UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

HEPATOPROTEÇÃO DOS ANTIOXIDANTES MELATONINA E N-ACETILCISTEÍNA NA HIPÓXIA INTERMITENTE

DARLAN PASE DA ROSA

Porto Alegre, janeiro de 2013.

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“O destino não é uma questão de sorte, é uma questão de escolha. Não é algo para se esperar, é algo para se conquistar.”

William Jennings Bryan

Apresentação

Esta tese compõe-se de três experimentos que utilizam o modelo de hipóxia intermitente, simulando a apneia do sono. No primeiro experimento, desenvolvemos o protocolo desse modelo animal que apresentasse alterações hepáticas e de estresse oxidativo. O segundo experimento, teve por objetivo investigar as sinalizações moleculares inflamatórias envolvidas tanto no pulmão, quanto no fígado dos animais expostos. No terceiro experimento avaliamos o papel dos antioxidantes Melatonina e N-acetilcisteína, como tratamento, em fígados de camundongos expostos à hipóxia intermitente; neste, avaliamos as sinalizações moleculares inflamatórias e apoptóticas. Todos os experimentos tiveram como foco comum o modelo de hipóxia intermitente e a avaliação inflamatória observada no fígado dos roedores.

Resumo

Introdução: Apneia do sono é uma doença respiratória crônica com alta prevalência que causa múltiplas interrupções respiratórias, levando à hipóxia intermitente (HI). A HI culmina com a geração de radicais livres, estresse oxidativo, inflamação e esteatose hepática. A Melatonina (MEL) e N-acetilcisteína (NAC), são potentes antioxidantes, capazes de inibir esses radicais livres e o estresse oxidativo. **Objetivos**: Investigar o mecanismo de inflamação em um modelo de hipóxia intermitente que simule a apneia do sono, avaliando-se o fígado e o pulmão, as respostas aos tratamentos com MEL e NAC frente às alterações oxidativas e inflamatórias no fígado de camundongos. **Métodos**: Utilizamos 120 camundongos machos, adultos, divididos em três experimentos: avaliação do modelo experimental (n=36), avaliação inflamatória molecular em fígado e pulmão (n=12) e avaliação molecular em camundongos com uso de antioxidantes (n=72). Para a hipóxia intermitente foi utilizado o sistema de câmaras que mantêm os roedores em um equipamento que simula a apneia do sono, durante oito horas diárias. No primeiro experimento avaliaram-se as alterações hepáticas em dois momentos, com 21 dias de exposição e 35 dias de exposição à HI. Nos demais experimentos foram utilizados o mesmo sistema durante 35 dias de exposição. No terceiro experimento, a partir do 21° dia iniciaram-se a administração intraperitoneal dos antioxidantes (MEL-200uL/Kg) e NAC-10mg/Kg). **Resultados**: Foi verificado que o tempo de 21 dias de exposição, não foi encontrado alterações nos fígados dos camundongos. Nos animais expostos durante 35 dias à HI, contatou-se a presença de estresse oxidativo, com aumento de dano oxidativo a lipídios e ao DNA e a redução das defesas antioxidantes, e aumento significativo de metabólitos de óxido nítrico (NO), além da presença de lesão tecidual na histologia hepática. Nos pulmões e nos fígados dos camundongos submetidos à HI, contatou-se a presença de estresse oxidativo, e aumento de expressão de fatores de transcrição: hipóxia induzível (HIF-1α), nuclear (NF-κB) e necrose tumoral (TNF-α), como mediadores inflamatórios, bem como elevação da expressão da óxido nítrico sintase induzível (iNOS) e fator de

crescimento vascular endotelial (VEGF), como mediadores de resposta vascular, e Caspase 3 clivada, como enzima responsável pela apoptose. Nos animais que foram tratados com MEL e NAC, houve redução significativa, nos fígados, de todas proteínas que apresentaram-se elevadas expressões do grupo exposto sem tratamento, assemelhando-se aos controles. **Conclusão**: Sugerimos que o tempo necessário de hipóxia intermitente, que simule a apneia do sono, para lesão hepática e estresse oxidativo seja de 35 dias, nesse tempo de exposição sabemos que tanto o pulmão quanto o fígado possuem estresse oxidativo, inflamação e apoptose, e que o uso de Melatonina e N-acetilcisteína foram capazes de proteger os fígados dessas agressões.

PALAVRAS-CHAVE

Apneia do sono, hipóxia intermitente, inflamação, Melatonina, N-acetilcisteína.

Abstract

Introduction: Sleep apnea is a chronic respiratory disease with high prevalence causing multiple interruptions in breathing, leading to intermittent hypoxia (IH). The IH culminates with the generation of free radicals, oxidative stress, inflammation and hepatic steatosis. Melatonin (MEL) and N-acetylcysteine (NAC) are potent antioxidants, capable of inhibiting these free radicals and oxidative stress. **Objectives:** To investigate the mechanism of inflammation in a model of intermittent hypoxia that simulates sleep apnea, evaluating the liver and lung, responses to treatment with MEL and NAC front of oxidative and inflammatory changes in the liver of mice. **Methods:** We used 120 male mice, adults, divided into three experiments: evaluation of the experimental model (n = 36), inflammatory molecular assessment in liver and lung (n = 12) and molecular evaluation in mice with antioxidants (n = 72) . For intermittent hypoxia was used to maintain camera system rodents in a device that simulates sleep apnea during eight hours. The first experiment evaluated the hepatic changes in two stages, with 21 days of exposure and 35 days of exposure to IH. In other experiments we used the same system for 35 days of exposure. In the third experiment, from day 21 began intraperitoneally administration of antioxidants (MEL-200uL/Kg and NAC-10mg/Kg). **Results:** It was found that the time of exposure of 21 days, no changes were found in the livers of mice. In animals exposed for 35 days to IH, contacted the presence of oxidative stress, with increased oxidative damage to lipids and DNA and reduction of antioxidant defenses, and a significant increase of metabolites of nitric oxide (NO), and the presence of tissue injury in liver histology. In the lungs and livers of mice subjected to IH, contacted the presence of oxidative stress, and increased expression of transcription factors: hypoxia inducible (HIF-1α), nuclear (NF-κB) and tumor necrosis factor (TNF-α), such as inflammatory mediators and increase the expression of inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF), vascular response mediators, and cleaved Caspase 3 as enzyme responsible for apoptosis. In animals treated with NAC and MEL, a significant reduction in the livers of all proteins that were elevated expression of the exposed untreated, similarly to controls.

Conclusion: We suggest that the time required for intermittent hypoxia, simulating sleep apnea, to liver damage and oxidative stress is 35 days exposure at this time we know that both the lungs and the liver have oxidative stress, inflammation and apoptosis, and the use of Melatonin and N-acetylcysteine were able to protect the livers of these aggressions

KEYWORDS

Sleep apnea, intermittent hypoxia, inflammation, Melatonin, N-acetylcysteine.

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Lista de Abreviaturas e Siglas

SIGLA	Significado
ALT	Alanina aminotransferase
AP	Fosfatase alcalina
AST	Aspartato aminotransferase
ATP	Adenosina trifosfato
CAT	Catalase
CH	Radical centrado no carbono
CO2	Dióxido de Carbono
CPAP	Pressão positiva contínua de vias aéreas
Cu+	Cobre
DHGNA	Doença hepática gordurosa não alcoólica
DI	Índice de dano
EEG	Eletroencefalograma
EHNA	Esteatohepatite não alcoólica
EMG	Eletromiografia
eNOS	Óxido nítrico sintase endotelial
EO	Estresse oxidativo
ERN	Espécies reativas de Nitrogênio
ERO	Espécies reativas de Oxigênio
FA	Fosfatase alcalina
FADH2	Dinucleotídeo de Flavina e Adenina
Fe2+	Ferro
FEPPS	Fundação Estadual de Produção e Pesquisa
GPx	Glutationa peroxidase
GSH	Glutationa total
H2O2	Peróxido de Hidrogênio
HDL	Lipoproteínas de alta densidade
HE	Hematoxilina Eosina
HI	Hipóxia intermitente
HI 21	Hipóxia intermitente 21 dias
HI 35	Hipóxia intermitente 35 dias
HI+MEL	Hipóxia intermitente+Melatonina
HI+NAC	Hipóxia intermitente+N-acetilcisteína
HIF-1α	Fator induzível de Hipóxia 1 alpha
ICAM-1	Molécula de adesão intercelular 1
IH	Intermittent hypoxia
IH 21	Intermittent hypoxia 21 days
IH 35	Intermittent hypoxia 35 dias
IkB	Inibidor de proteínas kB
IKKα	IkB quinase alpha
IKKβ	IkB quinase Beta
IL	Interleucina
iNOS	Óxido nítrico sintase induzível
LDL	Lipoproteínas de baixa densidade
LPO	Lipoperoxidação
MEL	Melatonina
NAC	N-acetilcisteína
NAD	Dinucleotídeo de Nicotinamida e Adenina
NAFLD	Doença hepática gordurosa não alcoólica
NAPDH	Nicotinamida adenina dinucleotídeo fosfato
NF-kB	Fator de transcrição nuclear kB
nNOS	Óxido nítrico sintase neuronal

NO	Óxido nítrico
NOS	Óxido nítrico sintase
NREM	No rapid eye movement
O2.-	Ânion radical superóxido
OE	Oxidative stress
OH.	Radical hidroxil
ONOO-	Peroxinitrito
OSA	Obstructive sleep apnea
PDGF	Fator de crescimento derivado de plaquetas
PGI2	Prostaciclina
PHD	Proteína dominante prolil hidroxilase
PSG	Polissonografia
PVDF	fluoreto de polivinilideno
REM	Rapid eye movement
RL	Radicais livres
ROO.	Radical Peroxil
ROS	Espécies reativas de Oxigênio
SAHOS	Síndrome das apnéias e hipopnéias obstrutivas do sono
SAOS	Síndrome da apnéia obstrutiva do sono
SHI	Simulação de hipóxia intermitente
SHI+MEL	Simulação de hipóxia intermitente+Melatonina
SHI+NAC	Simulação de hipóxia intermitente+N-acetilcisteína
SM	Síndrome metabólica
SOD	Superóxido Dismutase
TAK1	Fator de crescimento transformante Beta de quinase ativada 1
TGF-β	Fator de crescimento tumoral Beta
TNF-α	Fator de necrose tumoral alpha
VAS	Vias aéreas superiores
VEGF	Fator de crescimento vascular endotelial
VEGFR2	Receptor de factor de crescimento vascular endotelial
XO	Xantina Oxidase

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1 Introdução

A Síndrome da Apneia Obstrutiva do Sono (SAOS) é um distúrbio respiratório do sono que leva ao fechamento total das vias áreas superiores. Tais alterações respiratórias levam à hipóxia intermitente (HI), que pode resultar em despertares transitórios do sono, ocasionando fragmentação do sono (Dempsey et al., 2010). Um estudo de prevalência verificou que 21,3% das pessoas analisadas apresentavam índice de apneia entre 5 e 14,9, enquanto 16,9% apresentavam índice de apneia superior ou igual a 15. Em pacientes portadores de obesidade, a prevalência chegou a 50% (Tufik et al., 2010).

Sugere-se que o estresse oxidativo, induzido pela HI, seja a primeira causa de dano em múltiplos organismos na SAOS (Zhou et al., 2012). A geração de espécies reativas de oxigênio, na SAOS, pode ser iniciada pela mudança de pressão de oxigênio, por promover momentos de isquemia e reperfusão (Lavie 2003, Lavie 2005, Suzuki et al., 2006, Lavie 2009), através da ativação da xantina oxidase, que se dá com a falta de oxigênio. Quando em presença de oxigênio, na reperfusão, gera o ânion superóxido e o peróxido de hidrogênio, contribuindo para o estresse oxidativo (Aalto et al., 1993, Zhang et al., 1994, Szabo et al., 1996).

A SAOS é associada à resistência insulínica e esteatose hepática (Byrne et al., 2009), que juntamente com o estresse oxidativo, contribui para a sua grande associação com a esteato-hepatite não alcoólica (EHNA) (Singh et al., 2005, Tanne et al., 2005, Tatsumi et al., 2005, Jouet et al., 2007, Kallwitz et al., 2007, Zamora-Valdes et al., 2007, Kheirandish-Gozal et al., 2008, Norman et al., 2008). A ativação do fator de hipóxia induzível (HIF-1α), que está ativado na HI, pode ser o responsável pela dislipidemia na SAOS (Drager et al., 2010). A SAOS contribui ainda para o aumento de ácido graxo livre e redução de beta-oxidação que resulta em acúmulo de gordura no fígado (Tanne et al., 2005, Jouet et al., 2007, Kallwitz et al., 2007, Jun et al., 2008, Mishra et al., 2008, Norman et al., 2008, Polotsky et al., 2009, Ulitsky et al., 2010). Dessa forma, a HI parece atuar nos dois “hits” da EHNA, tanto no acúmulo de gordura no hepatócito, quanto no aumento de estresse oxidativo.

A HI, na SAOS, ativa fatores inflamatórios como o fator nuclear kappa B (NF-kB) (Savransky et al., 2007, Ryan et al., 2009), sendo esse o regulador mestre do processo inflamatório e sua ativação conduz à ativação de fator de necrose tumoral (TNF-a), interleucinas 1 e 6, indução de óxido nítrico sintase induzível (iNOS), entre outros. Esses podem ser também ativados pelo fator de hipóxia induzível (HIF-1a) (Greenberg et al., 2006, Htoo et al., 2006, Yamauchi et al., 2006, Selmi et al., 2007), que resulta em apoptose (Carmeliet et al., 1998).

O uso de antioxidantes pode retardar ou evitar o dano causado por ERO. A Melatonina (MEL) é produzida na glândula pineal, e é conhecida como "hormônio da escuridão" devido a sua máxima produção ocorrer no escuro. Esse hormônio tem o papel antioxidante, neuroprotetor, cardioprotetor e anticarcinogênico. A MEL tem a capacidade de passar as membranas biológicas, alcançando todos os compartimentos subcelulares, protegendo-os dos radicais livres. A N-acetilcisteina (NAC), é um composto tiólico que contém um grupo sufidril, o qual caracteriza o papel de antioxidante sendo amplamente utilizado na clínica (Ziment 1988).

2 Revisão da literatura

2.1 Vigília e Sono

A vigília ocorre entre o despertar ao final do sono e antes do início do sono. Além de breves períodos de acordar, cinco a quinze vezes por hora, em geral nas trocas de estágio é acompanhada de movimentos corpóreos mais ou menos sutis. O despertar é diferente do acordar, sendo breve, com duração entre três e quinze segundos, correspondendo ao aparecimento de ondas alfa no eletroencefalograma (EEG). O acordar dura mais de quinze segundos e se acompanha de aumento de tono muscular, evidenciado por maior atividade na eletromiografia (EMG) (AASM, 1999).

No sono, o EEG mostra uma sucessão ordenada e cíclica de ondas cerebrais de diferentes amplitudes e frequências que caracterizam os estágios do sono. Estudo recente das propriedades taxonômicas dos estágios do sono concluiu que, do ponto de vista estatístico, as regras atuais de classificação de estágios resultam em padrões de EEG heterogêneos, podendo-se identificar três ou quatro tipos para cada estágio (Muller et al., 2006).

Atualmente, avalia-se a qualidade do sono objetivamente através da polissonografia (PSG), método que combina eletroencefalograma, eletro-oculograma, eletromiograma aos registros de respiração, saturação de oxigênio arterial e eletrocardiograma. Em adultos, o sono noturno é formado por quatro a seis ciclos de períodos de sono REM (do inglês, "rapid eye movement"), alternado com períodos de sono não REM (NREM, do inglês, “no rapid eye movement”). Cada ciclo dura de 90 a 110 minutos (Punjabi 2008).

Em humanos, o sono NREM é convencionalmente dividido em quatro estágios, de acordo com o aparecimento em sequência de ondas características no EEG, que vão do mais superficial, o estágio 1, até o mais profundo com ondas delta, o estágio 4 (Dempsey et al., 2010).

No sono REM, ocorre redução acentuada ou perda completa do tônus muscular. Também são observados abalos musculares e movimentos oculares

rápidos, resultantes de descargas de neurônios pontino-geniculado-occipitais (PGO). Elas nascem na ponte, propagam-se para o núcleo geniculado lateral e alcançam o córtex occipital, gerando as imagens dos sonhos. Os sonhos ocorrem regularmente durante todo o período de sono REM. Predominam no terço final da noite, ocupando de uma a duas horas do sono do adulto (Dempsey et al., 2010).

2.2 Apneias do Sono

Apneias e hipopneias do sono são perturbações respiratórias que ocorrem durante o sono, com duração de mais de dez segundos, causam hipoxemia e terminam com despertar autonômico ou cortical. O diagnóstico de síndrome das apneias e hipopneias obstrutivas do sono (SAHOS) exige a ocorrência de sintomas de sono perturbado, como sonolência, associado a cinco ou mais apneias ou hipopneias (AH) por hora de sono, sendo causadas por colapso da faringe (AASM, 1999, Dempsey et al., 2010).

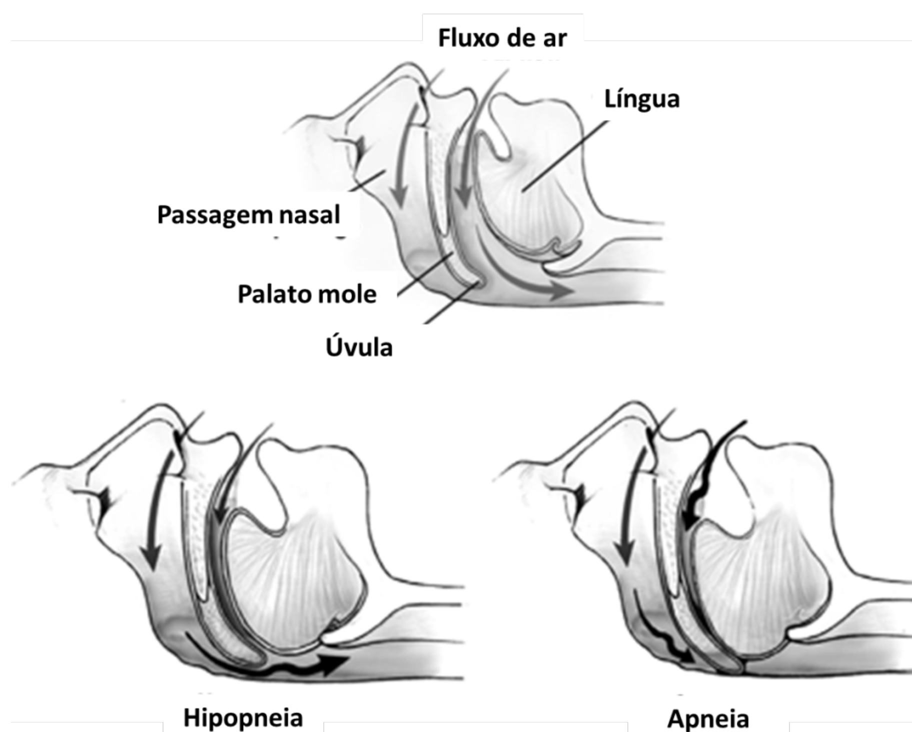
Além de sonolência diurna, as apneias do sono, por seu impacto sobre o sistema nervoso central, causam alterações psicossociais e distúrbios de memória, pensamento, comunicação e aprendizado (Redline et al., 1997, Musso et al., 2012). Em modelo animal, a hipóxia intermitente (HI) causa morte dos neurônios corticais por apoptose (Xu et al., 2004). As causas do prejuízo mental tanto podem ser fragmentação do sono pelos despertares, quanto episódio de HI durante as apneias, ou ambos.

Pacientes com apneia do sono apresentam pouco ou nenhum problema com a sua respiração e com a permeabilidade das vias aéreas superiores (VAS), enquanto acordados. A grande maioria dos portadores dessa doença apresenta sistemas de controle ventilatório que são capazes de regular sua ventilação alveolar e gasometria arterial com variações pequenas em relação à vigília. Além disso, quando acordado, tais sistemas de controle saudáveis possuem feedback suficientemente sensível e controles para assegurar a coordenação precisa da parede torácica e das vias aéreas superiores, com o recrutamento dos músculos respiratórios, de modo a proporcionar diâmetro máximo das vias aéreas, resistência das vias aéreas e volumes pulmonares

baixas e ótimo comprimento muscular-respiratórias, independente da condição ventilatória (Dempsey et al., 2010). Durante o sono, esses indivíduos, mesmo com o controle da respiração normal, estão, entretanto, sujeitos a sofrer apneias.

As perturbações respiratórias do sono são comumente divididas em centrais, denotadas por ausência ou redução acentuada na sinalização motora central para os músculos respiratórios; obstrutivas, compostas de esforços respiratórios contra via aérea superior fechada; ou mistas, quando ocorre alternância entre obstrutiva e central (Dempsey et al., 2010).

FIGURA 1 ILUSTRAÇÃO DO BLOQUEIO DA PASSAGEM DO AR PELAS VIAS AÉREAS SUPERIORES NA HIPOPNEIA E NA APNÉIA



FONTE: (adaptado de Somers et al., 2008).

A atividade elétrica de neurônios bulbares responsáveis pela inspiração mostra redução da amplitude na transição do estado vigília para o sono NREM, geralmente acompanhada de ligeira a moderada hipoventilação (2 a 8 mmHg $PaCO_2$) e aumentada de duas a cinco vezes a resistência das vias aéreas

superiores (Lydic et al., 1979, Dempsey et al., 1986, Henke et al., 1990, Lo et al., 2007).

No que tange ao aparelho cardiovascular do paciente com SAHOS, o aumento de incidência de hipertensão arterial sistêmica é comprovado com estudos em grande número de pacientes, sendo controlado para os fatores de confusão conhecidos (Nieto et al., 2000, Peppard et al., 2000). A hipertensão arterial pode ser a base para as demais consequências cardiovasculares das apneias e está comprovadamente relacionada à HI (Fletcher 2001, Wolk et al., 2003).

