol (a selective β2-adrenergic agonist) to modulate SGLT1 protein has never been investigated in lung and other tissues.

Severe sepsis induces massive infiltration of inflammatory cells and an increase of thiobarbituric acid reactive substances (TBARS) level in lung. Oxidative stress mediated by capsase leading to lung apoptosis in severe lung injury by sepsis [13]. However, investigation of oxidative stress levels and antioxidant status are required with varying degrees of sepsis severity and with changes of SGLT1 activity in the lung. Several studies have shown that sepsis, among other complications, promotes ventilatory dysfunction [14, 15] as well as an impairment on the cardiorespiratory responses to chemoreflex activation in awake rats [16]. Although the direct relationship between ASL glucose concentration and bacterial proliferation was well characterized [8], the effects of lung SGLT1 protein to modulate histopathological damages, oxidative stress and survival rate were not considered in sepsis.

Thus, the aims of the present study were to investigate the lungs of rats with sepsis with intranasally treatments of phlorizin or salbutamol, mainly observing: i) the SGLT1 protein localization in alveolar cells; ii) histopathological damages; iii) respiratory function and protein concentrations on bronchoalveolar lavage (BAL); iv) oxidative stress, and v) survival rate. Here, we demonstrate the importance of SGLT1 modulation by acute lung inflammation during sepsis development.

Methods

All animal procedures were approved by the Ethics Committee for Animal Research of the Federal University of Uberlandia (Approval No. 45/2015). CLP was performed in male Wistar rats (weighing ~260g) to trigger sepsis-induced lung injury. Rats underwent an aseptic midline laparotomy, and the portion of the cecum was exteriorized and placed outside of the abdominal cavity. The cecum was partially ligated using a 4.0 silk tie, perforated nine times with a 22-gauge needle and then gently squeezed to extrude a small amount of feces from the perforation. The cecum was returned into the abdominal cavity and the laparotomy was closed using a 4.0 silk sutures. Sham animals underwent the same procedure; however, cecum was not ligated and perforated [16]. Animals were caged and allowed free access to water and standard rodent chow diet (Nuvilab CR-1; Nuvital, Curitiba, Brazil). Twenty-four hours after surgery, rats were anesthetized by an intraperitoneal injection of ketamine (90 mg/kg) and xylasine (10 mg/kg). To demonstrate the effects of β2-adrenergic agonist on SGLT1 activity, CLP rats received salbutamol (0.15 mg/kg; 100 µL; CLP-salb). For inhibition of SGLT1 activity, CLP rats were submitted to phlorizin (10^{-3} M; 100 µL; CLP-phlo), whereas saline 0.9% was used as a control (vehicle; 100 µL; CLP-sal). Sham rats received saline 0.9% (vehicle; 100 µL; Sham). All treatments were performed intranasally two hours before sample collection, using a micropipette, and under anesthesia (ketamine (90 mg/kg) and xylasine (10 mg/kg), intraperitoneally). Animals were kept in dorsal recumbency throughout the experimental procedures. Body temperature was maintained at 37.5 ± 1.5ºC with a heating blanket. A 14-G cannula was used for tracheostomy and bronchoalveolar lavage (BAL) using a fluid-filled tube. Bronchoalveolar lavage and lung were obtained from anesthetized rats. All efforts were made to minimize animal suffering.

Measurement of sepsis and drugs effects on the pulmonary function

Following anesthesia, the cervical region accessed after a middle incision on the ventral surface of the neck, to expose the trachea. A cathether (PE-250) was gently inserted into the

trachea. During the experimental procedure, rats were allowed to breath spontaneously. Afterward, the cannula was connected with spirometer sensor (SpirometerFE141, ADInstruments, Sydney, Australia) for pulmonary function analysis. Data are collected in volts and converted into mL. Tidal volume signal (mL) was processed in Power Lab system (ADInstruments, Sydney, Australia) with LabChart program, and respiratory rate (breath/min) and minute-volume (mL) were obtained. We have manually analysed tidal volume/body weight (mL/g) and minute-volume/body weight (mL/g).

Bronchoalveolar lavage (BAL) collection

Under anesthesia, the trachea was accessed and a 19-gauge needle was gently inserted into the trachea. One mL of physiological saline was slowly injected and gentle aspirations were performed to collect ~300 µL of bronchoalveolar lavage (BAL). The BAL was immediately stored in nitrogen and after at -20 ℃ for further analysis. Finally, the left ventricle was sectioned and lungs were exsanguinated and collected for further analyses.

Histopathological analysis of lung

Histopathological analyses of lung samples were performed by hematoxilin-eosin staining. The trachea was clamped at end-expiration of bronchoalveolar lavage (BAL). Lungs were removed *en bloc* and abdominal aorta and vena cava were sectioned to quickly kill the animal by exsanguination. The lower and middle lobes of the right lung were fixed with 4% formaldehyde in phosphate buffer (PB) prior to paraffin inclusion to preserve pulmonary architecture. Lung sections were deparaffinized in xylol/xylene and rehydrated with a graded series of ethanol. To evaluate histopathological patterns, tissues were cut into 5-µm sections for hematoxylin-eosin (HE). Lungs were examined by light microscope (Leica ICC50). Photomicrographs at magnifications of x400 were obtained from non-overlapping fields per section. Airway damage was quantified using a scoring system protocol [17]. Briefly, scores of 0 to 4 were used to represent the severity of atelectasis and bronchial inflammation with 0 standing for no effect and 4 for maximum severity effect. To represent scores of inflation, score 1 was used to hypoinsuflation, score 2 to normoinsuflation and score 3 to hyperinsuflation. Hyperinflation was characterized by large-volume gas-exchanging air spaces (structures with morphology distinct associated with alveoli higher than 120 µm)[17, 18]. Furthermore, the extent of each scored characteristic per field was stated with a scale between 0 to 4, with 0 standing for no visible evidence and 4 for complete involvement per field (quadrants). Specific scores to atelectasis, bronchial inflammation and inflation were calculated as the product of