Em roedores, modelos de HI foram capazes de provocar hipertensão arterial sistêmica, resistência à insulina e dislipidemia. Estudos em humanos demonstraram que pacientes obesos com SAHOS podem ter risco aumentado de desenvolver esteato-hepatite não alcoólica e dano hepático crônico (Savransky et al., 2007). HI durante doze semanas em camundongos não causou esteatose, mas determinou aumento de transaminase, colesterol, triglicérides e glicemia. Além de aumento no volume dos hepatócitos, houve acúmulo de glicogênio hepático e evidência de estresse oxidativo (Savransky et al., 2007).

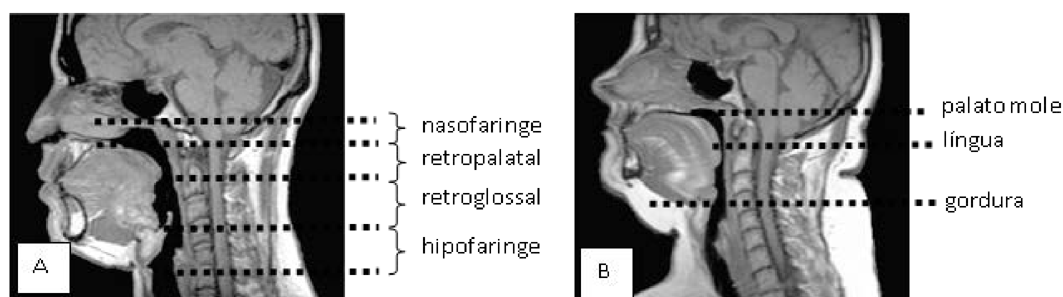
2.3 Alterações Anatômicas da SAOS

As vias aéreas superiores (VAS) fazem parte de uma estrutura complexa e necessária para a realização da deglutição, vocalização e respiração. Sua obstrução é mais frequente em seres humanos, em parte, porque o osso hioide não está firmemente ligado às estruturas esqueléticas, como em outros mamíferos, o osso hioide é anexado ao processo estiloide do crânio (Young et al., 2004).

Além do arco hioide, alguns pesquisadores atribuem mudanças anatômicas no desenvolvimento evolutivo, contribuindo para frequência elevada de SAOS em humanos. Isso ocorre principalmente quanto à posição da laringe em relação à orofaringe, a qual, ao separar o palato mole da epiglote, estreita a orofaringe, fazendo com que a língua invada o espaço disponível (Lieberman et al., 1999, Lieberman et al., 2001, Davidson 2003).

A região oral da faringe é dividida em nasofaringe, retropalatal, retroglossal e hipofaringe (Figura 2A). Pode ocorrer o fechamento (bloqueio da passagem de ar) em uma ou mais regiões da faringe em indivíduos que apresentam a SAOS, sendo que essas regiões podem apresentar um estreitamento até mesmo durante a vigília (Horner et al., 1989, Schwab et al., 1995, Morrell et al., 1998, Schwab et al., 2003). Embora a região retropalatal da orofaringe seja o local mais comum de bloqueio (Figura 2B), o estreitamento da via aérea é um processo dinâmico e muito variado, inclui as regiões retroglossal e hipofaringe (Hudgel 1986, Morrison et al., 1993). Em indivíduos portadores de obesidade com SAOS, ocorre o fechamento das VAS principalmente na velofaringe, enquanto em pacientes portadores de obesidade com SAOS e recesso na mandíbula, o colapso ocorre em ambas as velo e orofaringe (Watanabe et al., 2002).

FIGURA 2 RESSONÂNCIA MAGNÉTICA DA IMAGEM SAGITAL DE INDIVÍDUO NORMAL (A) E DE SAOS (B).



FONTE: (Adaptado de Watanabe, Isono et al. 2002)

Destacam-se as quatro regiões da via aérea superior (nasofaringe, região retro- palatal, retroglossal, hipofaringe) e tecidos moles das VAS (palato mole, língua, gordura) e estruturas crânio-faciais (mandíbula). Nota-se que o paciente em apneia apresenta as VAS menores, enquanto na região retropalatal e retroglossal o palato mole e a língua são maiores, e a quantidade de gordura subcutânea é maior. Adaptado de Schwab, 1995 (Adaptado de Schwab et al., 1995).

2.4 ALTERAÇÕES METABÓLICAS NA SAOS

Apesar de sua importante associação com obesidade e problemas cardiovasculares, a apneia do sono tem sido tratada como anormalidade respiratória e não como doença sistêmica (Vgontzas et al., 2005, Schulz et al., 2008). O risco cardiovascular é maior quando a apneia do sono se associa às alterações glicolipídicas (Gruber et al., 2006).

A obesidade, tanto central quanto visceral, está associada com maior risco para SAOS (Shinohara et al., 1997, Tufik et al., 2010), demonstrando que há outros fatores, além da causa puramente mecânica, que podem contribuir para a patogênese dos distúrbios do sono. Depósitos de gordura visceral representam uma fonte rica de mediadores humorais e citocinas inflamatórias, portanto podem ter impacto sobre as respostas neurais do controle respiratório (Schwartz et al., 2008).

Estudos mostram correlação entre apneia do sono, inflamação e resistência à insulina (intolerância à glicose) e a contribuição destas condições para aterosclerose e doença cardiovascular, assim como, a presença de dislipidemia (aumento sérico de triglicerídeos e redução do colesterol HDL) (Schulz et al., 2008). Diversos estudos apontam que a SAOS está independentemente associada com níveis aumentados de colesterol total, LDL e triglicérides, enquanto outros não relatam essa relação (Newman et al., 2001, Drager et al., 2005, Tsioufis et al., 2007, Drager et al., 2010, Drager et al., 2010). O tratamento com CPAP (pressão positiva contínua em vías aéreas) pode ter um efeito benéfico sobre o perfil lipídico nesses pacientes (Robinson et al., 2004, Dorkova et al., 2008, Tokuda et al., 2008), no entanto, a maioria desses estudos não foram desenhados especificamente para avaliar o perfil lipídico, ignorando fatores importantes de confusão, tais como dieta, atividade física e composição corporal (Drager et al., 2010).

O tecido adiposo libera muitos fatores humorais, incluindo clássicas citocinas pró-inflamatórias como o fator de necrose tumoral α (TNF- α) e interleucina 6 (IL-6), os quais estão aumentados em pacientes com SAOS e podem ser reduzidos com terapia de CPAP (Yokoe et al., 2003, Minoguchi et

al., 2004). A liberação de citocinas pró-inflamatórias pode ter relação direta com a falta de oxigenação adequada e não com obesidade.

Obesidade, dislipidemia, hiperglicemia, resistência à insulina e hipertensão arterial caracterizam a síndrome metabólica, reconhecidamente associada com aumento do risco de doenças cardiovasculares e diabete tipo II (Grundy et al., 2004, Eckel et al., 2005, Tasali et al., 2008).

Estudos mostram que pacientes com SAOS apresentam alto risco de desenvolver síndrome metabólica (Schulz et al., 2008) e seis vezes maior predisposição para alterações glicolipídicas (Gruber et al., 2006). A gravidade da SAOS (Chin et al., 1999), tem forte associação com hipertensão (Nieto et al., 2000, Peppard et al., 2000) e diabete (Resnick et al., 2003, Punjabi et al., 2004) e embora os mecanismos fisiopatológicos da síndrome metabólica (SM) ainda não estejam completamente esclarecidos (Eckel et al., 2005, Kono et al., 2007), devido ao efeito de confusão com a obesidade (Lam et al., 2007), tudo indica a existência de elo comum entre a SAOS e a SM (Coughlin et al., 2004, Vgontzas et al., 2005) e estudos ainda sugerem a apneia do sono como manifestação da síndrome metabólica (Ip et al., 2002, Coughlin et al., 2004, Sasanabe et al., 2006). A presença concomitante da SAOS e da SM é chamada de “síndrome Z” (Wilcox et al., 1998, Venkateswaran et al., 2007), que se caracteriza pela concomitância entre apneia do sono, diabete, obesidade visceral, hipertensão e dislipidemia.

Embora a obesidade presente nos pacientes portadores de SAOS seja o principal fator determinante para a resistência à insulina e diabete, alguns estudos sugerem que a hipóxia e a fragmentação do sono podem causar e/ou agravar alguns dos fenótipos associados à síndrome metabólica, como, por exemplo, a resistência à insulina (Tassone et al., 2003, Punjabi et al., 2004).

Em pacientes diabéticos, a ERO prejudica a captação de glicose no músculo e gordura (Rudich et al., 1998, Maddux et al., 2001) e leva à diminuição da secreção de insulina pelas células β pancreáticas (Matsuoka et al., 1997). O aumento do estresse oxidativo também é subjacente à

fisiopatologia da hipertensão arterial (Nakazono et al., 1991) e aterosclerose (Ohara et al., 1993) que afetam as paredes vasculares das células.

O aumento de mediadores pró-inflamatórios estimulados pelo HIF-1α e pelo estresse oxidativo também estimulam a resistência insulínica (Yuan et al., 2001, Cai et al., 2005). A HI aumenta a expressão do gene de leptina, o qual age tanto em nível central quanto periférico, para inibir a secreção de insulina (Halaas et al., 1995, Schwartz et al., 1996, Barzilai et al., 1997, Kulkarni et al., 1997, Sivitz et al., 1997, Seufert et al., 1999, Rosmond 2003, Kojima et al., 2009). O desenvolvimento de resistência insulínica durante a HI, apesar dos altos níveis de leptína, sugere um fenômeno inexplicável de resistência à leptina (Unger et al., 2010).

A diabetes é a manifestação final da resistência à insulina e uma falha descompensada de secreção de insulina das células beta pancreáticas. A HI aumenta a apoptose e a proliferação das células beta pancreáticas (Yokoe et al., 2008, Xu et al., 2009).

2.5 Estresse Oxidativo

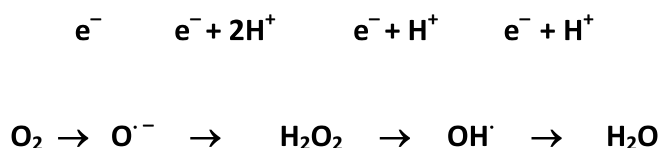
A obstrução das vias aéreas superiores leva à diminuição da oxigenação do sangue e hipóxia intermitente (HI), caracterizada por episódios repetidos de hipóxia/reoxigenação. A principal fonte de geração de radicais livres nas células durante a hipóxia foi relatada como semelhante à isquemia e à reperfusão, quando ocorre diminuição do potencial redox mitocondrial, causando uma produção de ERO a partir da cadeia de transporte de elétrons, principalmente no nível III do citocromo (Guzy et al., 2006).

2.5.1 Radicais livres

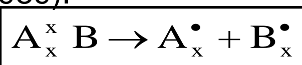
O consumo de oxigênio pelos organismos aeróbios está intimamente ligado à otimização da extração de energia dos diversos substratos energéticos. Seu alto potencial oxidante pode ser verificado pela comparação da eficiência com que ocorrem as reações do metabolismo aeróbio. Por exemplo, a oxidação completa da glicose pode liberar energia suficiente para ressíntese de 38 ATP, enquanto no sistema anaeróbio apenas 2 ATP são

obtidos. No primeiro caso, as células transformam glicose ($C_6H_{12}O_6$) em água e dióxido de carbono (CO_2), graças à oxidação por oxigênio molecular. A redução completa de uma molécula de oxigênio à água requer quatro elétrons. Redução tetraeletrônica do O_2 evita as reações intermediárias e ocorre na cadeia respiratória acoplada à fosforilação oxidativa, pelo sistema citocromo-oxidase, que forma ATP (Del Maestro 1980).

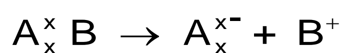
É preciso, porém, ressaltar que nem sempre o oxigênio se transforma diretamente em água. Em cerca de 5% do processo, o oxigênio tem tendência forte de receber um elétron de cada vez, redução monoeletrônica do oxigênio, formando, durante as reações, uma série de intermediários tóxicos e reativos que são as ERO (Meneghini et al., 1987).



O rompimento das ligações entre as moléculas também pode levar à formação de RL. Na fissão homolítica, cada elemento fica com um elétron desemparelhado, formando, assim, dois RL. Na fissão heterolítica, não há formação de RL, pois um elemento fica com os dois elétrons (com carga negativa) e o outro elemento fica sem nenhum elétron (com carga positiva) (Halliwell 1989).



Fissão Homolítica



Fissão Heterolítica

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 têm uma série de etapas durante as quais se consome uma espécie

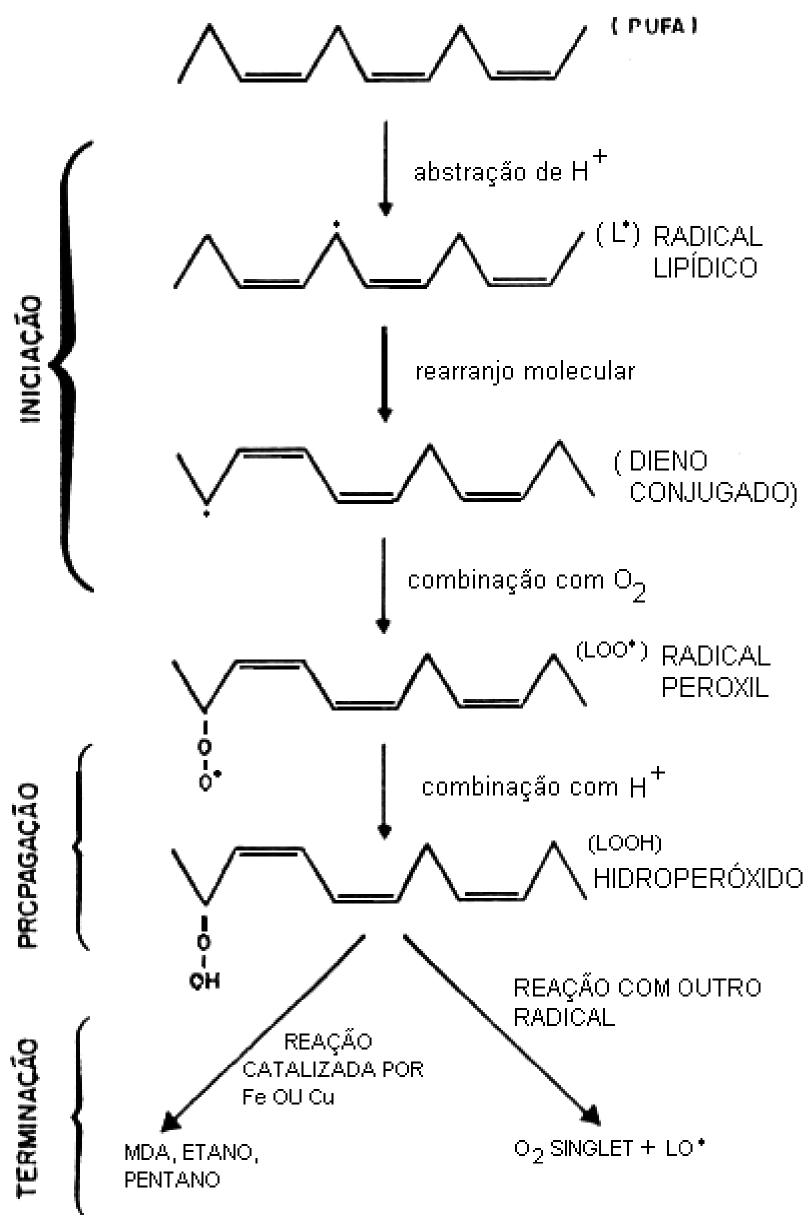
intermediária, os reativos convertem-se em produtos e os intermediários são regenerados, permitindo que o ciclo recomece. As etapas do processo de LPO são estas: iniciação, propagação e terminação.

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 hidroxil ($OH^{\cdot}$); 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. 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 ($ROO^{\cdot}$) 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 ele abstraiu, produzindo um lipídio hidroperóxido. Os lipídios hidroperóxidos decompõem-se numa reação catalisada por complexos de ferro e cobre, produzindo aldeídos como o malondialdeído, hidrocarbonetos voláteis (como o gás pentano) e outros produtos (Halliwell 1989). Foi sugerido que reações de degradação, as quais ocorrem durante a LPO, podem originar oxigênio *singlet*, acelerando este processo (Halliwell 1989).

Além da perda da fluidez da membrana, há também desarranjo dos receptores e potenciação da lise celular. O dano dos RL a enzimas que contêm enxofre e outras proteínas culmina em sua inativação, ligações cruzadas, de maneira aleatória, e desnaturação (Machlin et al., 1987).

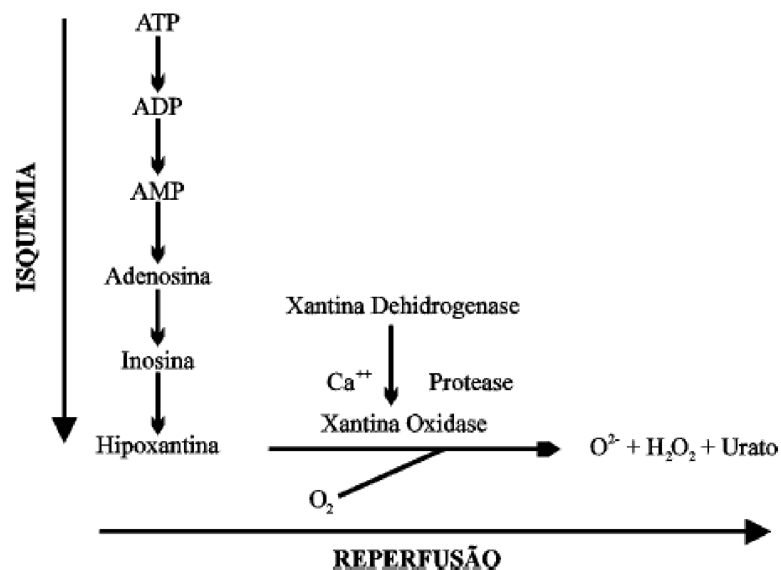
FIGURA 3 REAÇÕES EM CADEIA DA LIPOPEROXIDAÇÃO



FONTE: (adaptado de Del Maestro 1980).

Além disso, a liberação de ERO pode ser aumentada em condições de hipóxia através da ativação da xantina oxidase (XO) (Sohn et al., 2003), Nicotinamida adenina dinucleotídeo oxidase (NAPDH) (Jones et al., 2000), e fosfolipase A2 (Neidlinger et al., 2005). Devido à alteração do sistema de formação de ATP, na hipóxia, que altera da via aeróbica para a via anaeróbica, ativa-se a xantina oxidase que em presença de oxigênio (reoxigenação) gera radicais livres (Sohn et al., 2003).

FIGURA 4 MECANISMO PROPOSTO PARA GERAÇÃO DE RADICAIS LIVRES DE OXIGÊNIO APÓS PERÍODOS DE ISQUEMIA E REPERFUSÃO



FONTE: (Aalto et al., 1993, Zhang et al., 1994, Szabo et al., 1996).

Algumas outras ERO também são conhecidas como radicais livres (RL), tais como: átomo de hidrogênio, íons de metais de transição, e também os radicais de nitrogênio como o peroxinitrito: o óxido nítrico, o dióxido de nitrogênio, etc. Portanto, 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, Halliwell et al., 1984, Southorn et al., 1988).

2.5.2 Óxido Nítrico

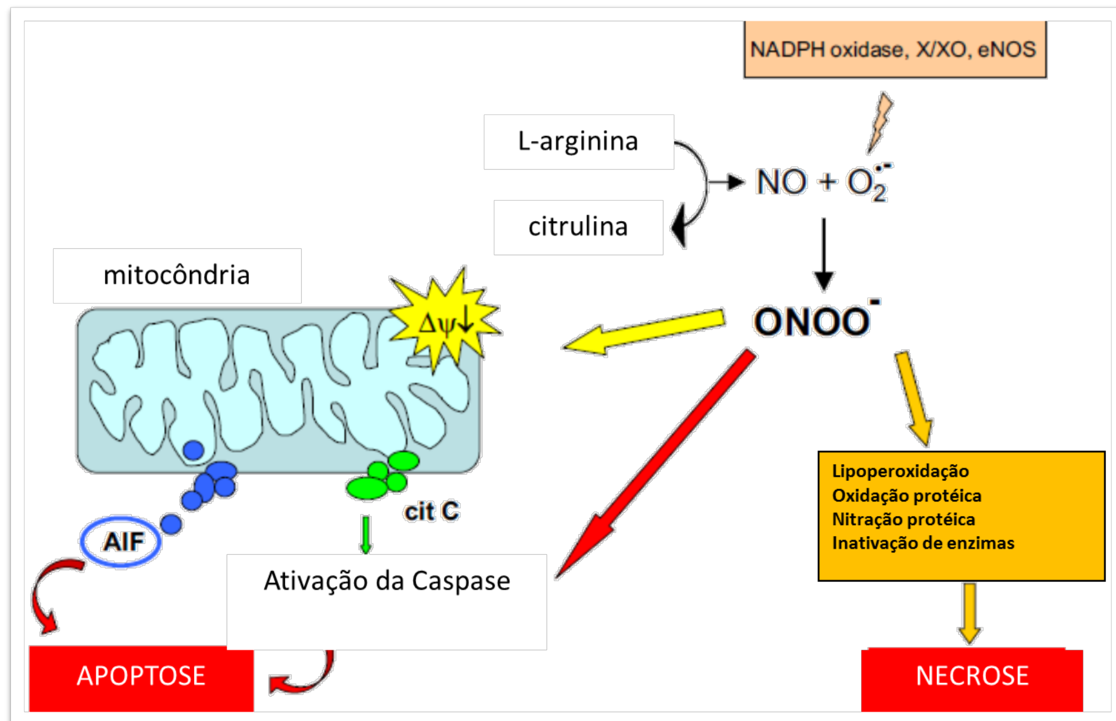
Óxido Nítrico (NO) é uma pequena molécula hidrofóbica que atravessa membranas celulares sem a necessidade de canais ou receptores (Pacher et al., 2007). Ele é gerado pela NO sintase (NOS), através da conversão de L-arginina a citrulina. Três tipos de NOS foram identificadas: NO sintase endotelial (eNOS), que é constitutiva do endotélio vascular e conhecida por ser fortemente ativada pela entrada de cálcio através da membrana; NO sintase induzível (iNOS), que foi identificado pela primeira vez nos macrófagos e em seguida, em outras células, incluindo hepatócitos, é conhecido por *up*-regulada por citocinas pró-inflamatórias e/ou lipopolissacarídeos, e é capaz de gerar

níveis baixos de NO em comparação às outras isoformas; e a NO sintase neuronal (nNOS) (Gross et al., 1995, Liaudet et al., 2000).

O NO exerce seus efeitos fisiológicos controlando o tono vascular, célula de adesão, a permeabilidade vascular e plaquetas de adesão (Ignarro et al., 1985, Radomski et al., 1987, Archer et al., 1994, Droge 2002). Ele também exerce, potencialmente, efeitos tóxicos, embora muitos deles sejam, provavelmente, mediados por produtos de oxidação incluídos na definição de espécies reativas de nitrogênio (ERN). Em particular, o NO é capaz de reagir rapidamente com $O_2^{\bullet-}$ para formar peroxinitrito ($ONOO^-$), contribuindo, também, para o estresse oxidativo.

Ele pode reagir com proteínas (nitração direta da tirosina ou reações com aminoácidos específicos), lipídios (peroxidação lipídica), podendo ser avaliado pela técnica de TBARS (Buege et al., 1978), e ácidos nucléicos (modificações oxidativas nas bases nucléicas), que pode ser avaliada pela técnica de teste cometa (Speit et al., 1999, Picada et al., 2003). Essa espécie reativa de nitrogênio (ERN) pode também interagir com mitocôndrias, atingindo compartimentos extra-mitocondriais ou ser produzida localmente através da interação do NO e $O_2^{\bullet-}$, resultando em reações oxidativas diretas de componentes principais da cadeia respiratória ou de radical livre mediando danos. A persistente geração de níveis significativos de $ONOO^-$ pode levar à indução de morte celular, tanto por apoptose quanto por necrose (Novo et al., 2008).

FIGURA 5 GERAÇÃO E REAÇÃO DO PEROXINITRITO.



FONTE: (adaptado de Novo et al., 2008)

2.8 ANTIOXIDANTES

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

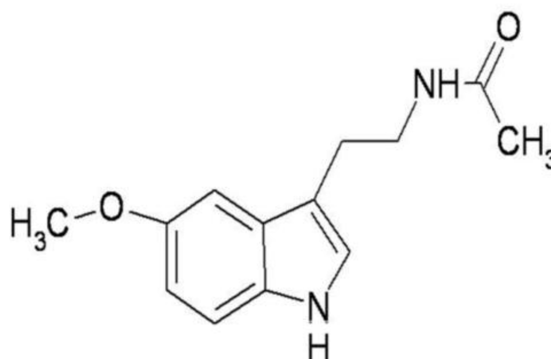
A defesa do organismo contra as ERO vai desde prevenção da formação das ERO, interceptação dos radicais formados, a reparo das células danificadas. Os sistemas que previnem a formação de ERO são considerados biomoléculas ligantes de metais (Fe^{2+} e Cu^{+}), ou seja, são os quelantes. A presença de proteínas quelantes é de vital importância aos seres vivos, pois previne as células de processos oxidativos catalisados por íons metálicos. Pigmentos especializados previnem a ação da radiação ultravioleta, a melanina e os carotenoides impedem a ação do oxigênio *singlet*. As enzimas que controlam os níveis de ERO são Glutationa peroxidase (GPx), Superóxido dismutase (SOD) e Catalase (CAT), sendo a interceptação a desativação das ERO, quando elas são destruídas de forma a impedir a oxidação posterior de outras moléculas. A desativação final de um composto com um ou mais

elétrons não emparelhados consiste na formação de outro produto não radical. O interceptador (antioxidante) mais eficiente deve combinar propriedades ótimas, as quais reagem com RL iniciais, tais como, radicais peroxil ($ROO^{\bullet}$) e, posteriormente, interagem com compostos hidrossolúveis para a sua própria regeneração (Halliwell 2007).

Os compostos hidrossolúveis transferem a função radical para longe do sítio-alvo potencial e são chamados “scavengers” de RL. A combinação de uma substância com um RL leva à formação de um não radical ou um radical menos lesivo como, por exemplo, tocoferóis e carotenoides. As substâncias que funcionam como “quenchers” de oxigênio *singlet* são aquelas que absorvem a energia de excitação e a liberam em forma de calor ou movimento (Halliwell 2007).

A Melatonina (N-acetil-5-metoxitriptamina) é produzida na glândula pineal, e é conhecida como “hormônio da escuridão” devido sua máxima produção ser no escuro (Arendt 2005). Estudos Experimentais e clínicos mostram que a Melatonina tem papel antioxidante, neuroprotetor, cardioprotetor e anticarcinogênico (Acuna-Castroviejo et al., 2007). Entretanto, o mecanismo exato da ação da Melatonina não é claramente compreendido (Raza et al., 2008).

FIGURA 6 ESTRUTURA MOLECULAR DA MELATONINA



FONTE: (Srinivasan et al., 2011).

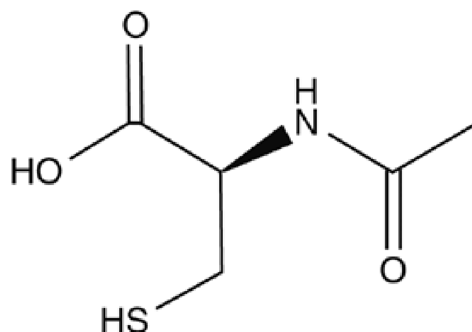
A melatonina parece proteger, experimentalmente, ratos induzidos a dano oxidativo (El-Missiry et al., 2007). Uma molécula de Melatonina pode prender quase dois radicais peroxil, protegendo o DNA e os eritrócitos (Zhao et al., 2008). Ela estabiliza as membranas de encontro aos radicais livres (Garcia et al., 1997, Garcia et al., 1998).

A Melatonina estimula determinadas enzimas oxidativas, como as glutationas peroxidase (Marin et al., 1997). Algumas outras vantagens da Melatonina é que ela pode prontamente cruzar membranas biológicas (Costa et al., 1995), e assim alcançar todos os compartimentos sub-celulares, protegendo-os dos radicais livres, e ela não possui nenhuma toxicidade (Millan-Plano et al., 2003).

A N-acetilcisteina (NAC) é um composto tiólico que contém um grupo sulfidril formado pela seguinte fórmula química $C_5H_9NO_3S$, seu peso molecular é igual a 163,2 e é amplamente usado na clínica médica (Ziment 1988). A NAC é um mucolítico que teve suas primeiras aplicações no tratamento de doenças congestivas e obstrutivas pulmonares associadas à hipersecreção. A NAC também é usada no tratamento da síndrome da angústia respiratória no adulto e em casos de imunodeficiência adquirida na infecção por HIV (Sarnstrand et al., 1995). Sua atividade antioxidante é dada principalmente por dois mecanismos:

- Reduzir diretamente o H_2O_2 e o $O_2^{\bullet -}$ a espécies menos reativas formando radicais sulfúricos ou cisteína;
- Promover a biossíntese de GSH que funciona como "scavenger" de radicais livres ou como substrato no ciclo redox da glutationa (Aruoma et al., 1989).

FIGURA 7 ESTRUTURA MOLECULAR DA N-ACETILCISTEÍNA



FONTE: (Tepel 2007).

O aumento da expressão da enzima antioxidante superóxido dismutase pode proteger as células contra apoptose na HI (Xu et al., 2009).

2.6 Apneia do Sono e Dano Hepático

O grau de esteatose hepática está diretamente ligado ao índice de apneia/hipopneias por hora, sendo reconhecido como um fator de risco (Tanne et al., 2005, Shpirer et al., 2010), independentemente de índice de massa corporal, sugere desempenhar um papel na patogênese de EHNA, aumenta a resistência à insulina e deve possuir uma contribuição direta da hipóxia na lesão hepática (Tanne et al., 2005).

2.7.1 Doença Hepática Gordurosa Não alcoólica (DHGNA)

Doença hepática gordurosa não alcoólica abrange um grande espectro de doenças, variando de esteatose sem inflamação à esteatohepatite não alcoólica (EHNA) e cirrose hepática (Day et al., 1998, Browning et al., 2004). A progressão da DHGNA é proposta com um modelo de dois “hits”, o primeiro envolvendo o acúmulo de triglicérides nos hepatócitos, sendo atribuída a ligação com a resistência insulínica e obesidade. O aumento de ácido graxo livre induz a biossíntese de triglicérides e esteatose hepática. A progressão da esteatose para EHNA é atribuído ao segundo “hit” que é o desenvolvimento de inflamação e fibrose (Day et al., 1998).

A DHGNA é atualmente a doença hepática crônica mais comum no mundo, afetando 30% da população geral adulta e de 60 a 70% dos diabéticos portadores de obesidade (Younossi et al., 2011). Segundo alguns autores, relaciona-se que SAOS predispõe o desenvolvimento de síndrome metabólica,

diabetes mellitus e doença cardiovascular, e que esta relação é pelo menos parcialmente independente da obesidade (Coughlin et al., 2004, Marin et al., 2005, West et al., 2006, Drager et al., 2011). Não se possui ainda tratamento eficaz para EHNA, porém, um tratamento eficaz para SAOS é o CPAP que pode melhorar as alterações metabólicas e cardiovasculares (Drager et al., 2007, Abe et al., 2010, Sharma et al., 2011).

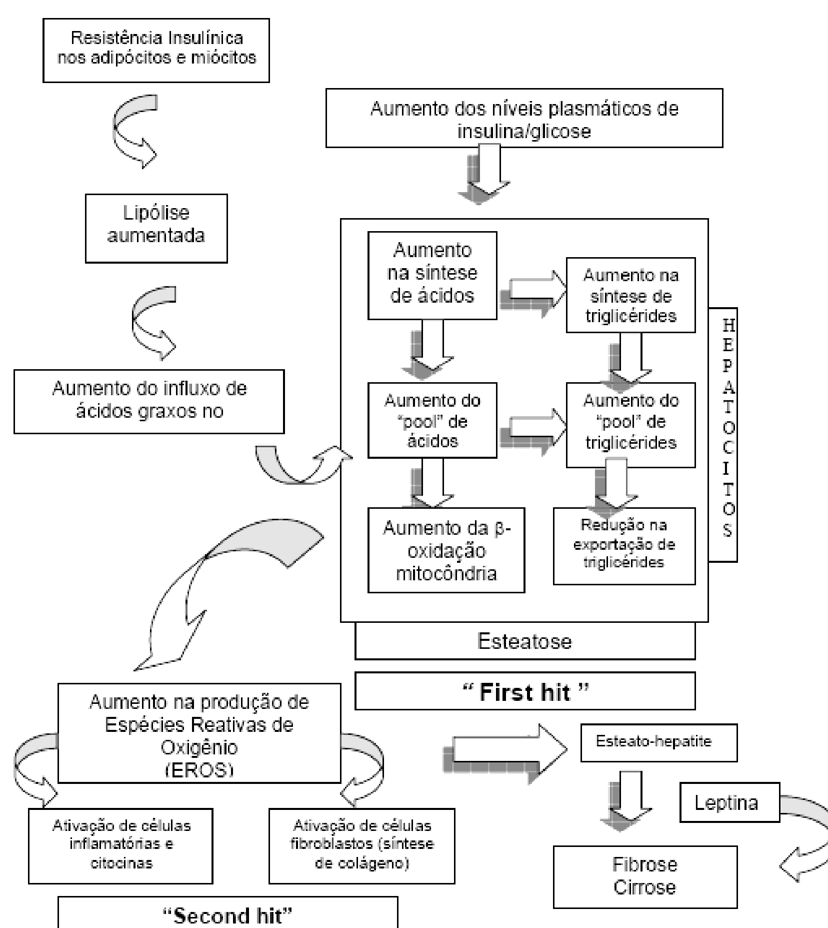
A imensa maioria dos pacientes com DHGNA, bem como com EHNA, é assintomática, porém com aumento de enzimas hepáticas ou a presença de fígado hiperecogênico na ultrassonografia sugere a doença (Marchesini et al., 2003). Cerca de 90% dos pacientes com essa enfermidade apresentam pelo menos uma manifestação da síndrome metabólica, sendo que aproximadamente 1/3 deles apresenta a síndrome completa que é definida com pelo menos três das seguintes características: obesidade central, hipertrigliceridemia, colesterol HDL (lipoproteína de alta densidade) baixo, hipertensão arterial e glicemia acima de 110 mg/dl (Torres et al., 2008).

Ainda não está totalmente esclarecida a relação entre esteatose, fibrogênese e a doença crônica no fígado. A teoria mais aceita atualmente foi proposta por Day e James (1998) (Day et al., 1998, Day et al., 1998) e se refere à teoria dos dois “hits”. O processo de formação da EHNA ocorre em duas etapas fundamentais: a deposição lipídica e a reação inflamatória (Day et al., 1998). A etapa inicial, segundo a teoria dos “hits” seria o acúmulo de ácidos graxos no hepatócito, suplantando sua capacidade de metabolização e exportação consequente à ação lipogênica da insulina. A hiperinsulinemia presente na obesidade e síndrome metabólica favoreceria a lipogênese hepática e aumentaria a lipólise periférica, aumentando excessivamente a quantidade de ácidos graxos para o fígado, condições estas favoráveis para a infiltração gordurosa hepática (McCullough 2006). Logo, a resistência insulínica seria a condição inicial ou o primeiro estímulo (“first hit”).

Os ácidos graxos livres no fígado são normalmente metabolizados pela β-oxidação mitocondrial, principal etapa do catabolismo dos ácidos graxos que ocorre na mitocôndria (a carnitina carrega o ácido graxo do citosol para a matriz mitocondrial, onde fragmentos de dois carbonos são removidos do

terminal carboxila da acil-CoA, produzindo acetil-CoA, NADH e FADH2), todavia, sob condições de desequilíbrio, a β-oxidação peroxissomal é ativada, gerando peróxido de hidrogênio (H_2O_2). Estes, por sua vez, podem levar à formação do radical hidroxil (OH^-), altamente reativo, levando ao estresse oxidativo e consequentemente à inflamação e fibrose (Neuschwander-Tetri et al., 1990).

FIGURA 8 MODELO PROPOSTO DE FISIOPATOLOGIA DA EHNA



FONTE: (adaptado de Day et al., 1998).

2.7.2 Fator induzível de Hipóxia 1 alfa (HIF-1α)

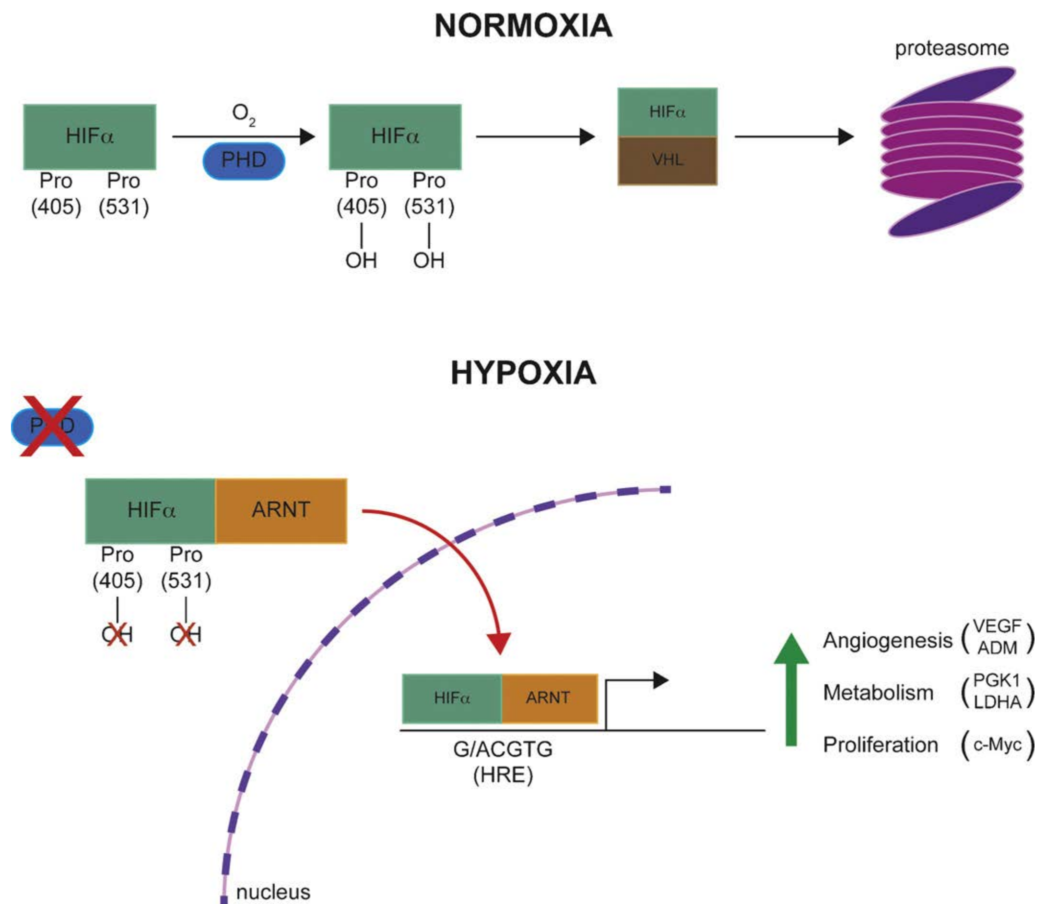
Células de mamíferos podem desenvolver uma série de adaptações para sobreviver à hipóxia aguda e até mesmo prolongada. A hipóxia reduz a capacidade de a célula manter o seu nível de energia, provinda da glicólise e

da fosforilação oxidativa (Semenza 2003). Em casos graves de hipóxia, provocará uma elevada taxa de mutação, o que pode ser explicado pela redução da atividade de reparo de DNA (Perou et al., 2000). Porém, nesses casos, a célula inicia uma cascata de eventos que levam à morte celular por apoptose, prevenindo assim o acúmulo de células com mutações induzidas por hipóxia (Reynolds et al., 1996). O regulador chave da resposta à hipóxia é o fator induzível de hipóxia 1 (HIF-1). O HIF-1 pode induzir a apoptose (Carmeliet et al., 1998, Moritz et al., 2002), prevenir a morte celular, ou até mesmo estimular a proliferação celular (Akakura et al., 2001).

HIF-1 é uma proteína heterodimérica composta de duas subunidades: a subunidade β, constitutivamente expressa, e uma subunidade α, em que a expressão e a atividade são controladas pela concentração de oxigênio intracelular (Chilov et al., 1999, Semenza 2000). Durante normóxia, HIF-1α é rapidamente degradada pelo sistema ubiquitina proteossomal, enquanto que a exposição a condições de hipóxia impede a sua degradação (Salceda et al., 1997, Huang et al., 1998, Kallio et al., 1999).

A hidroxilação dos resíduos de prolina (405 e 531), na normóxia, ocorre pela degradação dependente de oxigênio na subunidade alfa é mediada pela proteína dominante prolil hidroxilase (PHD) sob tensão de O_2 normal. Com a baixa tensão de oxigênio a degradação pela PHD fica comprometida, liberando o HIF-1α para translocar ao núcleo com a ligação ARNT/HIF-1β, pela interação HLH e PAS dominantes e com recrutamento de coativadores como a CBP/p300 (Shay et al., 2012).

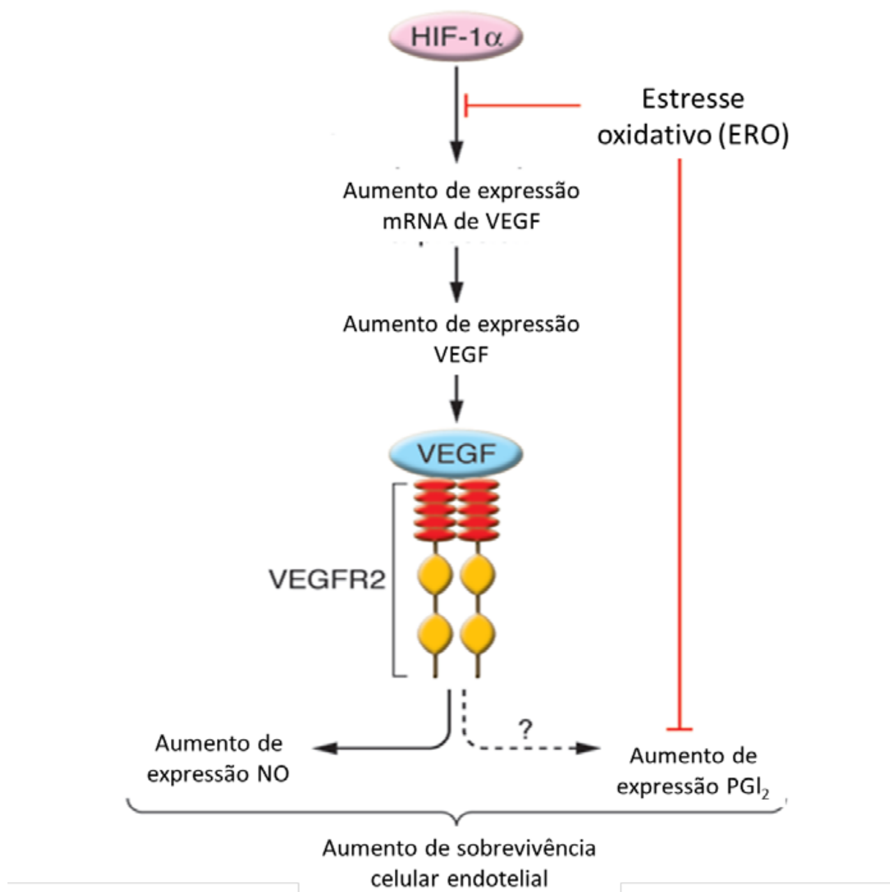
FIGURA 9 REPRESENTAÇÃO DA ATIVIDADE DO HIF-1A NA PRESENÇA DE OXIGÊNIO E NA HIPÓXIA.