severity and extent of each feature. The cumulative airway damage score was calculated as the sum of each score characteristic (atelectasis, bronchial inflammation and inflation).

Immunohistochemistry analysis

The high lobe of the right lung were fixed in 4% formaldehyde phosphate buffer (PB) followed by cryoprotection in increasing sucrose solutions (10%, 20% and 30%) in PB. Seven-μm-thick sections were placed on gelatin-coated slides (Sigma Chem. Co., St Louis, USA), and subjected to immunodetection using anti-rat SGLT1 antibody (1:100, Merck Milipore, Germany, catalog number 07-1417), followed by incubation with Alexa Fluor 488 (1:150, Molecular Probes, Eugene, Oregon, USA, catalog number A21441). F-actin staining was performed with rhodamin-phalloidin (1:200, Molecular Probes, Merck Milipore, Germany, catalog number R415). After washings, tissue sections were coverslipped and analyzed in a fluorescence microscope (LSM 510 meta).

Survival Rate

To analyze the effect of surgery in all treatments (sham rats, sepsis, salbutamol and phlorizin), survival rates were recorded every hour until the endpoint at 26h.

Oxidative Stress Marker Analysis

TBARS was measured in lower lobes of the left lung by reacting with malondialdehyde (MDA) and thiobarbituric acid (0.67% TBA). The organic-phase was evaluated with a fluorometer at 515 nm excitation and 553 nm emission. MDA standard curve allowed quantification of this compound in the samples by linear regression [19].

Catalase, Superoxide Dismutase (SOD) and Glucose 6-Phosphate Dehydrogenase Activity (G6PDH) antioxidants activity

The catalase activity of lung tissues was assayed as described previously [20]. Briefly, lung homogenate was added with H_2O_2 in 50 mM KP buffer (pH 7.0), and H_2O_2 decomposition was monitored at 420 nm. One unit of catalase activity catalyzed the degradation of 1 μmol of H_2O_2 per min. Activity of SOD measured by the inhibition of autoxidation of pyrogallol. This inhibition occurs in presence of SOD and was evaluated using a spectrophotometer at 420 nm. A calibration curve was constructed using SOD as standard. A 50% inhibition of pyrogallol was defined as one SOD unit (U). The results were calculated as U/mg protein. Glucose 6-phosphate dehydrogenase was monitored by the production of NADPH with a consequent

increase in absorbance at 340 nm. The reading was performed in a microplate, where the samples were incubated with Tris-HCl buffer (100 mM, pH 7.5), magnesium chloride (MgCl2 2 M), NADP + (0.5 mM), and glucose 6-phosphate (1 mM) [19].

Analytical Procedures

Protein concentration was performed by the Bradford colorimetric method based on the formation of complexes between the dye Coomassie Brillant Blue with the polypeptide chain. Five microlitres of lung extracts or BAL were placed in a microplate. Absorbance was obtained with a GENESYS 10S UV-VIS spectrophotometer in a wavelength of 595nm.

Statistical analysis

All values are reported as mean ± SEM. Number of animals is informed in legends. Comparisons of the means were performed by one-way analysis of variance (ANOVA), followed by mean comparisons through the Student-Newman-Keuls post-test (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA). Student´s t-tests were performed as appropriate. Survival rates were analyzed by Chi-square using log-rank (Mantel-Cox) test. Values of $P < 0.05$ were considered as statistically significant.

Results

CLP rats were acutely treated with saline (sal), salbutamol (salb), and phlorizin (phlo); thus, the following groups were studied: Sham, CLP-sal, CLP-salb and CLP-phlo.

The twenty-four hour-CLP did not change body weight ($P > 0.05$). Neither salbutamol nor phlorizin treatments altered body weight ($P > 0.05$; data not shown).

Effect of sepsis and phlorizin in respiratory function

Figure 1A shows the respiratory rate of rats under anesthesia. Sepsis and salbutamol treatment had no effect in respiratory rate (CLP-sal vs Sham and CLP-salb vs CLP-sal, $P > 0.05$). Differently, phlorizin treatment increased ($P < 0.05$) respiratory rate in CLP rats. Tidal volume was increased ($P < 0.05$) in CLP-sal and CLP-salb compared with Sham rats (Fig 1B). Treatment with phlorizin inhibited ($P > 0.05$) alteration in tidal volume promoted by sepsis. Differently, minute volumes in CLP-sal, CLP-salb e CLP-phlo were unchanged compared with Sham rats (despite de increase of ~40%, ~50% and ~25% in mean of CLP-sal, CLP-salb and CLP-phlo rats, respectively; Fig. 1C).
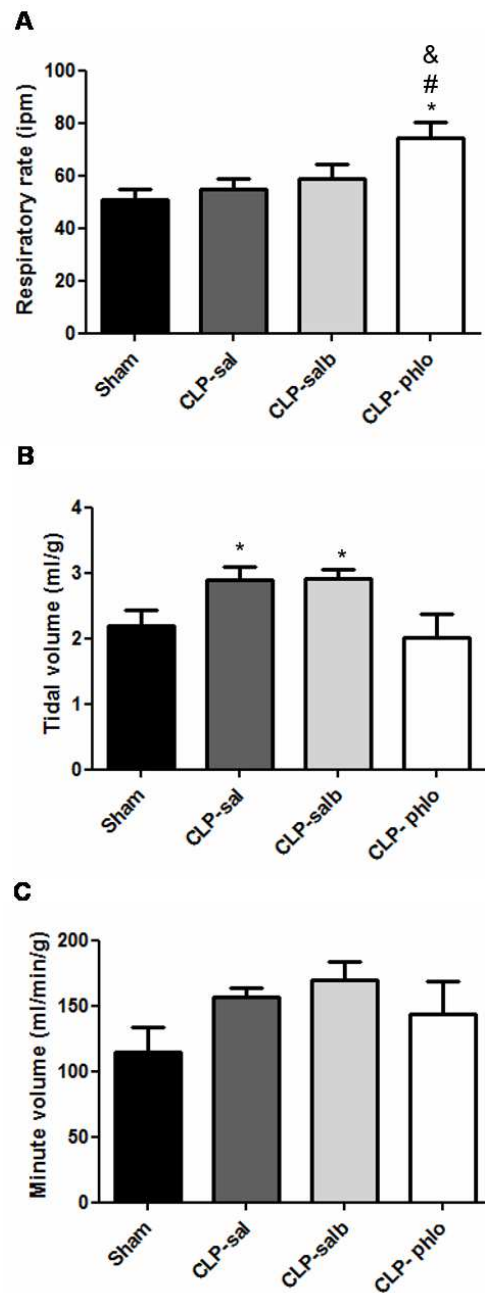
Figure 1

Figure 1. Pulmonary function. Respiratory rate (breath/min, A), tidal volume/body weight (ml/g, B) and minute-volume/body weight (ml/g, C) of Sham , CLP saline (CLP-sal), CLP salbutamol (CLP-salb) and CLP phlorizin (CLP-phlo) treated rats. Results are mean ± SEM of 6-7 animals; *$P < 0.05$ vs Sham; and #$P < 0.05$ vs CLP-sal; &$P < 0.05$ vs CLP-salb. One-way ANOVA followed by StudentNewmanKeuls post-test (A) and Student´s t-test for mean comparisons (B).

Effect of sepsis, salbutamol and phlorizin in oxidative stress in lungs

Oxidative stress analyses in lung of rats with sepsis are shown in Figure 2. Lipid peroxidation (TBARS, Fig 2.A) was unchanged in CLP-sal compared to Sham (despite the increase of ~30%, ~45% and ~45% in mean of CLP-sal, CLP-salb and CLP-phlo rats, respectively). Antioxidant defense systems were performed to assess catalase (Fig 2.B), SOD (Fig 2.C) e G6PDH (Fig 2.D) and enzyme activities in the lung. Catalase activity increased ($P > 0.05$) in CLP-sal compared to Sham rats. The salbutamol treatment (CLP-salb) did not change ($P > 0.05$) higher levels of catalase in CLP rats; however, phlorizin (CLP-phlo) reduced ($P < 0.05$) levels of catalase activity in CLP rats. Sepsis and treatments had no effect on SOD (Fig 3C) and G6PDH (Fig 3D) activities in the lung.
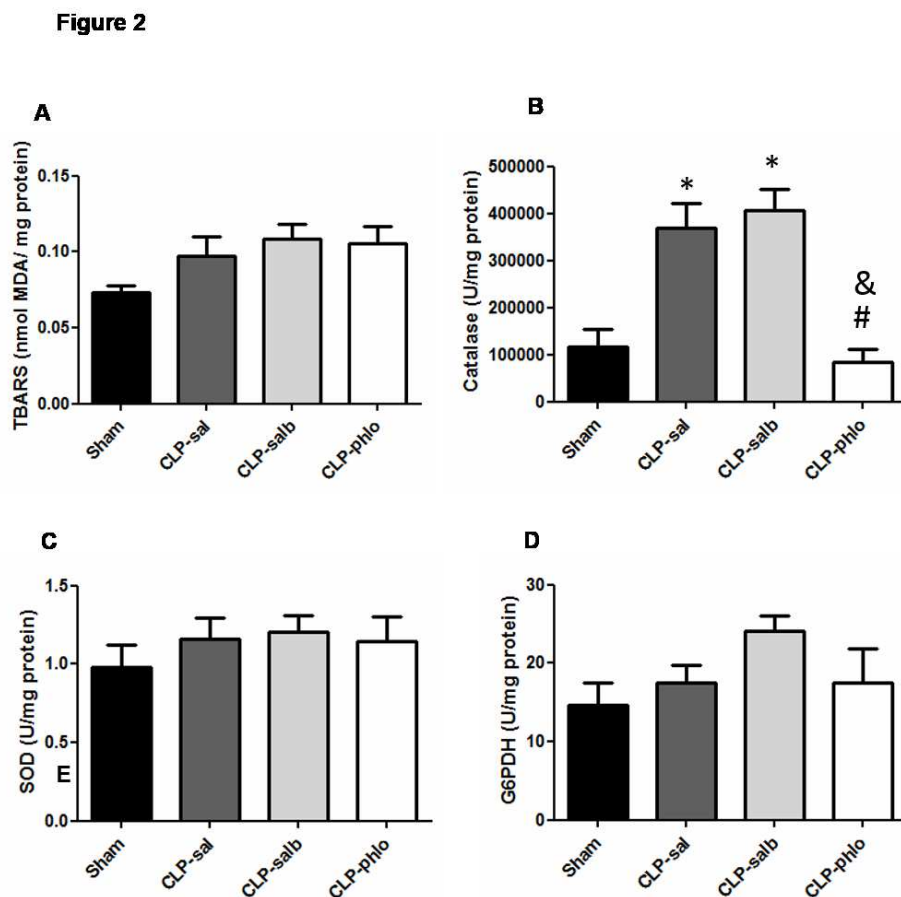
Figure 2