FONTE: (Shay et al., 2012)

A falta de O_2 ativa o HIF-1α, que estimula a eritropoietina (Satoh et al., 2006) e fator de crescimento vascular endotelial (VEGF) o que juntamente com os fatores de transformação de crescimento beta (TGF-β), de crescimento derivado de plaquetas (PDGF) e moléculas de adesão intercelular-1 (ICAM-1), participa da ativação de promotores e genes durante a hipóxia (Forsythe et al., 1996).

FIGURA 10 EXPRESSÃO DE NO POR VÍA DO HIF-1α



FONTE: (adaptado de Taraseviciene-Stewart et al., 2008).

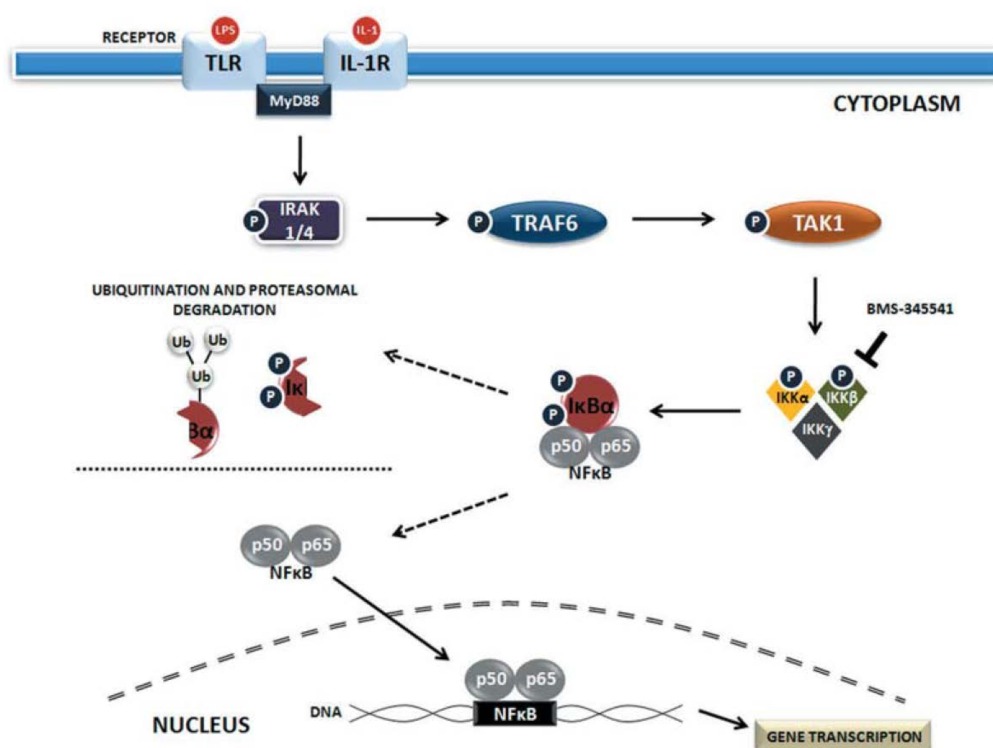
A expressão do gene VEGF é controlada pelo HIF-1α. Em células endoteliais, a síntese de prostaciclína (PGI_2) e óxido nítrico (NO) é um resultado da ativação do receptor de VEGF (VEGFR2). Ativação de VEGFR2 induz a produção de NO, que em conjunto com a PGI_2 promovem a sobrevivência das células endoteliais. Porém, ainda não está estabelecida a ligação entre a ativação de VEGFR2 e a síntese de PGL_2.

2.7.3 Fator nuclear kappa B

A família do fator nuclear kappa B (NF-kB) compõe-se de cinco membros: REL-a (p65), NF-kB1 (p50; p105), NF-kB2 (p52; p100), c-REL e REL-b (Ghosh et al., 1998). Essas subunidades, exceto a REL-b, são homo ou heterodímeros que formam o NF-kB. O mais comum ativado em reações inflamatórias é o heterodímero de p50 e p65 (Souza et al., 2012).

A transcrição nuclear do NF-kB é regulada pela ação inibitória do inibidor de proteínas kB (IkB), que é alvo para degradação pela via de fosforilação pela ação de quinases IkB (IKKα, IKKβ) (Nath et al., 2012). A sinalização inflamatória ativa uma cascata de evento, como a fosforilação do receptor TNF que leva à ativação do fator de crescimento transformante-b-quinase ativada 1 (TAK1) que fosforiza o complexo IKK e em seguida fosforiza a proteína IkBa, resultando em ubiquinação, dissociação de IkBa com NF-kB e degradação de IkBa pelo protessoma. O NF-kB se desloca para o núcleo e liga-se com a banda específica de DNA, inicializando a transcrição de múltiplos genes, incluindo citocinas, quimosinas e outros mediadores inflamatórios (Souza et al., 2012).

FIGURA 11 ATIVAÇÃO DO NF-KB



FONTE: (Souza et al., 2012).

O NF-kB é o regulador mestre do processo inflamatório e sua ativação na hipóxia leva à ativação de TNF-α, interleucinas 1 (IL-1) e interleucinas 6 (IL-6), entre outros. Muitos desses mediadores também são ativados pelo fator HIF

(Greenberg et al., 2006, Htoo et al., 2006, Selmi et al., 2007). Rius e colaboradores evidenciaram que o HIF-1α é suprimido, em qualquer estímulo, em ratos nulos de IKKβ. Demonstrando que o NF-kB pode regular a transcrição de HIF-1α (Rius et al., 2008).

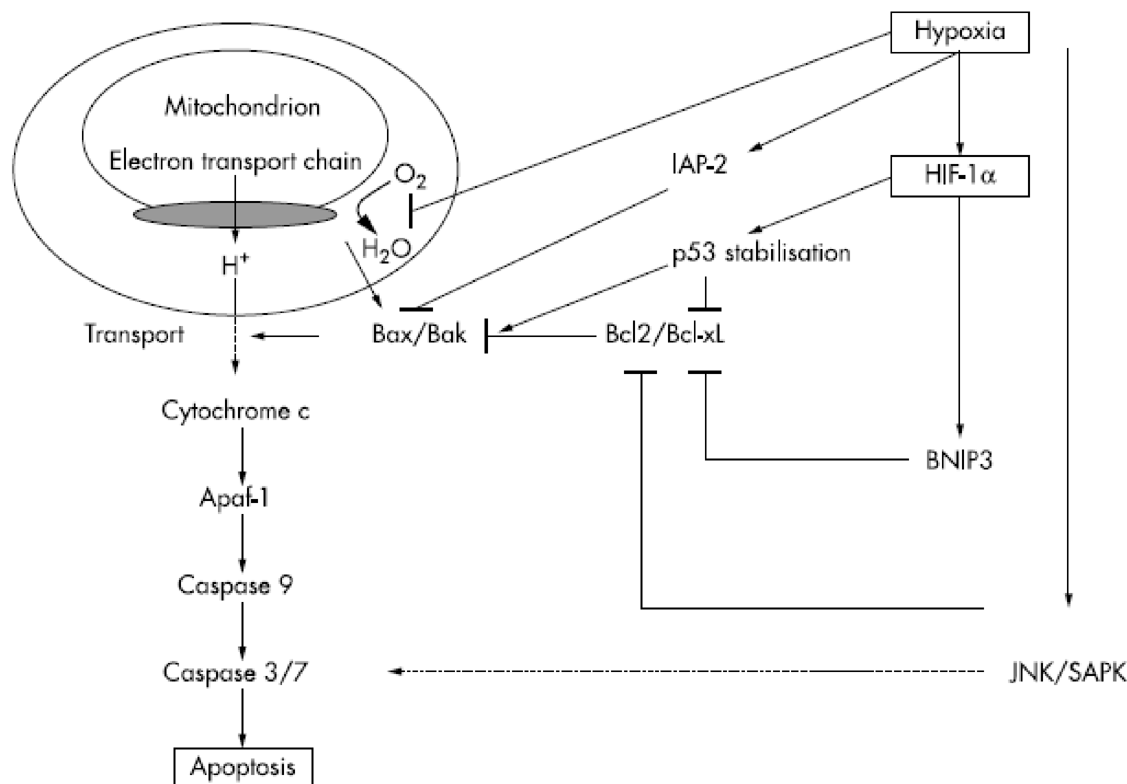
2.7.4 Caspases

São características da apoptose: a condensação da cromatina, exposição da fosfatidilserina à superfície da célula, encolhimento citoplasmático, formação de corpos apoptóticos e fragmentação do DNA (van de Schepop et al., 1996). A apoptose é um processo dependente de energia, diferentemente da necrose que também ocorre na ausência de ATP (McClintock et al., 2002). A apoptose é regulada por uma cascata de proteínas chamadas Caspases, sendo essas as proteínas executoras da apoptose e estão presentes em todas as células. Após a clivagem, Caspases tornam-se ativos e iniciam vias que levam à apoptose (Greijer et al., 2004).

A via de sinalização que leva à morte celular programada é mantida por reguladores positivos e negativos, esse equilíbrio entre tais fatores decide se a célula sofre apoptose ou se sobrevive. As proteínas que podem alterar-se para a sobrevivência são as proteínas antiapoptóticas Bcl-2 e Bcl-xL, enquanto que as proteínas proapoptóticas Bax, Bad, Bak e Bis induzem à morte celular programada (Gross et al., 1999).

Um importante regulador de apoptose após a lesão do DNA é a proteína p53, que pode induzir a Bax e Bak, que regula a liberação de citocromo C da mitocôndria, iniciando assim a cascata que conduz à apoptose (Wei et al., 2001). O citocromo C liga-se ao fator de ativação de protease apoptótica 1 (apaf-1) ativando a Caspase 9, que por sua vez cliva as Caspases 3 e 6 (Li et al., 1997, Nicholson et al., 1997) levando à morte celular.

FIGURA 12 REPRESENTAÇÃO ESQUEMÁTICA DE VIAS DE SINALIZAÇÃO DE APOPTOSE INDUZIDA POR HIPÓXIA.



As linhas cheias indicam uma interação direta, a linha tracejada uma interação indireta.
FONTE: (Greijer et al., 2004).

Em células tumorais, o HIF-1α pode induzir a apoptose por meio de dois mecanismos, aumentando a estabilidade do produto do gene supressor de tumor p53 ou pela produção de BNIP3 (Bcl2/adenovírus E1B19 kDa interagindo proteína 3) e NIX, um homólogo de BNIP3, que agem inibindo as proteínas antiapoptóticas Bcl-2 e BcL-xL (Boyd et al., 1994).

Tendo em vista essas considerações citadas, pretendemos com esse trabalho desenvolver em modelo de hipóxia intermitente, por meio de simulação de apneia do sono, a avaliação dos danos causados no pulmão e fígado, e a ação dos antioxidantes no tecido hepático.

3 Objetivos

3.1 OBJETIVO PRINCIPAL

Avaliar no modelo de hipóxia intermitente, simulando apneia do sono em camundongos, o estresse oxidativo, o processo inflamatório e o uso dos antioxidantes Melatonina e N-acetilcisteína.

3.2 OBJETIVOS SECUNDÁRIOS

Respondido no Experimento I – *Hepatic oxidative stress in an animal model of sleep apnoea: effect of different duration of exposure* – Publicado na *Comparative Hepatology – Biomed Central,* 2011, 10**:1**

∴ Definir um modelo experimental de hipóxia intermitente em camundongos o qual mimetiza as alterações hepáticas;

∴ Avaliar a integridade hepática pelas enzimas Alanina e Aspartato aminotransferases e Fosfatase alcalina.

∴ Avaliar os danos oxidativos, aos lipídios de membrana e ao DNA no fígado.

∴ Quantificar a atividade antioxidante das enzimas SOD, GPx e CAT no fígado.

∴ Quantificar a Glutationa total no fígado.

∴ Quantificar os metabólitos do óxido nítrico no fígado.

∴ Verificar alterações histológicas hepáticas por meio da coloração de HE.

Respondido no Experimento II – *Simulating sleep apnea by exposure to intermittent hypoxia induces inflammation in the lung and liver* – Publicado na *Mediators of Inflammation - Hindawi, 2012, Article ID 879419, 8 pages*

∴ Verificar o processo inflamatório e apoptótico nos tecidos pulmonar e hepático.

∴ Avaliar o dano oxidativo aos lipídios de membrana no pulmão e no fígado.

∴ Quantificar a atividade antioxidante das enzimas SOD e CAT em ambos os tecidos analisados.

∴ Quantificar, no pulmão e no fígado, as expressões das proteínas NF-κB pela porção p65, HIF-1α, TNF- α, iNOS, VEGF e Caspase 3 pela porção clivada.

Respondido no Experimento III – *Antioxidants inhibit the inflammatory and apoptotic processes in an intermittent hypoxia model of sleep apnea* – Enviado à publicação para Liver International – Wiley

∴ Avaliar a ação dos antioxidantes Melatonina e N-acetilcisteína sobre os danos no fígado de camundongos expostos à hipóxia intermitente.

∴ Avaliar a integridade hepática avaliada pelas enzimas hepáticas AST, ALT e fosfatase alcalina.

∴ Avaliar a lipoperoxidação hepática e quantificar a atividade antioxidante da enzima SOD no modelo experimental.

∴ Verificar a ação da MEL e NAC sobre as alterações histológicas do fígado pela coloração de hematoxilina e eosina.

∴ Quantificar as expressões das proteínas HIF-1α, NF-κB pela porção p65, IKB- α, Caspase 3 e 6 pelas porções clivadas, TNF-α, iNOS, VEGF e TGF-β.

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RESEARCH Open Access

Hepatic oxidative stress in an animal model of sleep apnoea: effects of different duration of exposure

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Abstract

Background: Repeated apnoea events cause intermittent hypoxia (IH), which alters the function of various systems and produces free radicals and oxidative stress.

Methods: We investigated hepatic oxidative stress in adult mice subjected to intermittent hypoxia, simulating sleep apnoea. Three groups were submitted to 21 days of IH (IH-21), 35 days of IH (IH-35), or 35 days of sham IH. We assessed the oxidative damage to lipids by TBARS and to DNA by comet assay; hepatic tissue inflammation was assessed in HE-stained slides. Antioxidants were gauged by catalase, superoxide dismutase, glutathione peroxidase activity and by total glutathione.

Results: After IH-21, no significant change was observed in hepatic oxidative stress. After IH-35, significant oxidative stress, lipid peroxidation, DNA damage and reduction of endogenous antioxidants were detected.

Conclusions: In an animal model of sleep apnoea, intermittent hypoxia causes liver damage due to oxidative stress after 35 days, but not after 21 days.

Background

In obstructive sleep apnoea (OSA), pharyngeal occlusion occurs, typically for 10 to 40 seconds, causing a decrease of PaO_2 and an increase in $PaCO_2$, ending with an arousal [1]. Intermittent hypoxia due to OSA causes oxidative stress, a recognized mechanism in the nonalcoholic fatty liver disease (NAFLD), which may progress to non-alcoholic steatohepatitis (NASH) [2].

Intermittent hypoxia (IH) increases liver damage [3]. During hypoxia, activation of xanthine oxidase [4], NAPDH oxidase [5], and phospholipase A_2 [6] occurs, forming reactive oxygen species (ROS). Increased ROS and decreased antioxidant capacity [7-9] induce oxidative stress [10]. In hypoxia, superoxide anions are formed, which, together with nitric oxide (NO), the main vasodilator, produce peroxynitrite [11-13]. This reaction reduces the bioavailability of NO, attenuating NO-dependent vasodilation, capillary perfusion and expression of adhesion molecules [14-17].

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The formation of ROS in OSA is similar to what occurs in ischemia-reperfusion [18]. Oxidative stress leads to inflammation, recognised as a mechanism of the pathophysiology of OSA [19]. Excessive formation of ROS leads to lipid peroxidation in cell membranes, protein oxidation and DNA damage [20-22]. Several ROS are formed in hepatocytes through the activation of Kupffer cells and inflammatory cells [23].

Another group has exposed mice to IH and to a high-cholesterol diet for 6 months, revealing the involvement of OSA in non-alcoholic steatohepatitis (NASH) [3]. IH aggravates paracetamol-induced liver damage after 21 days [24]. To understand the mechanisms leading to NAFLD and NASH it may relevant to identify the time frame in which these phenomena occur. There are, however, no studies specifically investigating the duration of IH exposure that causes liver damage in an animal model of sleep apnoea. This knowledge will be relevant to help design future studies.

The aim of the present study was to establish the duration of exposure to intermittent hypoxia necessary and sufficient to trigger liver damage and oxidative stress in mice.

Methods

The experimental procedures complied with the rules established by the "Research in Health and Animal Rights" according to the Commission of Research and Ethics in Health of the Research and Postgraduate Group of the Hospital de Clínicas de Porto Alegre.

Thirty-six male CF-1 mice (8-11 weeks old) from Fundação Estadual de Produção e Pesquisa (FEPPS) were employed. They were kept at the Animal Experimentation Unit of the Research Center of the Hospital de Clínicas of Porto Alegre in plastic boxes measuring 30 × 19 × 13 cm lined with wood chips, in a 12-hour dark/light cycle (light from 7 a.m. to 7 p.m.) at a temperature of 22 4°C. The mice were given food (Purina-Nutripal, Porto Alegre, RS, Brazil) and water *ad libitum*.

The animals were randomly divided into three groupings (n = 12): group SIH, sham intermittent hypoxia, which underwent the simulated procedure; group IH-21, exposed to hypoxia for 21 days; and group IH-35, exposed hypoxia for 35 days.

IH procedures were described in detail before [25]. In brief, during five weeks, 7 days per week, 8 hours a day, from 9 a.m. to 5 p.m., in the lights-on period, the rodents were placed in the cages (Figure 1). A mixture with 90% nitrogen and 10% CO_2 was released in the hypoxia chamber, for 30 seconds. The gas mixture reduced the oxygen fraction from 21% to approximately 8% and the CO_2 fraction to 6%. Subsequently, a fan insufflated room air in the chamber for 30 seconds, restoring the oxygen fraction to 21%. Each hypoxia/normoxia cycle lasted for 60 seconds; in 8 hours, 480 IH periods occurred, equivalent to an apnea index of 60 per hour.

The SIH group was housed in an adjacent cage and underwent the same fan activity as the IH group, but no gas was introduced in the cage during the hypoxia cycle (Figure 1).

On the 21st or 35th day, the animals were killed. They were first anaesthetised with ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (50 mg/kg ip). Blood was collected from the retro-orbital vein with the aid of a heparinised glass capillary [26] to complete the hepatic integrity (AST, ALT and ALP) test and comet assay. We removed the liver of animals for histological analysis; the rest were frozen -80°C for later biochemical analysis. The animals were euthanized by exsanguination under deep anaesthesia [27,28].

Nine millilitres of phosphate buffer (140 mM KCL, 20 mM phosphate, pH 7.4) per tissue gram was added, and tissue was homogenised in an Ultra Turrax at 4°C. Next, it was centrifuged for 10 minutes at 4,000 rpm (2150.4 g). The samples were stored again at -80°C for posterior analyses.

We used the Bradford method to quantify protein, with bovine albumin as the standard (Sigma®). The samples were measured spectrophotometrically at 595 nm, and values expressed in mg/g liver [29] were used to calculate values of TBARS (thiobarbituric acid-reactive substances) and antioxidant enzymes.

The amount of aldehydes generated by lipid peroxidation is measured by the TBARS method, which measures the amount of substances reacting with thiobarbituric acid. The samples were incubated at 100°C for 30 minutes after addition of 0.37% thiobarbituric acid in 15% trichloroacetic acid and centrifuged at 3000 rpm (1612.8 g) for 10 minutes at 4°C. Absorbance was determined spectrophotometrically at 535 nm [30].

The analysis of SOD is based on the inhibition of the reaction of the superoxide radical with adrenaline [31].

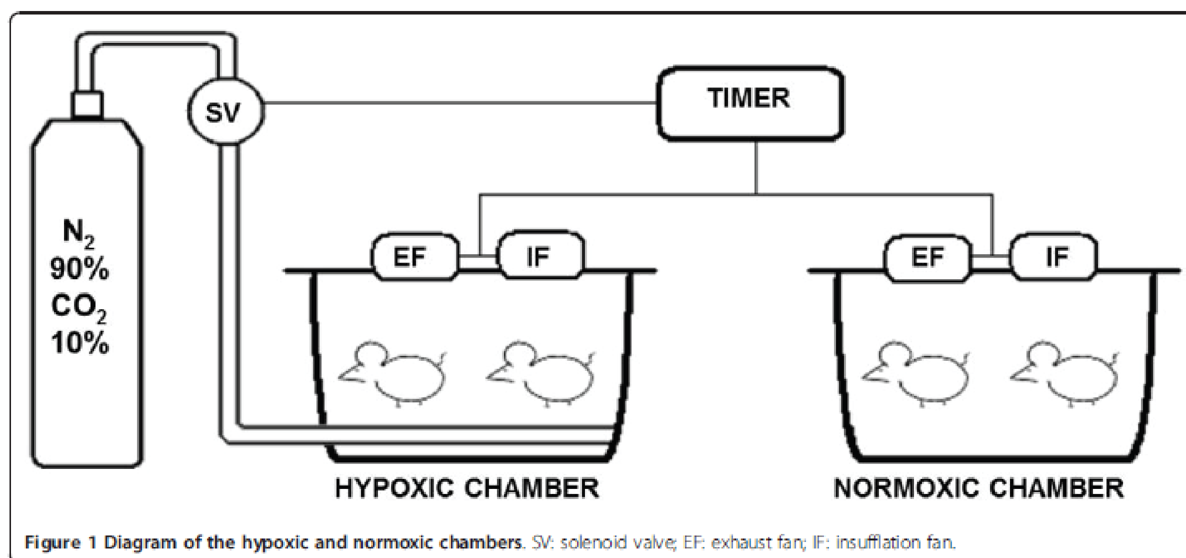

Figure 1 Diagram of the hypoxic and normoxic chambers. SV: solenoid valve; EF: exhaust fan; IF: insufflation fan.