Figure 2. Oxidative stress in lungs. Lipid peroxidation (TBARS, A), catalase (B), SOD (C) and G6PDH (D) were analyzed in lungs of Sham, CLP saline (CLP-sal), CLP salbutamol (CLP-salb) and CLP phlorizin (CLP-phlo) treated rats. Results are mean ± SEM of 5 animals; *P <0.05 vs Sham; and #P <0.05 vs CLP-sal; &P < 0.05 vs CLP-salb. One-way ANOVA followed by StudentNewmanKeuls post-test.

Effect of sepsis, salbutamol and phlorizin on subcellular distribution of SGLT1 protein in lung

Immunodetection of F-actin (red color) and SGLT1 (green color) in pulmonary alveoli is shown in Figure 3. This figure shows the F-actin immunodetection in pulmonary alveoli of sham, CLP-sal, CLP-salb and CLP-phlo rats (Fig 3 A, E, I, M; respectively), from which the squared marked alveolar septum was amplified and analyzed for F-actin (Fig 3 B, F, J, N) and SGLT1 (Fig 3 C,G,K, O), as well as for the merged image (orange to yellow colors, Fig 3 D, H, L, P). In lung alveolar cells of sham rats (Fig 3 A to D), SGLT1 protein expression was detected in luminal membrane and in the intracellular region (cytoplasm as a reserve pool). Sepsis promoted an increase of SGLT1 protein in the intracellular region, despite the presence of SGLT1 in plasma membrane. The intracellular presence of SGLT1 in alveolar cells of lung from CLP-sal rats was detected in central part of the cell and near the plasma membrane (Fig 3 E to H). The β2-adrenergic agonist salbutamol (Fig 3 I to L) promoted a strong enhanced expression of SGLT1 content in luminal membrane of alveolar cells. The co-expression of SGLT1 and F-actin reinforced the salbutamol-induced SGLT1 translocation, as evinced by the yellow color. Phlorizin treatment blocked the SGLT1 staining in plasma membrane, suggesting effective inhibition of SGLT1 function, which was associated with SGLT1 detection mainly in the intracellular region.
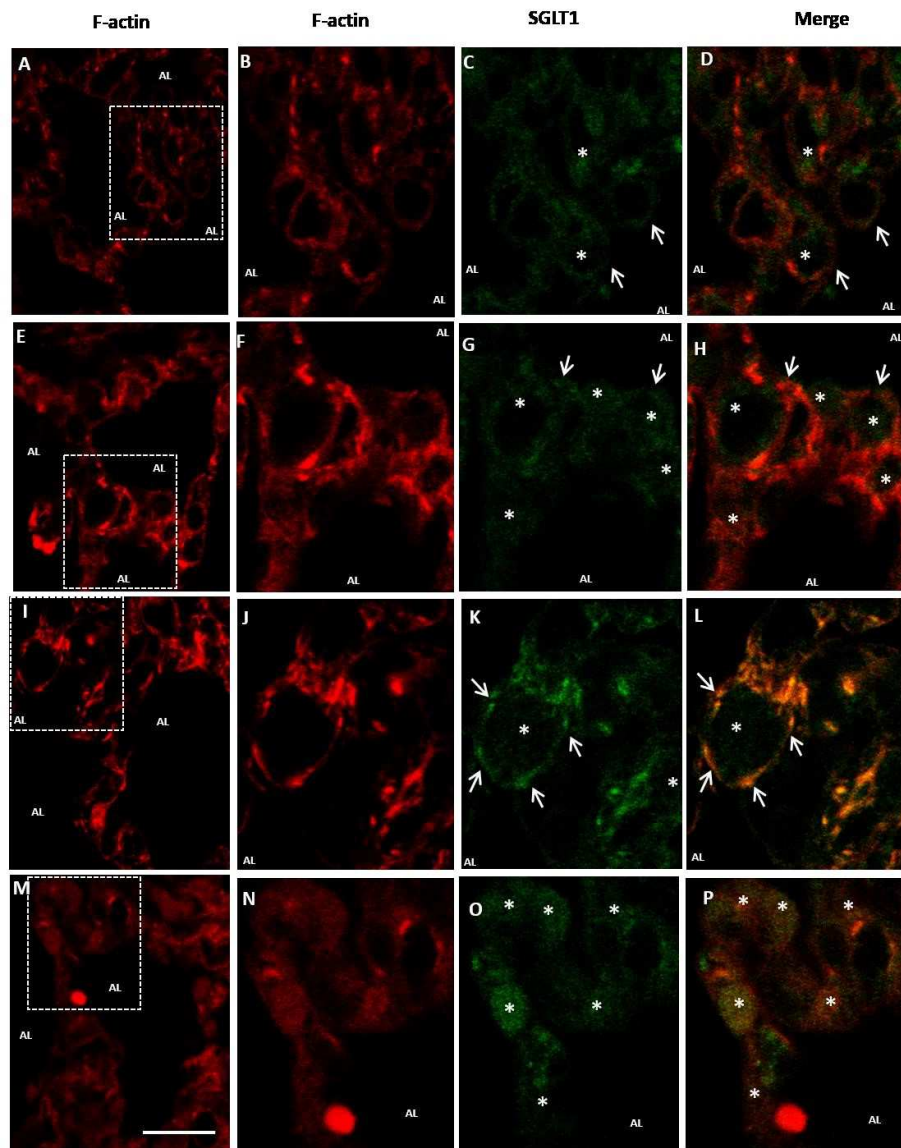
Figure 3. SGLT1 stained of lung tissue. Alveolar structures in lung from Sham (A to D), CLP saline (CLP-sal, E to H), CLP salbutamol (CLP-salb, I to L) and CLP phlorizin (CLP-phlo, M to P) treated rats. Sections (A, E, I and M) were immunostained with anti-F-actin antibody (red). White enclosed boxes showing an alveolar septum and alveolar lumen, taken with a greater resolution, are presented in the next sections: (B, F, J and N) F-Actin (red), (C, G, K and O) SGLT1 (green) and (D, H, L and P) merged photomicrographs for colocalization of SGLT1 and F-actin (yellow to orange). White arrows indicate the presence of SGLT1 in the luminal membrane. White asterisks indicate SGLT1 in cytoplasm. Description: (AL) alveolar lumen of the rat pulmonary section. Magnification, x600, scale bar, 10 μm. Images are representative of 2-3 animals in each group.

Effects of CLP and salbutamol on alveolar and bronchiolar structures in rats

To determine whether sepsis, salbutamol and phlorizin promoted airway damage in lungs, lung sections were stained with hematoxylin-eosin (Fig 4A-H). Scores of atelectasis (Fig 4.I), bronchial inflammation (Fig 4.J), inflation (Fig 4.L) and airway damage (Fig 4.M) were also showed in Figure 4. The alveolar and bronchiolar structures remained unaltered in Sham rats without atelectasis and bronchial inflammation. Besides, CLP increased scores of atelectasis ($P < 0.05$), bronchiolar inflammation ($P<0.05$) and did not change ($P > 0.05$) score of inflation. Salbutamol treatment reduced ($P<0.05$) scores of bronchial inflammation and increased score of inflation ($P<0.05$). Besides, salbutamol treatment did not promote changes ($P > 0.05$) in score of atelectasis in CLP rats. Phlorizin treatment increased ($P < 0.05$) scores of atelectasis and bronchial inflammation compared with CLP-sal rats.
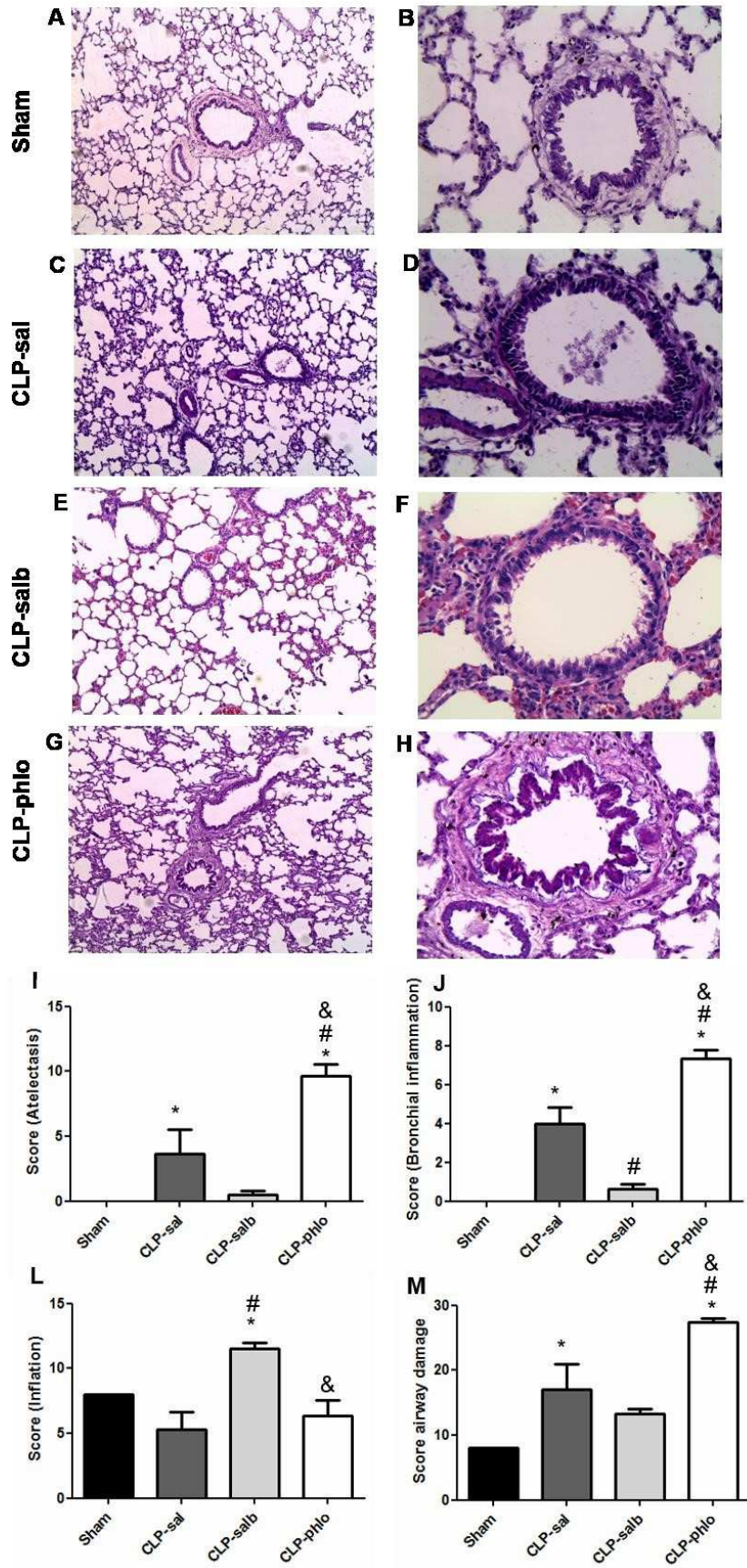
Figure 4