The auto-oxidation rate of epinephrine, which is progressively inhibited by increasing amounts of SOD in the homogenate, was monitored spectrophotometrically at 480 nm. The amount of enzyme that inhibited 50% of epinephrine auto-oxidation was defined as 1 U of SOD activity.

The analysis of CAT activity is based on measuring the decrease in hydrogen peroxide [32]. Catalase activity was determined by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM phosphate buffer saline (pH 7.2) and 0.3 M hydrogen peroxide. The enzyme activity was assayed spectrophotometrically at 240 nm.

The activity of GPx is based on the consumption of NADPH in the reduction of oxidised glutathione [33]. The glutathione peroxidase activity was determined by the oxidation rate of NADPH in the presence of reduced glutathione and glutathione reductase. Sodium azide was added to inhibit catalase activity. The GPx activity was measured with a spectrophotometer at 340 nm.

Total glutathione (GSH), a water soluble non-enzymatic antioxidant, [34] was measured as described previously [35], in a reaction medium consisting of a solution of 300 mM phosphate buffer ($Na_2HPO_4 \cdot 1H_2O$) and a solution of dithionitrobenzoic acid (DTNB). The reaction products were read at 412 nm.

The alkaline comet assay was carried out as described in [36], with minor modifications [37]. The liver tissue samples (200-250 mg) were placed in 0.5 mL of cold phosphate-buffered saline (PBS) and finely minced in order to obtain a cell suspension; the blood samples (50 μL) were placed in 5 μL of anti-coagulant (heparin sodium 25.000 UI- Liquemine®). Liver and blood cell suspensions (5 μL) were embedded in 95 μL of 0.75% low melting point agarose (Gilco BRL) and spread on agarose-precoated microated microscope slides. After solidification, slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA an 10 mM Tris, pH 10.0), with freshly added 1% Triton X-100 (Sigma) and 10% DMSO for 48 h at 4°C. The slides were subsequently incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min, at 4°C. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 15 min to perform DNA electrophoresis. The slides were then neutralized (0.4 M Tris, pH 7.5), stained with silver and analyzed using microscope. Images of 100 randomly select cells (50 cells from each of two replicate slides) were analyzed from each animal. Cells were also visually scored according to tail size into five classes ranging from undamaged (0) to maximally damage (4), resulting in a single DNA damage score to each animal, and consequently to each studied group. Therefore, the damage index (DI) can range from 0 (completely undamaged, 100 cells × 0) to 400 (with maximum damage, 100 × 4). Damage frequency (%) was calculated based on the number of tailed versus tailless cells.

The levels of nitrates and nitrites were measured by the reaction of the samples with Griess reagent. Aliquots of 50 μL were incubated with enzyme cofactors and nitrate reductase for 30 minutes at room temperature for the conversion of nitrate to nitrite. The nitrite formed was then analysed by reaction with the Griess reagent, forming a coloured compound that was measured by spectrophotometer at a wavelength of 540 nm [38].

For histological evaluation, part of the liver was preserved in 10% formalin for 24 hours, embedded in paraffin, and cut into 6-μm thick sections with a microtome. Sections were stained with hematoxylin and eosin.

The results are expressed as mean ± standard error. We used ANOVA and the Student-Newmann-Keuls or Student's t-test for comparing groups. The significance level was 5% ($p < 0.05$).

Results

The circulating levels of the liver enzymes aspartate aminotransferase (AST), alanine amino transferase (ALT), and alkaline phosphatase (ALP), parameters of liver damage, showed no significant difference between the IH-21 group and the SIH. The IH-35 group showed significantly increased levels ($p < 0.05$) compared to the sham intermittent hypoxia group (Table 1).

Lipid peroxidation measured by the TBARS technique showed no oxidative damage in group IH-21 compared to SIH. However, there was significant damage in the lipid peroxidation in liver subjected to hypoxia for 35 days (Figure 2). Evaluation of the antioxidant enzymes showed a significant decrease in the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in liver tissue with intermittent hypoxia for 35 days (Table 2). The quantification of total endogenous glutathione in the liver showed a significant decrease in the 35-day hypoxia group compared with the sham intermittent hypoxia (Figure 3). These results demonstrate that IH induced a decrease in the endogenous antioxidant defence.

Table 1 Enzymes indicating hepatic integrity: AST, ALT and alkaline phosphatase.

Enzymes	SIH	IH-21	IH-35
AST (U/L)	124.4 ± 6.5	94.36 ± 7.05	145.8 ± 7.2[a]
ALT (U/L)	45.5 ± 4.0	48.50 ± 2.85	55.6 ± 1.3[b]
AP (U/L)	97.7 ± 3.1	84.25 ± 1.98	122.6 ± 2.4[c]

Data are presented as mean ± standard error (n = 12 animals/group). [a] IH-35 vs SIH, p = 0,04; [b] IH-35 vs SIH, p = 0,03; [c] IH-35 vs SIH, p < 0,0001. SIH: sham intermittent hypoxia group; IH-21: intermittent hypoxia for 21 days; IH-35: intermittent hypoxia for 35 days; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase.

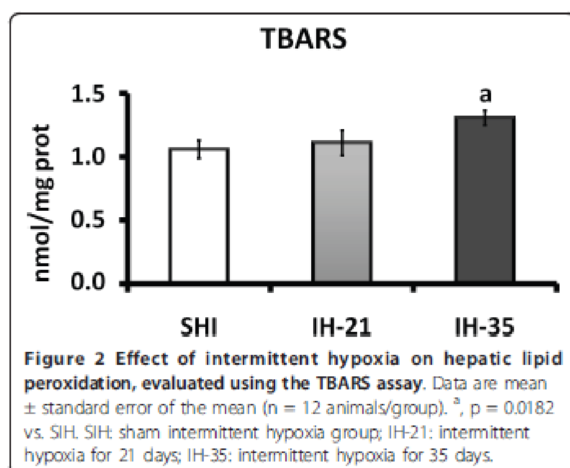

Figure 2 Effect of intermittent hypoxia on hepatic lipid peroxidation, evaluated using the TBARS assay. Data are mean ± standard error of the mean (n = 12 animals/group). [a], p = 0.0182 vs. SIH. SIH: sham intermittent hypoxia group; IH-21: intermittent hypoxia for 21 days; IH-35: intermittent hypoxia for 35 days.

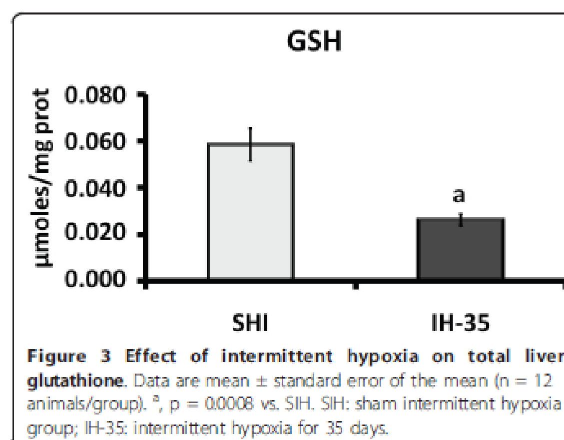

Figure 3 Effect of intermittent hypoxia on total liver glutathione. Data are mean ± standard error of the mean (n = 12 animals/group). [a], p = 0.0008 vs. SIH. SIH: sham intermittent hypoxia group; IH-35: intermittent hypoxia for 35 days.

The assessment of DNA damage by the comet assay showed that the damage in blood did not differ between groups, but the liver tissue exhibited a significant increase in DNA damage in group IH-35 compared with SIH (Table 3).

In the assessment of metabolites of nitric oxide in liver tissue of mice subjected to IH for 35 days, we noted a significant increase in NO in these animals compared with SIH (Table 4).

Several histological liver changes were also observed in animals of the IH-35 group - ballooning, steatosis, necrosis and the presence of neutrophils -when compared with mice under sham intermittent hypoxia (Figures 4 and 5).

Discussion

We report for the first time that 35 but not 21 days of exposure to IH, simulating an OSA of 60 events per hour, reducing for 6% the concentration of oxygen, causes hepatic damage. This is also the first report to combine the description of enzyme, lipid, DNA, oxidative, and nitrosative hepatic damage. We used an experimental model that produces levels of hypoxia comparable to those observed in patients with severe OSA [24,39]. Although our findings cannot be immediately translated to the clinical setting, they are in agreement with the literature indicating an OSA-NASH association [40,41].

Table 2 Activities of liver antioxidant enzymes

Enzymes	SIH	IH-35	p value
SOD (USOD/mg prot)	4.63 ± 0.26	3.16 ± 0.25	0.0005
GPx (mmol/min/mg prot)	1.00 ± 0.11	0.52 ± 0.06	0.0028
CAT (pmol/mg prot)	1.06 ± 0.04	0.79 ± 0.03	0.0003

Data are mean ± standard error (n = 12 animals/group). SIH: sham intermittent hypoxia group; IH-35: intermittent hypoxia for 35 days. SOD: superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase.

Two mechanisms are proposed for the morbidity caused by OSA: the activation of inflammatory factors and oxidative stress [42,43], which also can be modulated by genetic, lifestyle and environmental factors [43,44]. Oxidative stress plays an important role in various diseases as well as in OSA, which causes an effect similar to ischemia-reperfusion [18] in which there is activation of xanthine oxidase, leading to the formation free radicals and further imbalance between oxidants and antioxidants [4-6].

The analysis of liver integrity showed that the liver tissue of mice subjected to intermittent hypoxia was damaged, but only after 35 days, as demonstrated by the significant increase in circulating AST, ALT and alkaline phosphatase. The present results demonstrate damage both at cytoplasmic and mitochondrial level, confirmed by the presence in the histological examination of ballooning, steatosis, necrosis and the presence of neutrophils in the liver, similar to what is observed in NASH [45].

In the evaluation of hepatic lipid peroxidation, we observed a significant increase in lipid oxidative damage in animals that were subjected to hypoxia for 35 days, as indicated by the TBARS test, but not in group IH-21.

Table 3 Comet assay on peripheral blood and liver tissues from mice subjected to hypoxia

	SIH		IH-35	
Tissue	Damage index[a]	Damage frequency[b]	Damage index	Damage frequency
Blood	15.3 ± 4.4	7.6 ± 1.3	19.3 ± 4.1	8.0 ± 1.4
Liver	38.1 ± 5.1	14.8 ± 1.8	114.7 ± 32.3**	43.2 ± 11.3**

Data are presented as mean ± standard error (n = 6 animals/group). SIH: sham intermittent hypoxia group; IH-35: intermittent hypoxia for 35 days. [a], Damage index: can range from 0 (completely undamaged, 100 cells × 0) to 400 (with maximum damage, 100 × 4). [b], Damage frequency: calculated based on the number of cells with tails versus those with no tail. **, p < 0.01, statistically significant difference from sham intermittent hypoxia group (t-test).

Table 4 Quantification of nitric oxide metabolites in liver tissue

Metabolites	SIH	IH-35	p value
$NO_{2(\mu mol/L)}$	2.128 ± 0.202	3.405 ± 0.112	0.0001
$NO_{3(\mu mol/L)}$	0.018 ± 0.002	0.050 ± 0.003	0.0001

Data are mean ± standard error of the mean (n = 12 animals/group). SIH: sham intermittent hypoxia group; IH-35: intermittent hypoxia for 35 days; NO_2: total nitrate; NO_3: nitrites.

This damage can be caused by the increase of free radicals in the liver tissue. Similar data have been reported in other studies of intermittent hypoxia [46-48] and by our laboratory in other experimental models of hepatic oxidative damage [49-54].

As we did not observe liver damage in animals exposed to IH for 21 days, by the liver enzyme, histological, or lipid peroxidation assays, we concluded that this duration of IH causes no damage to the organ. Therefore, dosages of antioxidant enzymes, comet assay and nitrites metabolites were not conducted in the IH 21 group.

Comet assay in liver tissue revealed a significant increase in DNA damage in the IH-35 group in comparison to the SIH group. No evidence of damage was observed in blood tissue. The rate of DNA damage detected by the comet assay depends on the tissue or organ analyzed [55]. Here, the DNA damage was observed only in the tissue most susceptible to lesions produced by IH. In the alkaline version used, the comet assay detects a broad spectrum of DNA lesions, including single strand breaks [56,57].

Previous comet assay and TBARS data have demonstrated increased formation of free radicals in sleep apnoea patients [11]. Possibly, the formation of superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), which

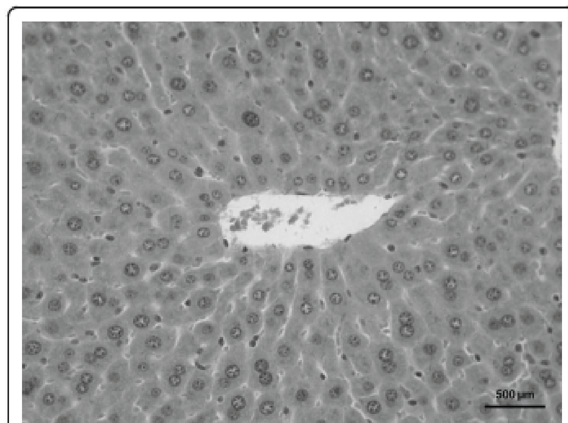

Figure 4 Photomicrograph of the mouse liver in sham intermittent hypoxia condition. A normal histological pattern was observed. Hematoxylin and eosin.

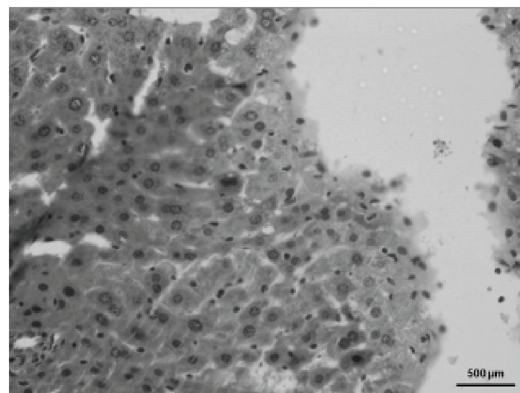

Figure 5 Photomicrograph of the mouse liver in intermittent hypoxia for 35 days. It was observed cellular ballooning, steatosis, necrosis and the presence of neutrophils. Hematoxylin and eosin.

appear to be increased in individuals with OSA, is due to the conversion of xanthine dehydrogenase (type D) into its oxidase (type O) form in hypoxia, followed by the activation of the oxidase form during reoxygenation (normoxia) by the hypoxanthine formed during hypoxia. This xanthine oxidase activity generates $O_2^{\cdot-}$, H_2O_2, and uric acid [4,11].

Our evaluation of the endogenous antioxidant liver enzymes SOD, GPx and CAT showed that their activities were significantly decreased in mice after 35 days under intermittent hypoxia. Quantification of total glutathione revealed significant decreases in the group exposed to intermittent hypoxia compared to SIH, demonstrating a reduced hepatic antioxidant defence in these animals.

The increase in TBARS and decrease in endogenous antioxidants observed in the present study further promotes oxidative stress, contributing to aggravation of the liver tissue injury. This kind of pathological synergy is evidenced in experimental models of liver damage induced by xenobiotic agents that cause oxidative stress such as carbon tetrachloride and toluene [49,50,52,54,58], by surgical procedures such as ligation of the common bile duct [51,53] or by thymoquinone [59].

The increased nitric oxide metabolites nitrite and nitrate in the livers of IH-35 mice confirms findings by other authors, who demonstrated a significant increase of nitric oxide in animals exposed to IH simulating OSA (6 min/6 min) during 120 days [48], and to hypobaric hypoxia during 32 days [60]. The increase of NO, along with increased free radicals, may generate nitrosative stress caused by the reaction products of these two substances, such as peroxide nitrite ($OONO^{\cdot}$) formed by the reaction between NO and $O_2^{\cdot-}$ [11]. Much evidence indicates that oxidative and nitrosative

stress have important roles in the complication of hypoxia [61].

OSA is usually accompanied by arterial hypertension, pulmonary hypertension, myocardial infarction and stroke, which may be due to changes in nitric oxide production [62]. Veasey et al. had demonstrated irreversible basal forebrain nitrosative damage as a possible cause for residual sleepiness in OSA [63].

It is increasingly clear that IH is capable of causing liver tissue damage. This was here demonstrated by several lines of evidence: elevated circulating levels of liver enzymes, NO increase, damage to lipids and DNA, and reduced endogenous antioxidant defences. Further translational research is necessary to completely correlate these findings with the NASH pathology.

Conclusions

The present results suggest that a model of intermittent hypoxia for 35 days, simulating sleep apnoea, is useful to investigate liver injury by oxidative and nitrosative stress. Exposure to intermittent hypoxia during 21 days may be insufficient to produce hepatic damage.

Acknowledgements

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Authors' contributions

DPR conducted the animal studies. DPR and JGS collected tissues and performed analyses. DPR and DM wrote the manuscript. JNP, NPM and DM reviewed the manuscript. DPR and DM designed the study and reviewed the manuscript. All the authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Simulating Sleep Apnea by Exposure to Intermittent Hypoxia Induces Inflammation in the Lung and Liver

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hypoxia (IH). IH can lead to the formation of free radicals that increase oxidative stress, and this mechanism may explain the association between central sleep apnea and nonalcoholic steatohepatitis. We assessed the level of inflammation in the lung and liver tissue from animals subjected to intermittent hypoxia and simulated sleep apnea. A total of 12 C57BL/6 mice were divided into two groups and then exposed to IH ($n = 6$) or a simulated IH (SIH) ($n = 6$) for 35 days. We observed an increase in oxidative damage and other changes to endogenous antioxidant enzymes in mice exposed to IH. Specifically, the expression of multiple transcription factors, including hypoxia inducible factor (HIF-1α), nuclear factor kappa B (NF-κB), and tumor necrosis factor (TNF-α), inducible NO synthase (iNOS), vascular endothelial growth factor (VEGF), and cleaved caspase 3 were shown to be increased in the IH group. Overall, we found that exposure to intermittent hypoxia for 35 days by simulating sleep apnea leads to oxidative stress, inflammation, and increased activity of caspase 3 in the liver and lung.

1. Introduction

Obstructive sleep apnea (OSA) consists of sleep-disordered breathing. Cyclic episodes result in the momentary closure, partial or complete, of the upper airway at the level of the pharynx. The repeated pauses in breathing can lead to intermittent hypoxia (IH) and increased reactive oxygen species (ROS) [1].

The increase of ROS in OSA is likely due to the repeated oxygen depletion followed by the hyperoxia that develops to restore oxygen pressure (PO_2). A similar phenomenon is observed in ischemia followed by reperfusion [2–5]. In ischemia/reperfusion, xanthine oxidase generates free radicals in the presence of oxygen, contributing to oxidative stress [6–8].

OSA is associated with chronic liver diseases, such as nonalcoholic steatohepatitis (NASH) [9–16]. Savransky and colleagues demonstrated that IH can act as a "second hit" to liver disease by amplifying the tissue damage induced by a high dose of paracetamol [17, 18]. The injury mechanism, triggered by OSA, appears to be related to the formation of peroxynitrite, depletion of glutathione, and apoptosis of hepatocytes [18].

In OSA, inflammatory factors, such as nuclear factor kappa B (NF-κB), are activated at a systemic level [17, 19]. NF-κB is a master regulator of the inflammatory process, by inhibiting its inhibitor IKK-b, and its activation leads to the increased expression of tumor necrosis factor (TNF-α), interleukins 1 and 6, and inducible nitric oxide synthase (iNOS). Alternatively, these factors can be activated by

hypoxia inducible factor (HIF-1α) [20–23], which results in apoptosis [24].

Several studies have shown that OSA is associated with inflammation, NASH, oxidative stress, and apoptosis. This is the first experimental study that evaluated the inflammatory process in the lung and liver with intermittent hypoxia, suggesting that there is a recruitment of inflammatory mediators recognized during ischemia and reperfusion. Here, we investigate the molecular mechanism involved in the lung and liver injury in an animal model of OSA.

2. Methods

The experiments were approved and completed according to the Research and Ethics Committee of the Research and Postgraduation at the Hospital de Clínicas de Porto Alegre, Brazil.

A total of 12 C57BL/6 mice (8–11 weeks old) were housed in plastic boxes (30 × 19 × 13 cm) at the Animal Experimentation Unit of the Hospital de Clínicas de Porto Alegre. The mice were kept on a 12-hour light/dark cycle (lights on from 7 AM to 7 PM) at 22 ± 4°C and given free access to food (Purina-Nutripal, Porto Alegre, RS, Brazil) and water.

The mice were randomly divided into two experimental groups ($n = 6$ per group). The groups consisted of mice exposed to intermittent hypoxia for 35 days (IH group) and mice that underwent a simulation of the IH procedure (SIH group).

The mice were placed in intermittent hypoxia chambers 8 hours a day (9 AM to 5 PM) for 5 weeks (Figure 1). The animals were exposed to a gas mixture consisting of 90% nitrogen and 10% carbon dioxide for 30 seconds. The gas mixture reduces the oxygen fraction in the chambers by 6 ± 1%. In sequence, the gas release is then blocked and fans are triggered to restore ambient air for the remaining 30 seconds. The SIH group was housed in a cage and subjected to the same adjacent fan activity as the IH group but no gas was introduced into the cage [25].