Figure 4. Hematoxylin-eosin stained of lung tissue. Alveolar and bronchiolar structures in lung from Sham, CLP saline (CLP-sal), CLP salbutamol (CLP-salb) and CLP phlorizin (CLP-phlo) treated rats. Hematoxylin-eosin stained sections (**A-H**), Sham (**A-B**), CLP-sal (**C-D**), CLP-salb (**E-F**) and CLP-phlo (**G-H**) magnification, x400, and severity of atelectasis (**I**), bronchial inflammation (**J**), inflation (**L**) and airway damage scores (**M**);Images are representative of 4-5 animals in each group. *P <0.05 vs Sham; and #P <0.05 vs CLP-sal; &P < 0.05 vs CLP-salb. One-way ANOVA followed by StudentNewmanKeuls post-test.

Effect of salbutamol and phlorizin on BAL protein concentration of diabetic rats

Figure 5 shows total protein concentration measured in BAL. Sepsis, salbutamol and phlorizin did not change (P> 0.05) the BAL total protein concentration in rats.
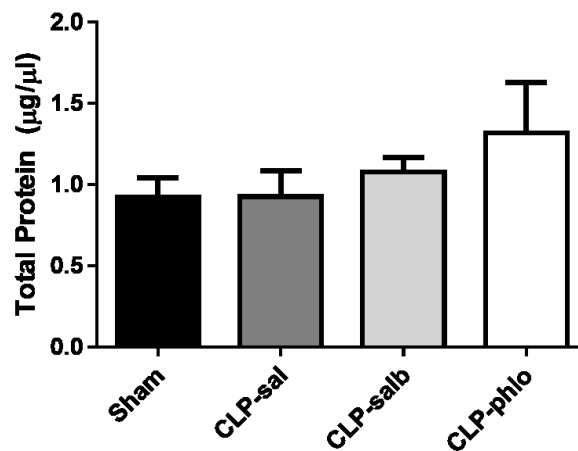
Figure 5

Figure 5. Total protein concentration in bronchoalveolar lavage. Total protein concentration was analyzed in BAL from Sham, CLP saline (CLP-sal), CLP salbutamol (CLP-salb) and CLP phlorizin (CLP-phlo) treated rats. Results are mean ± SEM of 5-6 animals; One-way ANOVA followed by StudentNewmanKeuls post-test.

Effect of phlorizin on survival rate in septic rats

As demonstrated in Figure 6, the survival rates of Sham, CLP-sal and CLP-salb rats were 100% 24h after CLP or simulated surgery. Phlorizin treatment can markedly decrease the 1-hour (25 h) and 2-hour (26 h) survival rates in animals 24 following CLP (P < 0.05). The Log-rank

(Mantel-Cox) test showed Chi-square of 9.167 for phlorizin treated CLP rats (vs. Sham, CLP-sal and CLP-phlo).
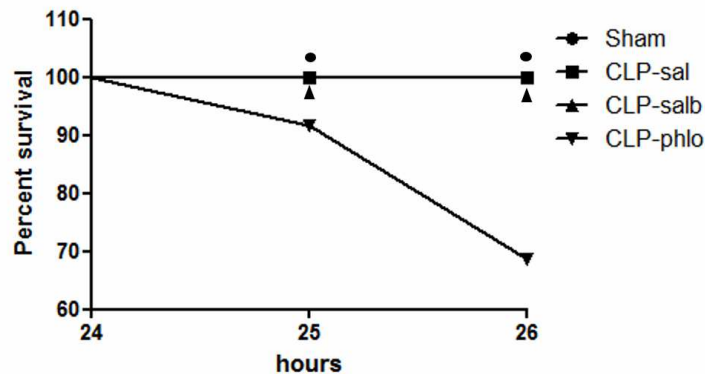
Figure 6

Figure 6. Survival analyses. Kaplan-Meier curve was constructed with survival rates of CLP saline (CLP-sal), CLP salbutamol (CLP-salb) and CLP phlorizin (CLP-phlo) treated rats. Results are mean of 7-12 animals per group; *P <0.05 vs Sham, CLP-sal and CLP-salb. Chi square = 9,167 using log-rank (Mantel-Cox) test.