After 35 days, the animals were deeply anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (50 mg/kg) and the liver and lungs were removed. The organs were immediately frozen in liquid nitrogen and kept at −80°C for subsequent analysis. The animals were euthanized by exsanguination under deep anesthesia [26, 27].

The organs were cut and divided for biochemical and protein analyses. For analysis of oxidative stress, 100 mg of tissue was added to 0.9 mL of buffer (140 mM KCl, 20 mM phosphate, pH 7.4) and homogenized with a micropestle in microtubes. After centrifugation at 2150.4 g for 10 minutes in a refrigerated centrifuge (4°C), the supernatant was discarded and the pellet was stored at −80°C for further analysis. For western blotting, a nuclear extraction protocol was used. Briefly, 100 mg of tissue was added to 0.6 mL of lysis buffer (25 mM HEPES, 1% Triton X-100, 2 mM EDTA, 0.1 mL NaCl, 25 mM NaF, 1 mM sodium orthovanadate, and a protease inhibitor cocktail) and homogenized with a micropestle in microtubes. After centrifugation at 15,000 g for 10 minutes at 4°C, the supernatant was discarded and the pellet was stored at −80°C for further analysis.

2.1. Oxidative Stress

2.1.1. Proteins. The protein concentration in the homogenate was measured spectrophotometrically at 595 nm using the Bradford method. The values are expressed in mg/mL [28] and were used in the calculations for the TBARS and antioxidant enzymes.

2.1.2. Assessment of Lipid Peroxidation. The TBARS technique consists of heating the homogenate with thiobarbituric acid to produce a colored product that is subsequently measured at 535 nm using a spectrophotometer. The change in color is due to the presence of malondialdehyde and other substances produced from lipid peroxidation in the biological material.

Briefly, 0.25 mL of 10% trichloroacetic acid (TCA), 0.10 mL of homogenate, 0.067 mL of 0.67% thiobarbituric acid (TBA), and 0.033 mL of distilled water were added to a tube, stirred, and then heated at 100°C. After the tubes cooled, 0.20 mL of n-butyl alcohol was added to extract the pigment. The tubes were then stirred and centrifuged for 10 minutes at 1110 g. A 0.20 mL aliquot of the supernatant was added to a 96-well plate. The absorbance of the samples was quantified on a spectrophotometer at 535 nm. The TBARS concentration was expressed in nmol per mg protein [29].

2.1.3. Determination of Superoxide Dismutase (SOD). The technique used to measure SOD was based on the level of inhibition caused by the reaction of the enzyme with O^{-2}. We used adrenaline in an alkaline medium to produce adrenochrome and O^{-2} [30].

In a 96-well plate, we measured SOD activity in the reaction medium (50 mM glycine-NaOH, pH 10) and three samples containing different concentrations of homogenate. After addition of 10.5 μL epinephrine (60 mM, pH 2.0), the reaction was monitored for 2 min at 480 nm. The enzymatic activity was expressed in units SOD/mg protein.

2.1.4. Determination of Catalase (CAT). Catalase enhances the decomposition of hydrogen peroxide into water and oxygen. The rate of decomposition of hydrogen peroxide is directly proportional to enzyme activity and follows pseudo-first-order kinetics with respect to hydrogen peroxide.

The decrease in absorption at 240 nm was determined after adding 7 μL of 300 mM H_2O_2 to the reaction medium (50 mM phosphate regulator). The catalase concentration was expressed as pmol/mg protein [31].

2.2. Western Blots. A total of 50 mg of protein was added to a buffer (60% glycerol, 2 M Tris, SDS, and 10% Pyrroline 0.5%) and incubated for four minutes at 100°C. After electrophoresis was performed [32] on a 9–12% polyacrylamide gel, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane [33]. The membrane was washed with

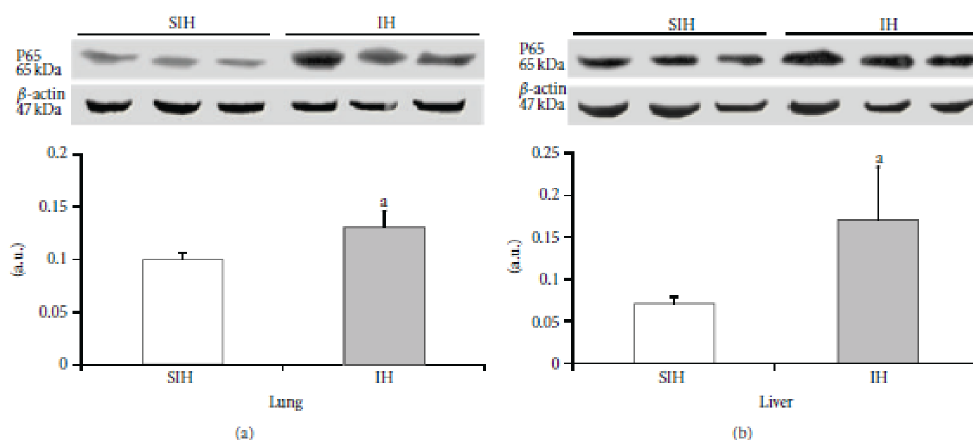

FIGURE 1: The effect of intermittent hypoxia on the expression of phosphorylated NF-κB in the liver ((a), $P = 0.0247$) and lung ((b), $P = 0.0033$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

PBS contained 0.5% Tween 20 and then incubated in a blocking solution (5% skim milk powder and 0.5% Tween 20 in cold PBS) for 30 minutes. After washing, the membrane was incubated overnight at 4°C with the primary antibody. Next, the membrane was washed and incubated in the secondary antibody (HRP) for two hours at room temperature. After another wash, the protein was visualized using chemiluminescent detection (Chemiluminescent HRP Substrate), film, and a transiluminator (L-Pix Chemi molecular imaging—Loccus Biotechnology). β-actin was used as a loading control. The results were quantified using LabImage 1D (Loccus Biotechnology) and are expressed as arbitrary units.

2.3. Statistical Analysis. For analyzing the result, the Student's t-test was performed using SPSS version 18.0 (Statistical Package for Social Science). The results are represented as the mean ± standard error of the mean. The statistical significance level was set as $P < 0.05$.

3. Results

Lipid peroxidation, a marker of oxidative damage, was significantly increased in the lung (14%) and liver (29%) of the IH group when compared with the SIH group (Table 1).

The activity of endogenous SOD was significantly lower in lung tissue (56%) and higher in liver tissue (87%) from IH animals when compared with the control group (Table 1). The activity of CAT was significantly higher in both organs (32% in the lung and 184% in the liver) from the IH group when compared with the SIH group (Table 1).

The activated (phosphorylated) p65 subunit of NF-κB was increased by 30% in the lung and 39% in the liver of IH mice when compared with SIH mice.

The expression of HIF-1α and TNF-α was significantly increased in the IH group when compared with the SIH group (Figures 2 and 3). In the lung tissue, HIF-1α increased by 96% and TNF-α increased by 38%. In the hepatic tissue, HIF-1α was increased by 19% and TNF-α was increased by 48%.

The expression of iNOS and VEGF was significantly higher in the IH group when compared with controls (Figures 4 and 5). There was a 35% increase in iNOS and a 22% increase in VEGF in the lung tissue. The liver showed a 79% increase in iNOS levels and a 71% increase in VEGF. Cleaved caspase 3 was increased by 237% in the lung and 182% in the liver of IH animals when compared to the SIH group (Figure 6).

4. Discussion

Animal models that use intermittent hypoxia can help elucidate the mechanism of damage to various systems caused by sleep apnea. Independent of body mass index, the respiratory disturbance index is directly related to the degree of liver damage and is recognized as a risk factor for nonalcoholic fatty liver disease (NAFLD) [10, 34]. It has been proposed that the development of NASH is produced in two phases consisting first of the accumulation of triglyceride, which is attributed to insulin resistance and obesity, and then the presence of inflammation and fibrosis [35], which is correlated with oxidative stress and hepatic lipid peroxidation [36, 37].

Our research group has described [38] oxidative damage to membrane lipids measured by TBARS and changes in endogenous antioxidant enzymes in the liver tissue that indicate the role of oxidative stress in our model system. These data are in agreement with the results described in other model systems [38–41]. Oxidative stress occurs through xanthine oxidase by producing the superoxide anion radical ($O_2^{-\bullet}$) and hydrogen peroxide [42, 43]; it is suggested

Table 1: The effect of intermittent hypoxia on hepatic lipid peroxidation as shown by the TBARS assay and liver antioxidant enzyme activity.

		SIH	IH	P value
Liver	$TBARS_{(nmol/mg\ prot)}$	2.90 ± 0.23	3.76 ± 0.15	0.0389
	$SOD_{(USOD/mg\ prot)}$	3.13 ± 0.53	5.86 ± 0.70	0.0118
	$CAT_{(nmol/mg\ prot)}$	0.82 ± 0.17	2.33 ± 0.09	0.0015
Lung	$TBARS_{(nmol/mg\ prot)}$	4.57 ± 0.10	5.22 ± 0.10	0.0116
	$SOD_{(USOD/mg\ prot)}$	7.27 ± 0.99	4.64 ± 0.22	0.0272
	$CAT_{(nmol/mg\ prot)}$	2.62 ± 0.18	3.48 ± 0.13	0.0042

Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.
SIH: sham intermittent hypoxia group; IH: intermittent hypoxia.
SOD: superoxide dismutase; CAT: catalase.

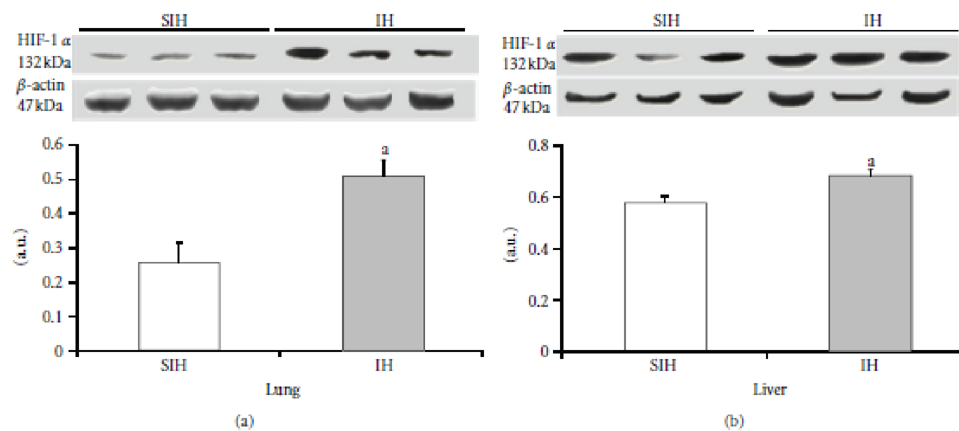

Figure 2: The effect of intermittent hypoxia on the expression of HIF-1α in the liver ((a), $P = 0.0227$) and lung ((b), $P = 0.0086$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

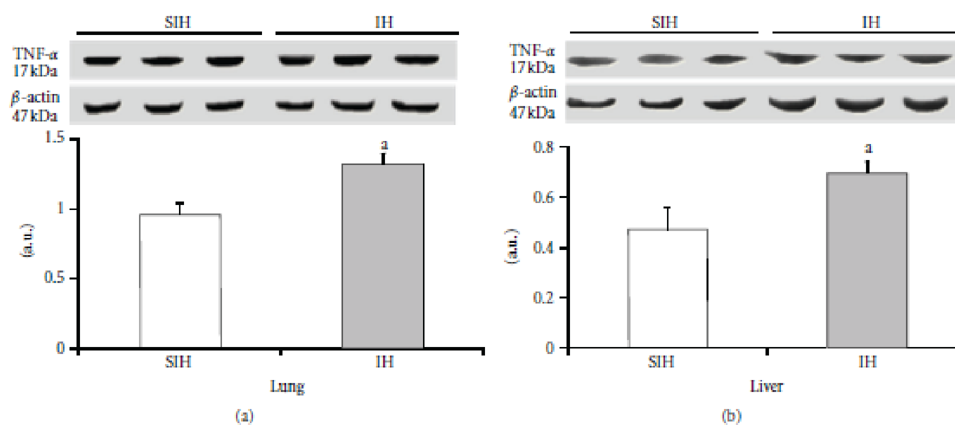

Figure 3: The effect of intermittent hypoxia on the expression of TNF-α in the liver ((a), $P = 0.0382$) and lung ((b), $P = 0.0171$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

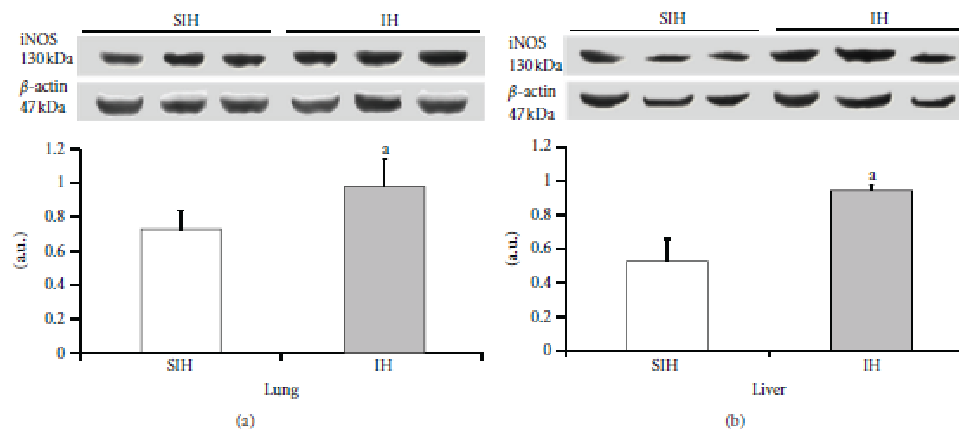

Figure 4: The effect of intermittent hypoxia on the expression of iNOS in the liver ((a), $P = 0.0091$) and lung ((b), $P = 0.0107$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

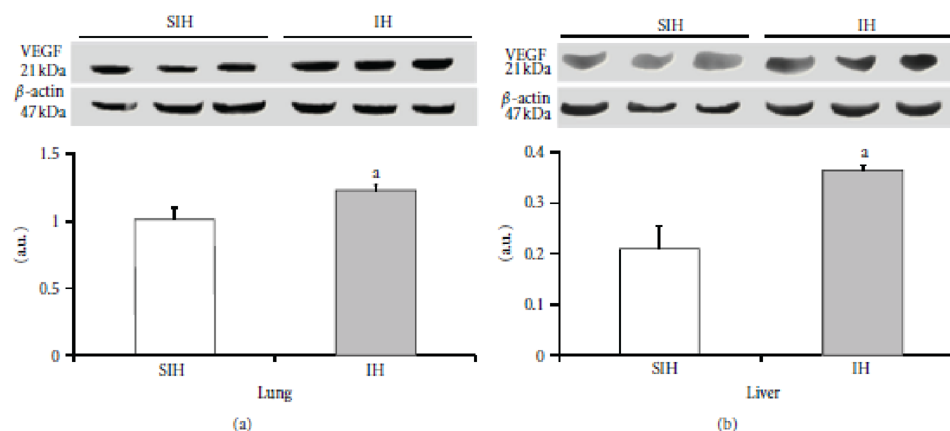

Figure 5: The effect of intermittent hypoxia on the expression of VEGF in the liver ((a), $P = 0.0062$) and lung ((b), $P = 0.0184$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

that the $O_2^{-\bullet}$ and H_2O_2, formed by the activity of xanthine oxidase, act independently on the activity of SOD and CAT [44]. Nitrosative stress includes the formation of nitric oxide (NO) that binds $O_2^{-\bullet}$ to form the radical peroxynitrite [38, 42, 45, 46].

HIF-1α regulates the concentration of oxygen, and it can be the initiator of inflammation in intermittent hypoxia [47, 48] or stimulated by oxidative stress [49]. This protein is correlated with chronic alcohol use and the presence of NAFLD [50]. HIF-1α also stimulates macrophages, increases the production of VEGF and iNOS [51, 52], reduces apoptosis [24, 53], and stimulates cell proliferation [54].

It is suggested that inflammatory activity is dependent on NF-κB [55], indicating that NF-κB can regulate HIF-1α transcription [56]. Although indirect, inhibition of IKK experimentally prevents the activation of NF-κB and was found to prevent the development of steatosis and NASH [57]. In the present study, we observed an increase in the expression of HIF-1α in the liver and the lung of mice exposed to hypoxia.

The stimulation of TNF-α leads to phosphorylation of IκB, which results in activation of NF-κB. Activation of NF-κB causes it to translocate to the nucleus and promote the transcription of numerous proinflammatory genes [58].

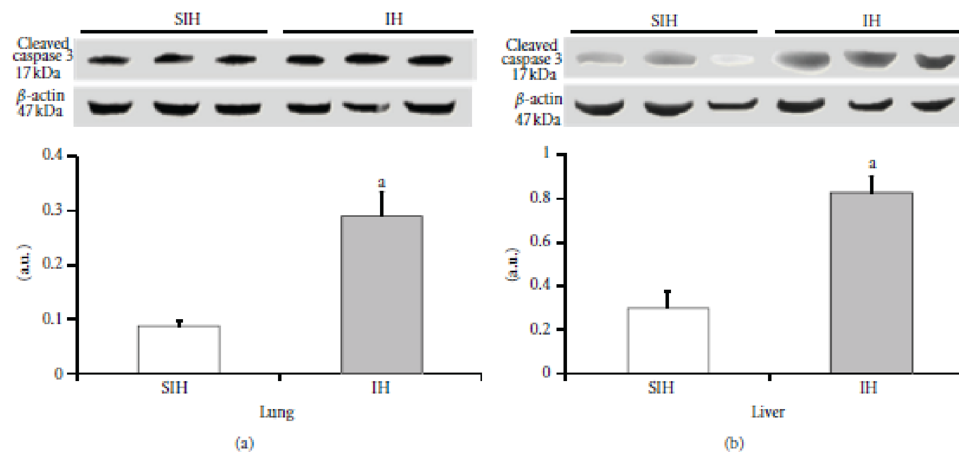

FIGURE 6: The effect of intermittent hypoxia on the expression of cleaved caspase 3 in the liver ((a), $P = 0.0022$) and lung ((b), $P = 0.0003$). Results are reported as mean ± standard error, $n = 6$ per group. P value according to Student's t-test.

Here, we showed that TNF-α and NF-κB were increased in animals exposed to intermittent hypoxia.

VEGF is essential for the initiation of angiogenesis, and it has a strong effect on vascular elements in response to hypoxia [59, 60]. In this study, we found increased expression of VEGF in both organs when mice were subjected to intermittent hypoxia.

In our previous work, we found that there is an increase in nitric oxide metabolites (NO) after exposure to intermittent hypoxia [38]. In the present study, we evaluated an enzyme responsible for NO production, iNOS, and found that the levels of this enzyme were increased in the lung and liver of animals exposed to hypoxia.

Apoptosis in all cells is regulated by caspases. After cleavage, caspases become active and initiate pathways that lead to apoptosis [61]. We found that cleaved caspase 3 expression is increased in the liver and lung of the IH group, demonstrating that there was activation of this apoptotic cascade.

Thus, the data suggest that intermittent hypoxia leads to liver and lung damage that can result from a cascade of signals initiated by oxidative stress, inflammation, and apoptosis.

5. Conclusion

In mice, the cyclic oxygen deprivation observed in sleep apnea induces oxidative stress and activation of HIF-1α, which stimulates a cascade of inflammatory signaling, nitric oxide generation, angiogenesis, and apoptosis in the lung and liver.

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3° ARTIGO – submetido à Liver International (11/12/2012)

ANTIOXIDANTS INHIBIT THE INFLAMMATORY AND APOPTOTIC PROCESSES IN AN INTERMITTENT HYPOXIA MODEL OF SLEEP APNEA

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LIST OF ABBREVIATIONS

ALP - alkaline phosphatase

ALT - alanine aminotransferase

AST - aspartate aminotransferase

CAT – Catalase

EDTA - Ethylenediaminetetraacetic acid

H_2O_2 - hydrogen peroxide

HE - Hematoxylin and eosin

HEPES - (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HIF-1α - Hypoxia-inducible factor 1 alpha

IH – intermittent hypoxia

IH+MEL – intermittent hypoxia and treatment with melatonin

IH+NAC - intermittent hypoxia and treatment with N-acetylcysteine

iNOS - inducible NO synthase

KCl – potassium chloride

MEL – Melatonin

NAC - N-acetylcysteine

NaCl – sodium chloride

NASH - nonalcoholic steatohepatitis

NF-kB - nuclear factor kappa B

NO - nitric oxide

$O_2^{\bullet-}$ - superoxide anion radical

OSA - obstructive sleep apnea syndrome

PVDF - polyvinylidene difluoride

SIH – simulation of intermittent hypoxia

SIH+MEL – simulation of intermittent hypoxia and treatment with melatonin

SIH+NAC – simulation of intermittent hypoxia and treatment with N-acetylcysteine

SOD – superoxide dismutase

TGF-β - Transforming growth factor beta

TNF-alpha - tumor necrosis factor

VEGF - vascular endothelial growth factor

XO - xanthine oxidase

ABSTRACT

Background: Sleep apnea causes intermittent hypoxia (IH). We aimed to investigate the proteins related to oxidative stress, inflammation and apoptosis in liver tissue subjected to IH as a simulation of sleep apnea in conjunction with the administration of either melatonin (MEL, 200 µL/kg) or N-acetylcysteine (NAC, 10 mg/kg).

Methods: Seventy-two adult male Balb-C mice were divided: simulation of IH (SIH), SIH+MEL, SIH+NAC, IH, IH+MEL and IH+NAC. The animals were subjected to simulations of sleep apnea for eight hours a day for 35 days. The data were analyzed with ANOVA and Tukey tests with the significance set at $p<0.05$.