Discussion

Acute lung injury secondary to sepsis is a common endpoint of several pathophysiological processes [21]. Recently, we showed that reabsorption of ASL glucose contribute to airway sterility in lung and prevent pneumonia in diabetic condition [8], suggesting a protective role via SGLT1 in acute lung injury secondary to sepsis. The present study indicates that inhibition of SGLT1 function increased the severity of atelectasis and bronchial inflammation associated with significant decrease in survival rate of CLP rats, indicating that SGLT1 activation could prevent adverse effects of sepsis in lung.

Sepsis can be experimentally induced by a procedure known as CLP, which mimic human sepsis [22,23] (Wichterman et al., 1980). Several studies have shown that sepsis, among other complications, promotes ventilatory dysfunction [14,15] (Reddy et al., 2001; Benjamin et al., 2004) as well as impairment on the cardiorespiratory responses to chemoreflex activation in awake rats [16]. During resting breathing, we showed that sepsis promotes increase in tidal volume and maintenance of respiratory rate in anesthetized rats, which indicates increase in

metabolic demand in some tissues and organs [24]. These effects were not modified by salbutamol treatment. Surprisingly, the pre-treatment with phlorizin increased respiratory rate. The mechanisms by which occurs are still unclear. Most likely this result can not represent a 2-hour induced direct effect of phlorizin in regulation of phrenic nerve activity. Thus, we propose that phlorizin promote histological changes that prevent the expected increase in tidal volume, making it necessary to increase respiratory rate to sustain high metabolic rates.

Some evidences have been reported that acute lung damage secondary to sepsis increases oxidative stress (MDA contents, reactive oxygen species production) and inflammation [1; 25]. However, other studies revealed normal levels of oxidative stress in acute lung injury secondary to sepsis [26; 27]. The increased oxidative stress can stimulate peroxidase-glutathione and glutathione reductase systems in several territories [28], suggesting that this increase in the antioxidant system can blocked the oxidative enzymes. In the present study, sepsis did not cause changes in balance (measured by FRAP) between reactive oxygen species (ROS) and antioxidants. However, sepsis promoted increase of catalase activity in lung tissue, indicating that balance can be maintained due to high levels of this antioxidant enzyme. Absence of oxidative/antioxidative effect was similar in the salbutamol treatment. Other study using CLP rats showed a differential regulation of 8-iso prostaglandin F2alpha (8-ISO) and superoxide dismutase (SOD), which was corrected by salbutamol treatment [24]. Although these results are different from our present data, further evidences of catalase inhibition by phlorizin suggest that the reasons for this discrepancy of the salbutamol treatment may be due to the different dose, route of administration, and observation period after sepsis induction. Another important difference between our data and those reported elsewhere [24] is the type of biochemical investigation to measure the tissue oxidative stress (8-ISO vs. FRAP). Nevertheless, phlorizin reduced catalase activity in lung of rats with sepsis, suggesting an additional risk for lung damage development promoted by the oxidative stress under direct or indirect effects of the inhibitor of SGLT1 co-transporter. To our knowledge, this is the first study showing pulmonary function and oxidative/antioxidative effects of SGLT1 inhibitors in lung.

The correlation of SGLT1 function with ASL glucose concentration and microbial proliferation is well established in the literature [8]. High levels of SGLT1 in plasma membrane of alveolar cells by isoproterenol decreases ASL glucose concentration and microbial proliferation; and conversely, depletion of SGLT1 in plasma membrane by phlorizin increases ASL glucose concentration and microbial proliferation in the lungs of rats [8]. Apparently, the

present study indicates that acute lung injury secondary to sepsis maintained SGLT1 protein in the plasma membrane of pulmonary alveolar cells. Considering that inflammatory cytokines, such as IL-1β, might be mediating reduction of SGLT1 insertion in plasma membrane [29], we propose that unchanged SGLT1-mediated alveolar glucose uptake is sustained by translocation of SGLT1 via sympathetic nervous system. Bearing in mind the pro-inflammatory cytokines produced in the periphery can feedback to the brain, passing through the blood brain barrier at leaky points, such as organum vasculosum lamina terminalis (OVLT) and median eminence. The activation of inflammatory receptors in OVLT can stimulate sympathetic activity to several tissues [30]. As expected, after a 2-h phlorizin treatment, very low levels of SGLT1 in the plasma membrane were observed, which reinforces that glucose transport blockage through SGLT1 was certainly guaranteed [8; 31].