Results: IH there was a significant increase in oxidative stress and expression of HIF-1a. Additionally, we observed increase in the activation levels of NF-kB. This increase may be responsible for the increased expression of TNF-alpha and iNOS as well as the significant increase of VEGF signaling and expression of Caspase-3 and Caspase-6, which suggests an increase in apoptosis. In the groups treated with antioxidants, the analysis showed that the enzyme activity and protein levels were similar to those of the non-simulated group.

Conclusions: Thus, we show that IH causes liver inflammation and apoptosis, which may be protected with either MEL or NAC.

Keynote: sleep apnea, intermittent hypoxia, liver

INTRODUCTION

Hypopnea and sleep apnea are momentary and cyclic sleep-disordered breathing that can lead to either partial or complete upper airway closure referred to as obstructive sleep apnea syndrome (OSA). Such changes can result in intermittent hypoxia (IH), leading to transient arousals from sleep and eventual sleep fragmentation [1].

It has been suggested that oxidative stress induced by IH is the primary cause of damage in multiple organisms upon undergoing OSA [2]. The generation of reactive oxygen species (ROS) in OSA may be initiated by the change in oxygen pressure, which can cause instances of ischemia and reperfusion [3-6]. ROS formation occurs upon activation of xanthine oxidase, which is activated by the lack of oxygen and when in the presence of oxygen in reperfusion, and generates superoxide anions and hydrogen peroxide, both of which contribute to oxidative stress [7-9].

OSA is associated with chronic liver disease in nonalcoholic steatohepatitis (NASH) [10-17], which is linked to the presence of insulin resistance and hepatic steatosis without alcohol consumption [18]. Savransky and colleagues demonstrated that IH could act as a "second hit" of liver damage by showing amplified tissue damage after treatment with a high dose of paracetamol [19-20]. The injury mechanism triggered by OSA appears to be related to the formation of peroxynitrite, depletion of glutathione and apoptosis of hepatocytes [20].

IH leads to the activation of inflammatory factors such as nuclear factor kappa B (NF-kB) [19, 21], which is the master regulator of the inflammatory process. NF-kB activation leads to the activation of tumor necrosis factor (TNF-a) and interleukins 1 and 6 as well as the induction of inducible nitric oxide synthase (iNOS) and other enzymes. These proteins can also be activated by hypoxia-inducible factor (HIF-1α) [22-25], which results in apoptosis [26].

The use of antioxidants can either slow or prevent the damage caused by ROS. Melatonin (MEL) is produced in the pineal gland and is known as the

"hormone of darkness" because a majority of its production occurs in the dark. This hormone exhibits antioxidant, neuroprotective, anticarcinogenic and cardioprotective roles in the body. MEL has the ability to pass biological membranes and reach all subcellular compartments, protecting them from free radicals. N-acetylcysteine (NAC) is a compound that contains a sulfhydryl group, which confers antioxidant properties and is widely used in the clinic [27].

It was demonstrated that in the animal model used in this study, oxidative and nitrosative stress occur in the liver [28], which leads to the formation of lesions similar to those observed in induced by NASH [29]. It is known that antioxidants have the ability to either stop or reduce the damage caused by oxidative stress [30]. Based on this knowledge, we aim to investigate the oxidative and inflammatory changes in the liver in mice subjected to intermittent hypoxia as a simulation of sleep apnea and treated with antioxidants.

METHODS

The experimental procedures described are in accordance with the established "Research in Health and Animal Rights" guide from the Research and Ethics Committee of the Research and Graduate Hospital de Clínicas de Porto Alegre, Brazil.

We used 72 adult Balb-C mice (8-11 weeks old) and housed them in plastic cages (30x19x13 cm) in the Animal Experimentation Unit at the Hospital de Clínicas de Porto Alegre with a 12 hour light/dark cycle (light from 7 am to 7 pm) at 22 ± 4 °C. Food (Purina-Nutripal, Porto Alegre, Brazil) and water were freely available to the mice.

The mice were randomly divided into the following six experimental groups (n = 12 for each group): simulation of intermittent hypoxia (SIH), which underwent a simulation procedure; simulation of intermittent hypoxia followed by treatment with melatonin (SIH+MEL); simulation of intermittent hypoxia followed by treatment with N-acetylcysteine (SIH+NAC); intermittent hypoxia (IH), which were exposed to intermittent hypoxia for 35 days; intermittent hypoxia followed by treatment with melatonin (IH+MEL); and intermittent hypoxia and treatment with N-acetylcysteine (IH+NAC).

Sleep apnea was induced with the use of intermittent hypoxia chambers for 8 hours per day (9 am to 5 pm) during the rodents' sleep period for 5 weeks. In the chamber, the animals were exposed to a gas mixture of 90% nitrogen and 10% carbon dioxide for 30 seconds, which reduced the oxygen fraction from 21% (normal environment) to approximately 6%. Afterwards, the gas was blocked, and fans were used to replace the mixture with ambient air for the remaining 30 seconds. Each cycle lasted 60 seconds and was repeated for eight hours a day, totaling 480 episodes (equivalent to 60 apnea episodes per hour) [31].

The groups that were simulated (SIH, SIH+MEL and SIH+NAC) were housed in cages where they were subjected to the same fan cycles as the IH group, except that no gas was introduced into the cage [28].

Melatonin was administered intraperitoneally at a dose of 200 µg/kg diluted in 0.4 mL saline solution (0.9% NaCl) and 0.1 mL ethanol 1% [32] starting at day 21 of the gas treatment. Mice administered melatonin received a dose daily until they were euthanized for tissue analysis.

NAC was administered intraperitoneally at a dose of 10 mg/kg diluted in 0.5 ml of saline solution (0.9% NaCl) [33] starting at day 21 of the gas treatment. Mice administered NAC received a dose daily until they were euthanized for tissue analysis.

On day 35, the animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (50 mg/kg). The abdomen was shaved, followed by a laparotomy in which the liver was removed. Part of the left lobe was stored in formalin for histological analysis, and the remainder of the organ was frozen immediately in liquid nitrogen and kept at -80 °C for further analysis. The animals were euthanized by exsanguination under deep anesthesia at the end of the surgery [34-35].

To measure changes in oxidative stress, liver tissue was homogenized in phosphate buffer (140 mM KCl, 20 mM phosphate, pH 7.4) and centrifuged at 4 °C for 10 minutes at 2150.4 g. The supernatant was frozen and kept at -80 °C for further analysis.

For the western blots, cytosolic and nuclear extracts were prepared with lysis buffer (25 mM HEPES, 1% Triton X-100, 2 mM EDTA, 0.1 mL NaCl, 25 mM NaF, 1 mM sodium orthovanadate and protease inhibitor (Roche, Basel, Switzerland)) and centrifuged at 4 °C for 10 minutes at 15,000 g. The supernatants were frozen and kept at -80 °C for further analysis.

DETERMINATION OF OXIDATIVE STRESS

PROTEIN QUANTIFICATION

The protein concentrations in the homogenates were determined using the Bradford method with standard bovine albumin. The samples were measured spectrophotometrically at 595 nm, and the values were expressed as mg/mL [36). The calculated albumin curve was used to calculate the TBARS and antioxidant enzyme levels.

ASSESSMENT OF LIPOPEROXIDATION

The TBARS technique is performed by heating the homogenate with thiobarbituric acid, which results in the formation of a colored product (due to the presence of malondialdehyde and other substances from lipid peroxidation) that is measured in a spectrophotometer at 535 nm. The concentration of the TBARS obtained was expressed as nmol per mg protein [37].

DETERMINATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY

The measurement of SOD activity is based on the capacity of the sample to inhibit a reaction with O^{-2}. For this, we used adrenaline (60 mM, pH 2.0), which becomes adrenochrome in an alkaline medium (50 mM glycine-NaOH, pH 10) and produces the O^{-2} that the enzyme reduces. The enzymatic activity was expressed in units SOD/mg protein (amount of SOD required to inhibit the rate of reduction of adrenaline by 50%) [38].

DETERMINATION OF CATALASE (CAT) ACTIVITY

The enzyme catalase catalyzes the decomposition of hydrogen peroxide into water and oxygen; the rate of decomposition of hydrogen peroxide is

directly proportional to enzyme activity. The spectrophotometric measurement consisted of pipetting hydrogen peroxide into the reaction medium (regulatory phosphate solution), where it decomposed into oxygen. The test consists of measuring the decrease in absorption at 240 nm. The concentration was expressed as pmole / mg protein [39].

WESTERN BLOT

The nuclear and cytosolic extracts were separated by SDS-PAGE in 9 and 12% polyacrylamide gels, respectively [40]. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which was incubated with antibodies [41]. The detection of the chemiluminescent antibodies was performed with a commercially available kit (HRP Chemiluminescent Substrate) and exposed to film on an L Chemi-Pix molecular imaging (Loccus Biotechnology). To compare the protein levels, each gel was blotted for beta-actin. The results were quantified in LabImage 1D (Loccus Biotechnology) and expressed in arbitrary units.

HISTOLOGICAL ASSAY

For histological evaluation, a portion of the liver was preserved in 10% formalin for 24 hours, embedded in paraffin, and cut into 6 µm sections with a microtome. Hematoxylin and eosin (HE) staining was performed for histological visualization.

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS (Statistical Package for Social Science) software version 18.0 and analysis of variance (ANOVA) followed by post hoc Tukey tests. The results are represented as the means ± standard error of the mean, with the statistical significance level set at $p < 0.05$.

RESULTS

The plasma levels of the liver enzymes aspartate (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were significantly higher in the animals exposed to intermittent hypoxia compared to the controls.

In the IH group, the ALT levels were increased by 95%, the AST levels by 49.1% and the ALP levels by 22% compared to the SIH group. In the SIH+MEL group, there was a significant increase (85% in the ALT levels, 90% in the AST levels, and 18% in the ALP levels) compared to the simulated group; however, in mice exposed to IH and treated with MEL, there was a significant reduction in the levels of AST (30%) and ALT (44%), whereas treatment with NAC significantly reduced the AST levels (21%) compared to the animals in Group IH (TABLE 1).

In evaluating the amount of lipoperoxidation via the TBARS technique to determine the amount of oxidative damage to the membrane lipids, there was a significant increase in the SIH+MEL (170%), SIH+NAC (80%) and IH (133%) groups compared to the SIH group. When exposed to intermittent hypoxia and treated with either antioxidant, there was a significant reduction in the amount of oxidative damage in the IH+MEL (208%) and IH+NAC (136%) groups compared to the IH group (TABLE 2).

The amount of SOD activity was significantly reduced in the SIH+MEL (63%) and IH (133%) groups compared to the SIH group. In the mice exposed to intermittent hypoxia and treated with either NAC or MEL, the SOD activity levels were restored to the level of the control group (IH+MEL, 118%, and IH+NAC, 99%) (TABLE 2).

The liver histology showed the presence of steatosis, ballooning necrosis and the presence of neutrophils in the animals exposed to intermittent hypoxia. However, in the animals that were both exposed to IH and treated with antioxidants, we did not observe the same findings; instead, the parenchyma was returned to a state similar to that of the control (FIGURE 1).

The protein expression level of hypoxia-inducible factor 1 alpha (HIF-1α) was significantly higher (182%) in the intermittent hypoxia group compared to the other groups (FIGURE 2). To verify the activation of nuclear transcription factor kappa B (NF-κB), we analyzed the phosphorylation of the p65 subunit as well as the phosphorylation of the free form of its inhibitor IκB-α. Both proteins showed significantly increased expression in mice in the IH group (374% of

phosphorylated p65 and 135% of IκB-α) compared to the other groups (FIGURE 3).

The protein expression levels of tumor necrosis factor alpha (TNF-α), nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) were significantly higher in the IH group (206% for TNF-α, 251% for iNOS, 90% for VEGF and 193% for TGF-β) compared to all of the other experimental groups (FIGURE 4).

To assess the amount of apoptosis, we measured Caspases 3 and 6, which were significantly elevated in the IH group (455% for Caspase 3 and 151% for Caspase 6) compared to the other groups (FIGURE 3).

DISCUSSION

The implementation of isocapnic intermittent hypoxia as a simulation of sleep apnea for 35 days resulted in hepatocyte damage. One of the mediators of this type of injury is oxidative stress [28, 42-44], which acts through the generation of reactive oxygen species and decreased antioxidant defense. Another mechanism that causes hepatocyte damage is the activation of inflammatory and apoptotic factors.

The generation of oxidative stress can be initiated by several mechanisms, one of which is xanthine oxidase (XO). During hypoxia, this enzyme is activated to form hypoxanthine, which forms the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and uric acid in the presence of oxygen [45-46]. Nitrosative stress is another type of hypoxia-induced damage, which is caused by the formation of peroxynitrite (binding of $O_2^{-\bullet}$ with nitric oxide (NO)) and occurs primarily during sleep apnea [28, 45, 47-48].

Clinical studies have shown that the rate of sleep-disordered breathing is directly related to the degree of hepatic steatosis and is recognized as a risk factor for nonalcoholic steatohepatitis hepatitis (NASH) that is independent of the body mass index [11, 49]. This progression of steatosis to NASH is a result of inflammation accompanied by hepatocyte ballooning, necrosis, the appearance of Mallory bodies and perisinusoidal fibrosis or cirrhosis [50-51].

Oxidative stress is responsible for this progression, which has been referred to as the "second hit" of the disease by causing the development of inflammation and fibrosis [52]. IH alone may be a precursor of liver damage by initiating the development of steatosis, ballooning necrosis, apoptosis and oxidative stress, as observed in this study.

Hypoxia-inducible factor 1 alpha (HIF-1α) regulates the concentration of oxygen [53-54] and can initiate the development of inflammation in IH. It has been suggested that HIF-1α can also be activated by oxidative stress, which in turn can lead to increased oxidative stress [55]. This protein is correlated with the presence of hepatic steatosis [56] and stimulates macrophages as well as VEGF, iNOS and TGF-β [57-58].

It is suggested that inflammatory activity is dependent on the transcription factor NF-κB [59], indicating that NF-κB can regulate HIF-1α at the transcriptional level [60]. Though it still cannot be connected to the amount of steatosis, the inhibition of IKK was able to prevent the development of steatosis and NASH in an animal model [61].

Stimulation with TNF-α eventually leads to the phosphorylation of IκB, which results in the activation of NF-κB, which is free to translocate to the nucleus and orchestrate the transcription of numerous pro-inflammatory genes [62].

In response to the activation of HIF-1α, we stimulated angiogenesis [57-58]. Vascular endothelial growth factor (VEGF) is an essential protein for the initiation of angiogenesis and has a mitogenic effect on vascular elements in response to hypoxia [63-64]. Transforming growth factor beta (TGF-β) can be activated by stellate liver cells, contributing to the development of fibrosis. Several studies reported that the modulation of oxygen tension is an important regulator of the activation of TGF-β [65, 66].

Intermittent hypoxia can decrease intrapulmonary vasodilation, which reduces the lumen and increases the efficiency of gas exchange [67-68]. However, our group has shown that in other organs, there is an increase in vasodilators, especially nitric oxide (NO), and elevated levels of nitric oxide

metabolites (NO) in instances of intermittent hypoxia [28]. In the present study, we evaluated an enzyme responsible for NO production, inducible NO synthase, and observed high levels of enzyme expression in the liver in animals exposed to hypoxia. Previous studies suggest that this enzyme is activated by HIF-1α [57-58, 69].

HIF-1α can induce apoptosis, prevent cell death [26, 70] or even stimulate cell proliferation [71]. Apoptosis is regulated by a Caspase cascade that is present in all cells. After cleavage, Caspases become active and initiate pathways that result in apoptosis [72]. We observed increased expression levels of cleaved Caspases 3 and 6 in the IH group, demonstrating the activation of this apoptotic cascade.

We believe that injuries are initiated by histology-derived oxidative stress, with the initial inflammatory signaling cascade culminating in a positive feedback loop for oxidative stress and the activation of various inflammatory, apoptotic and angiogenic mediators.

We suggest that the use of antioxidants can reduce or even slow the progression of lesions in the liver tissue of mice subjected to isocapnic intermittent hypoxia by reducing both the histological findings, such as oxidative stress, and the inflammatory, apoptotic and angiogenic signaling cascades to the same levels as those of the controls.

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TABLE 1. The effects of intermittent hypoxia on hepatic integrity as determined by the levels of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase in the plasma.

	SIH	SIH+MEL	SIH+NAC	IH	IH+MEL	IH+NAC
AST (U/L)	83.9±5.6	159.6±10.8[a]	90.5±4.8[b]	125.1±12.5[c,d]	95.5±5.4[e,f]	103.2±3.8
ALT (U/L)	40.0±4.9	74.3±5.4[c]	49.9±4.1[g]	78.3±15.4[c]	54.5±4.8[f,g]	47.6±2.1[f]
ALP (U/L)	60.8±4.7	72.0±1.6[h]	78.6±2.7[c]	74.7±3.9[h]	85.6±1.5[a]	79.9±5.8[c]

The data are expressed as the means ± standard error of the mean (n = 12 animals / group).

a – vs SIH, $p<0.001$;

b – vs SIH+MEL, $p<0.001$;

c – vs SIH, $p<0.01$;

d – vs SIH+NAC, $p<0.05$;

e – vs SIH+MEL, $p<0.001$;

f – vs IH, $p<0.05$;

g – vs SIH+MEL, $p<0.05$;

h – vs SIH, $p<0.05$;

SIH: simulated intermittent hypoxia group; SIH+MEL: simulated intermittent hypoxia and melatonin; SIH+NAC: simulated intermittent hypoxia and N-acetylcysteine; IH: intermittent hypoxia; IH+MEL: intermittent hypoxia and melatonin; IH+NAC: intermittent hypoxia and N-acetylcysteine.

AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase.

TABLE 2. The effects of intermittent hypoxia on lipoperoxidation as measured by TBARS and endogenous superoxide dismutase (SOD) activity in liver tissue.

	SIH	SIH+MEL	SIH+NAC	IH	IH+MEL	IH+NAC
TBARS **(nmol/mg prot)**	0.36±0.05	1.08±0.10[a]	0.72±0.08[b, c]	0.71±0.20[b]	0.23±0.05[d]	0.30±0.04
SOD **(USOD/mg prot)**	12.43±1.55	7.90±0.89[b]	8.79±0.97	5.33±0.33[a]	12.16±0.91[e]	10.61±0.72[d]

The data are expressed as the means ± standard error of the mean (n = 12 animals / group).

a – vs SIH, p<0.001;

b – vs SIH, p<0.05;

c – vs SIH+MEL, p<0.01;

d– vs IH, p<0.01;

e – vs IH, p<0.001;

SIH: simulated intermittent hypoxia group; SIH+MEL: simulated intermittent hypoxia and melatonin; SIH+NAC: simulated intermittent hypoxia and N-acetylcysteine; IH: intermittent hypoxia; IH+MEL: intermittent hypoxia and melatonin; IH+NAC: intermittent hypoxia and N-acetylcysteine.

TBARS: substances reacting with thiobarbituric acid; SOD: superoxide dismutase.

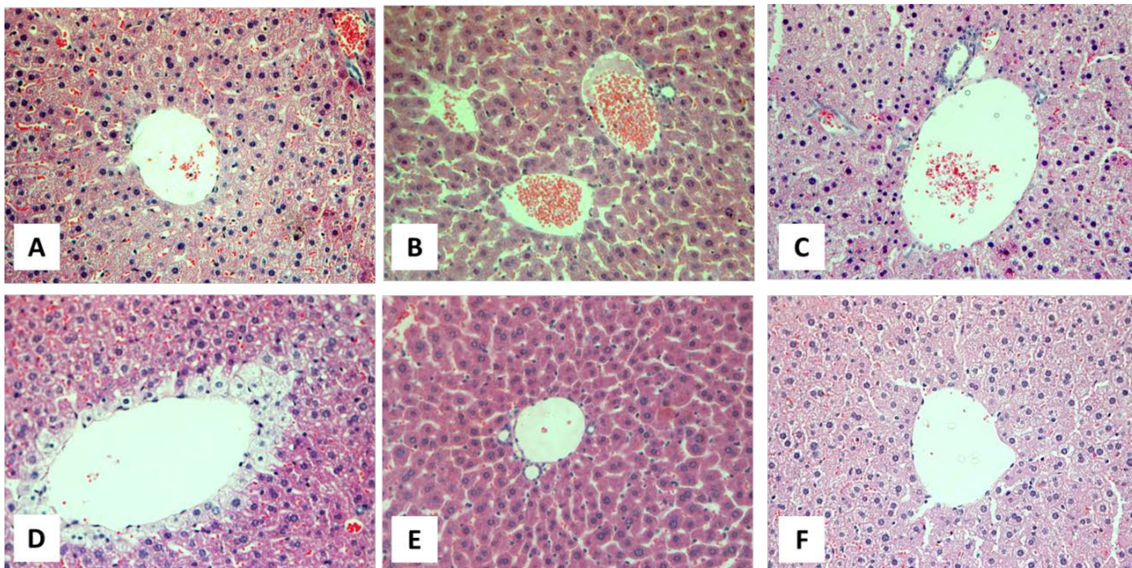

FIGURE 1. Photomicrograph of hematoxylin and eosin-stained mouse liver tissues. A. Simulation of intermittent hypoxia (SIH); B. Simulation of intermittent hypoxia followed by treatment with melatonin (SIH+MEL); C. Simulation of intermittent hypoxia followed by treatment with N-acetylcysteine (SIH+NAC); D. Intermittent hypoxia (IH); E. Intermittent hypoxia followed by treatment with melatonin (IH+MEL); F. Intermittent hypoxia followed by treatment with N-acetylcysteine (IH+NAC). The highlighted areas illustrate the presence of steatosis, ballooning necrosis and the presence of neutrophils. (400x).