We also demonstrated that salbutamol promoted SGLT1 translocation from the cytoplasm to plasma membrane of alveolar cells of lungs in septic condition. Although, the effects of β-adrenergic agonists on SGLT1 translocation has already been described in intestinal cells [32], and in acinar and ductal cells of salivary glands [33; 34]; and to the best of our knowledge, this is the first in vivo study evaluating the possible effects of salbutamol in SGLT1 co-transporter. Regarding that, only one study showed effect of β(2)-adrenoreceptor agonist (terbutaline) in glucose uptake mediated by SGLT1 translocation (via cAMP-PKA pathway) in ruminal epithelium of sheep [32]. We already expected a parallel regulation of salbutamol in lungs of rats with experimental sepsis. However, it is important to highlight that the potential benefits of β-adrenoreceptor agonists may also diminish with continued use since prolonged exposure can result in tolerance [35], indicating the importance of understanding the effects of several β(2)-adrenoreceptor agonists in SGLT1 translocation of lungs.

Histological damage analyses indicate that sepsis induces atelectasis, bronchial inflammation and airway damage, and corroborate expected morphological changes in lung following CLP [36]. Besides, by the first time, we showed that an SGLT1 inhibitor increased atelectasis and bronchial inflammation in rats with sepsis, which may play a pivotal role in ALI. Moreover, it is important to highlight that the increased severity of atelectasis, bronchial inflammation and airway damage associated with decreased tidal volume and the antioxidant system promoted by phlorizin can be related with significant decrease in survival rate of CLP rats. Strengthening the results about the importance of SGLT1, we also showed that treatment with the β2-agonist salbutamol reduced pulmonary atelectasis and bronchial inflammation. These data are in agreement with other study that showed reduction in inflammation scoring in

histopathological analysis and serum levels of TNF-α, IL-6, and IL-1β as a result of the administration of salbutamol in sepsis [36]. This increase of SGLT1 could be important to facilitate the influx of glucose/Na+/water into the cells, providing better conditions to fight against potential inflammation, while decreasing edema of the damaged area, and facilitating the process of breathing.

The present study could alert to the potential risk of several pulmonary damages in diabetic patients using dual SGLT1/SGLT2 inhibitors affected by acute lung injury secondary to sepsis. A randomized, doubled-blind placebo-controlled study to examine the safety and tolerability of canagliflozin indicated upper respiratory tract infection as adverse effects reported in >3% patients [37]. Federal Drug Administration (FDA) has recently approved canagliflozin for use in type 2 diabetes, while directing that a clinical outcome safety trial be undertaken [38]. Dual SGLT1/SGLT2 inhibitors, such as canagliflozin [39] and LX4211 [40], have been introduced in the diabetes pharmacopeia; however, bronchial inflammation, airway damage and reduction of survival rate should be considered in patients with sepsis. It is important to highlight that the lung effects of phlorizin have not been described in normoglycemic rats. Recently, in a elegant review study, dual SGLT1/SGLT2 inhibitors have been described as promising drugs for the treatment of cancer because SGLT1 and/or SGLT2 are overexpressed in various tumors, where they deliver glucose for euglycemic glycolysis [41]. The present data suggests that a second generation of dual SGLT1/SGLT2 inhibitors that do not enter the blood circulation and specific SGLT2 inhibitors may have reduced side effects due to the expression of SGLT1 in several organs.

In summary, we showd that sepsis induces atelectasis, bronchial inflammation, airway damage associated with increased tidal volume of the lung. Additionally, the salbutamol treatment of lungs of septic rats presented significant reduction of atelectasis, bronchial inflammation and airway damage associated with increased SGLT1 activity, suggesting a potential benefit in ALI secondary to sepsis. Besides, our data indicate that inhibition of SGLT function with phlorizin increased the severity of atelectasis and bronchial inflammation associated with changes in ventilation and inhibition of antioxidant system in lungs of septic rats. Finally, our study unravels new effects of SGLT1 inhibition, and suggests that septic patients submitted to the therapy of dual SGLTs inhibitors are at high risk of pulmonary damage.

Acknowledgments

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Anexo A

Universidade Federal de Uberlândia

– Comissão de Ética na Utilização de Animais –

CERTIFICADO

Certificamos que o projeto intitulado "Patofisiologia do tecido pulmonar em processo de sepse experimental em ratos", protocolo nº 045/15, sob a responsabilidade de Robinson Sabino da Silva – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica– encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADO pela COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS (CEUA) da UNIVERSIDADE FEDERAL DE UBERLÂNDIA, em reunião de 04/09/2015.

(We certify that the project entitled " Patofisiologia do tecido pulmonar em processo de sepse experimental em ratos ", protocol 045/15, under the responsibility of Robinson Sabino da Silva - involving the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata, for purposes of scientific research - is in accordance with the provisions of Law nº 11.794, of October 8th, 2008, of Decree nº 6.899 of July 15th, 2009, and the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and it was approved for ETHICS COMMISSION ON ANIMAL USE (CEUA) from FEDERAL UNIVERSITY OF UBERLÂNDIA, in meeting of 04/09/2015)

Vigência do Projeto	Início 10/09/2015 – Término 10/09/2017
Espécie / Linhagem / Grupos Taxonômicos	Ratos Wistar
Número de animais	120
Peso / Idade	250 g – 1,5 meses
Sexo	Machos
Origem / Local	Biotério
Número da Autorização SISBIO	-
Atividade(s)	-

Uberlândia, 17 de setembro de 2015

Prof. Dr. César Augusto Garcia
Coordenador da CEUA/UFU