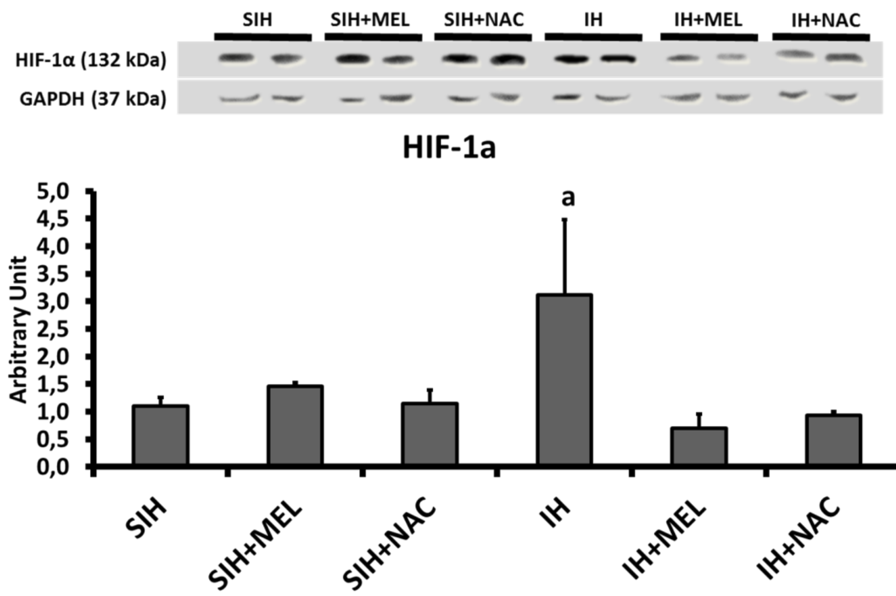

FIGURE 2. The expression of hypoxia-inducible factor 1 alpha (HIF-1 alpha).

a - $p < 0.006$

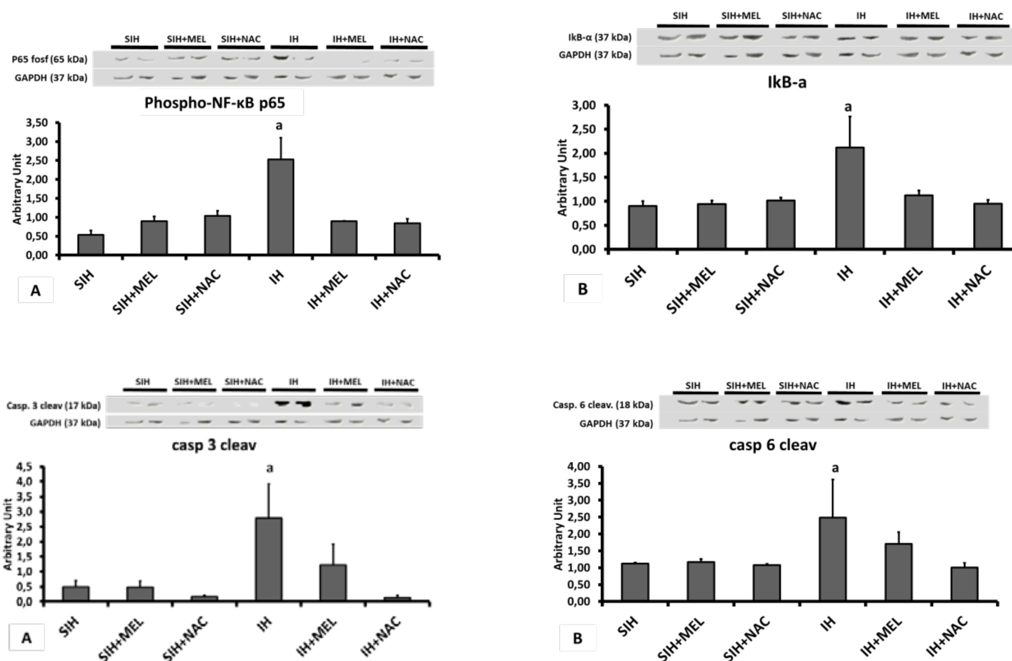

FIGURE 3. The expression levels of the phosphorylated p65 region of NF-ķB (A), phosphorylated IķB-alpha (B), cleaved Caspases 3 (C) and 6 (D).

P65 - a - $p < 0.011$

IKB - a - $p < 0.03$

Caspase 3 - $p < 0.05$

Caspase 6 - $p < 0.005$

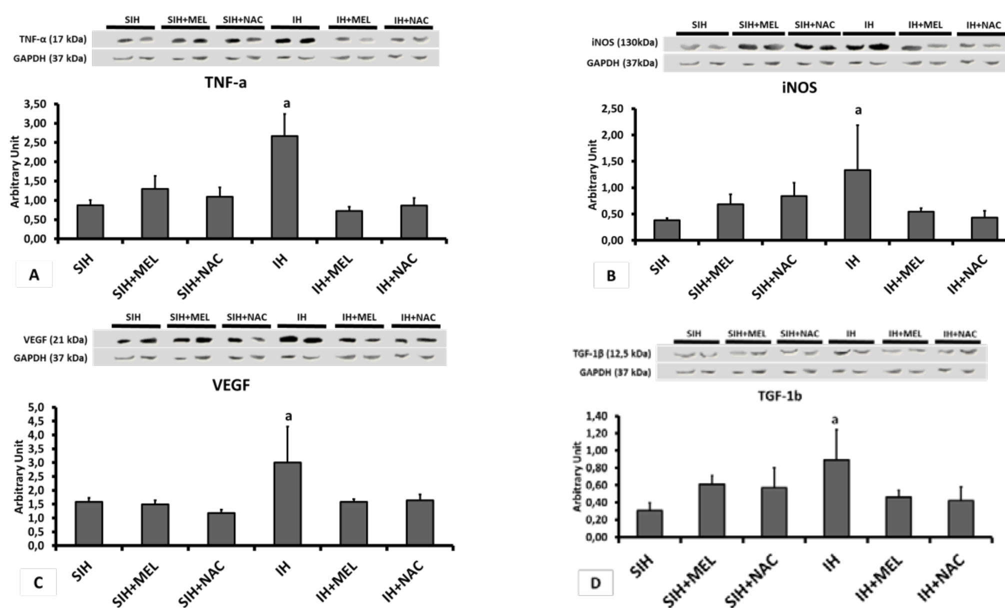

FIGURE 4. The expression levels of tumor necrosis factor TNF-alpha (A), inducible nitric oxide synthase - iNOS (B), vascular endothelial growth factor - VEGF (C) and transforming growth factor beta - TGF-β (D).

TNF - $p < 0.04$;

iNOS - $p < 0.05$;

VEGF - $p < 0.04$;

TGF - $p < 0.05$;

6 Considerações Finais

PRIMEIRO EXPERIMENTO - Publicado na Comparative Hepatology – BioMed Central (2011).

- ∞ Verificamos que em exposição durante 21 dias à hipóxia, não há alterações histológicas hepáticas;
- ∞ Após 35 dias de exposição à hipóxia intermitente, simulando a apneia do sono, há um aumento significativo das enzimas séricas hepáticas (AST, ALT e FA) e presença de necrose, esteatose, balonização e neutrófilos na histologia hepática, demonstrando dano ao tecido.
- ∞ Demonstramos que há dano oxidativo aos lipídios de membrana e ao DNA e redução significativa das enzimas antioxidantes nos camundongos expostos à HI por 35 dias.
- ∞ Houve aumento significativo dos metabólitos de óxido nítrico no grupo exposto por 35 dias à hipóxia.
- ∞ Consideramos que o modelo animal adequado para avaliação de dano hepático e estresse oxidativo com hipóxia intermitente em apnéia do sono é o de 35 dias de exposição.

SEGUNDO EXPERIMENTO - Publicado no Mediators of Inflammation - Hindawi (2012)

- ∞ Demonstramos que tanto o pulmão quanto o fígado de animais expostos à hipóxia intermitente apresentam o estresse oxidativo, a julgar pela lipoperoxidação e enzimas antioxidantes.
- ∞ Na avaliação do pulmão e do fígado de camundongos com apneia do sono houve aumento na expressão das proteínas HIF-1α, NF-κB, TNF-α, iNOS e VEGF e Caspase-3, que correspondem ao aumento da atividade inflamatória e apoptótica.

TERCEIRO EXPERIMENTO - submetido à Liver International (11/12/2012)

∞ A Melatonina reduziu as enzimas séricas hepáticas AST e ALT e reduziu os achados histológicos nos animais expostos à HI.

∞ A N-acetilcisteína reduziu a enzima sérica hepática AST e reduziu os achados histológicos nos animais expostos à HI.

Ambos os antioxidantes reduziram o estresse oxidativo nos camundongos do grupo da apneia no fígado.

∞ Melatonina e N-acetilcisteína reduziram as expressões das proteínas HIF-1α, NF-κB, TNF-α, VEGF, TGF-β, iNOS, Caspases 3 e 6.

∞ A Melatonina e a N-acetilcisteína parecem reduzir o estresse oxidativo, a esteatose, a inflamação e apoptose em fígado de roedores expostos à hipóxia intermitente

7 Apêndices

ARTIGO ENVIADO A PUBLICAÇÃO – com co-autoria

MELATONIN PREVENTS HYPERGLYCEMIA AFTER INTERMITTENT HYPOXIA

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Conflicts of interest disclosure

Renata S. Rivera-Kaminski: None

Denis Martinez: Owns a private sleep clinic; received research grants from government agencies; received six hundred dollars from Merck in a clinical research protocol.

Cintia Z. Fiori: None

Darlan P. Rosa: None

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ABSTRACT

To investigate whether the antioxidants melatonin and N-acetylcysteine are capable to prevent glycolipid metabolic changes caused by intermittent hypoxia in an animal model of sleep apnea in order to guide future investigations on the metabolic consequences of sleep apnea. Were realized comparisons of different interventions on six groups of Balb/c mice exposed during 35 days to isocapnic intermittent hypoxia (n=36) and to sham intermittent hypoxia (n=36). During 8 hours daily the intermittent hypoxia group underwent a total of 480 cycles of 30 seconds of progressive hypoxia to an inspired oxygen fraction nadir

of 7±1% followed by 30 seconds of normoxia. After day 21, mice were injected daily with vehicle (n=24), melatonin (n=24), or N-acetylcysteine (n=24). Glucose and lipids levels were measured. At day 35, glucose levels were significantly higher in the intermittent hypoxia group (141±38 mg/dL) than the sham group (75±17 mg/dL; $p<0.05$). The intermittent hypoxia groups receiving N-acetylcysteine and vehicle showed higher glucose levels than the group receiving melatonin (88±13 mg/dL). Lipid profile was unaffected by intermittent hypoxia and the antioxidants. The present results suggest that melatonin but not the antioxidant N-acetylcysteine prevents the already recognized increase in glucose levels after exposure to intermittent hypoxia. Although the mechanisms of the action of melatonin are still unclear, it is more likely a direct action of melatonin on the multifaceted glycemic control system than an antioxidant effect on the also complex oxidative stress reactions. Further exploration of the therapeutic role of melatonin in sleep apnea is warranted.

Keywords: Intermittent hypoxia, glucose, oxidative stress, melatonin, N-acetylcysteine, sleep apnea.

INTRODUCTION

Obstructive sleep apnea (OSA) affects up to one third of the adult population (Tufik *et al.*, 2010). It is characterized by intermittent asphyxia and recurrent arousals (Dempsey *et al.*, 2010). Exposure of healthy young men to intermittent hypoxia (IH) during four days promotes oxidative stress (Pialoux *et al.*, 2009).

OSA is a risk factor for cardiovascular disease (Somers *et al.,* 2008*)* and metabolic conditions such as insulin resistance, type-2 diabetes mellitus, and metabolic syndrome (Kono *et al.*, 2007). OSA is associated with poorer diabetes control, independent of confounding factors such as obesity (Aronsohn *et al.*, 2010). OSA is associated with fasting hyperglycemia. Diabetic subjects show an increase in reactive oxygen species generation and oxidative stress markers, simultaneously with decrease in antioxidant levels (Polotsky *et al.*, 2003).

Professional diabetes associations have recommended the investigation of OSA in the routine care of individuals with diabetes (Shaw *et al.*, 2008). Diabetes is a major risk factor for cardiovascular outcomes. If, however, individuals with diabetes are excluded, the apnea-hypopnea index represents the main risk factor for coronary artery disease (Martinez *et al.*, 2011) and protein damage (Klein *et al.*, 2010). OSA and diabetes are, therefore, complementary therapeutic targets.

Because the therapy of diabetes has limitations, investigating alternative methods to reduce glucose levels is of medical interest. OSA can be controlled by the use of continuous positive airway pressure. Treating OSA reverses metabolic disturbances, supporting a cause-effect relationship (Punjabi *et al.*, 2009). Treating OSA and the consequences of OSA may be an alternative preventive method and a novel therapeutic approach in patients with diabetes.

IH induces acute insulin resistance (Iiyori *et al.*, 2007) and rapidly normalized after cessation of each IH exposure (Yokoe *et al.*, 2008). Antioxidant

enzymes protect pancreatic islets against apoptosis but not β-cell proliferation induced by IH (Xu *et al.*, 2009).

In addition to effects on glycemic control, exposure to IH changes the lipid metabolism. The severity of increases in lipids is proportional to the severity of the hypoxic stimulus (Li *et al.*, 2007). IH increases adipose tissue lipolysis causing fatty free acid flux to the liver and reducing lipoprotein clearance (Li *et al.*, 2005). This up-regulates hepatic triglyceride biosynthesis via hypoxia-inducible factor-1alpha. Partial deficiency of functional hypoxia-inducible factor-1alpha in mice is protector against hypertriglyceridemia and hepatic lipid accumulation during IH (Li *et al.*, 2006).

Antioxidants reduce oxidative damage caused by OSA (Li *et al.*, 2006). Melatonin is a hormone released by the pineal gland during the dark portion of the day-night cycle (Macchi and Bruce, 2004). It regulates circadian rhythms but has been described as pleiotropic and antioxidant. Melatonin decreases molecular and cellular damage resulting from ischemia-reperfusion (Reiter and Tan, 2003) and IH (Bertuglia and Reiter, 2009). Protection induced by melatonin against impairment or damage caused by IH in diabetes may be related to the regulation of reactive oxygen species (Winiarska *et al.*, 2006) and to influences of melatonin in insulin secretion (Peschke and Mühlbauer, 2010). In humans, insulin and melatonin secretion are in phase opposition. Rodents have most of their activity in the dark period when melatonin and insulin are both elevated.

N-acetylcysteine is an antioxidant that has been tested for innumerous medical conditions, with ambiguous performance (Dodd *et al.*, 2008). In diabetic neuropathy, N-acetylcysteine was reported to inhibit reactive oxygen species and decreases free radicals (Kamboj *et al.*, 2010). Among its effects on pulmonary, immune, renal, and neuropsychiatric diseases, one of the most investigated scenarios is the use of N-acetylcysteine in interventionist cardiology (Marenzi *et al.*, 2006) due to its possible role in reducing free radicals and improving endothelial function.

To test the hypothesis that antioxidants may have a role in preventing the derangement caused on the glycolipid profile by IH, we performed an

experimental comparative study that evaluated the effect of the antioxidants melatonin and N-acetylcysteine on glucose, triglycerides, and cholesterol levels in mice subjected to IH for 35 days.

METHODS

Animals

At the institutional animal experimentation unit, two month-old Balb/C male mice received commercial standard chow (Purina-Nutripal, Brazil) and water *ad libitum*, under the observation of a veterinarian. Six mice were kept in each cage, measuring 30x19x13 cm, at an average room temperature of 22.4^{o}±0.6 C with a 12:12-h light-dark cycle (lights on at 0700 1900). All procedures followed the "Guide for the Care and Use of Laboratory Animals" and were approved by the institutional Animal Ethics Committee.

Intermittent Hypoxia System

The IH procedure has been described previously (Martinez *et al.*, 2008; Martinez *et al.*, 2010). In brief, during the light period, from 9 AM to 5 PM, a mixture of N_2 90% and CO_2 10% was released during 30 seconds in the cages, reducing gradually the oxygen fraction to 7±1% and increasing the CO_2 fraction to approximately 5±1%; afterwards, fans were activated during 30 seconds, introducing room air in the cage; 480 cycles of hypoxia-normoxia occurred per day. Thirty-six mice were allocated to the hypoxia procedure. The 36 control animals (sham) underwent the same manipulation as the hypoxia animals, except for insufflation of air in the cages instead of the gas mixture.

Antioxidants Administration

From day 21 to day 35, daily, before 7:00 PM, all animals received intraperitoneal injections of: 1) vehicle (n= 24), 2) melatonin (n= 24), or 3) N-acetylcysteine (n= 24). Melatonin was administered at a dose of 200µg/Kg diluted in 0.4 mL of 0.9% saline plus 0.1 mL of 1% ethanol. N-acetylcysteine was administered at a dose of 10 mg/kg dissolved in 0.5 mL 0.9% saline.

Groups of 12 mice were randomly allocated to six different protocols: 1) sham+vehicle injection; 2) sham+melatonin injection; 3) sham+N-acetylcysteine injection; 4) IH+vehicle injection (hypoxia+vehicle); 5) IH+melatonin injection (hypoxia+melatonin); 6) IH+N-acetylcysteine injection (hypoxia+N-acetylcysteine).

At day 35, after fasting for at least eight hours, mice were anesthetized with ketamine, 100mg/kg, and xylazine, 50mg/kg. After anesthesia was confirmed, euthanasia was accomplished by the withdrawal of approximately one milliliter of blood.

Biochemical Analyses

Enzymatic colorimetric assays were used to measure serum glucose (Glucose GOD-PAP - Roche/Hitachi 912/917/Modular: ACN 525), total cholesterol (“CHOL - Cholesterol CHOD-PAP” - Roche/Hitachi Modular: P/D: ACN 433), and triglycerides (TG – Triglycerides GPO-PAP” - Roche/Hitachi 912/917/Modular: ACN: 781).

Statistical Analyses

All data were analyzed using SPSS software, version 16 (SPSS, Chicago, IL, USA). Data are presented as mean ± standard error of mean in figures. One way analysis of variance (ANOVA) was used to compare the differences between groups, followed by Tukey’s b test, when necessary. Generalized estimating equations, with Bonferroni post-hoc test, were used to

analyze the effects of time, the differences between groups and the interaction between groups over time. Significance of a finding was assumed if P<0.05.

RESULTS

No difference in body weight existed between the experimental groups under hypoxia and under sham hypoxia at day 1 (P=0.13). At day 21 the average body weight of the three groups under IH was significantly reduced (P<0.001) as opposed to the three sham groups that significantly increased their weight (P= 0.005). At day 35, after 14 days being injected according with the protocol, both hypoxia and sham groups had lost weight (P<0.001; Fig. 1).

At day 35, glucose levels were significantly different between groups (ANOVA P<0.001), showing higher levels in hypoxia+vehicle and hypoxia+N-acetylcysteine groups, as compared to the remaining groups (P<0.01; Fig. 2). The average glucose level in sham+melatonin group is significantly lower than in the remaining sham groups combined (60±26 vs. 79±19 mg/dL; P= 0.033). No significant differences were seen among the six groups in terms of lipids levels.

DISCUSSION

The present results confirm previous reports of IH causing fasting hyperglycemia (Bertuglia and Reiter, 2009; Yokoe *et al.*, 2008). This is the first report of hyperglycemia linked to IH being prevented by melatonin. Effects of melatonin have been attributed to its ability to regulate reactive oxygen species (Grebe *et al.*, 2006). The possibility, however, of melatonin effect being due to its antioxidant properties is overshadowed by the fact that the other antioxidant, N-acetylcysteine, had null effect on glucose levels and body weight.

The faculty of melatonin to reduce glucose levels (Ríos-Lugo *et al.*, 2010) emerges, therefore, as the most likely explanation for the finding of normal glucose levels under hypoxia+melatonin group. In humans, melatonin reduces glucose, blood pressure, oxidative and nitrosative stress, and protects against the metabolic and functional impairment related with related to regulation of reactive oxygen species in the microcirculation (Grebe *et al.*, 2006). The fact that in humans the peaks of melatonin and insulin are in phase opposition may be a problem in the process of translating the present results to clinical practice.

Rats (Martinez *et al.*, 2008) and mice (Martinez *et al.*, 2010) lose weight under IH. The present data reproduces the previous findings in that, after 21 days of exposure to IH, a significant reduction in body weight was seen. The weight loss induced by the injections from day 21 to day 35 is probably due to manipulation stress since even the sham group receiving vehicle injections lost weight.

The elevated glucose levels in the hypoxia+N-acetyscisteine group were kept after eight hours or more of fasting. Although long fasting is almost certainly stressful for the mice, the metabolic changes that the animals underwent were similar for all the groups. Additionally, longer fasting time would allow longer time for the glucose level to lower. Therefore, this systematic cause of error is conservative, tending to eliminate the differences.

The finding of cholesterol and triglycerides levels similar in the sham experimental groups and under IH plus melatonin, N-acetylcysteine, or vehicle is surprising. Our results disagree from some reports (Li *et al.*, 2005) but are consistent with others that were unable to find changes in lipid profile under IH (Roche *et al.*, 2009). Hyperlipidemia depends on the severity of hypoxia. The nadir of inspired O_2 in the present study, 7%, is in between what is considered moderate and severe hypoxia and this may be the cause of no change in lipids.

The discovery of an action of melatonin on hypoxia-induced hyperglycemia serves as basis for future mechanistic studies. The present study, however, has limitations since it was designed to explore glucose and lipid levels, but no changes were seen in lipid profile. The initial objective of

assessing lipid levels consumed most of the small volume of blood withdrawn from each mice. The positive result became limited to a description of the effects of antioxidants on glycemia. Further research is necessary to elucidate the mechanisms of the finding of hyperglycemia prevention elicited only by melatonin. To explore the complex system of glycemic control it is necessary to gauge, at least, insulin, norepinephrine, and glucagon levels; those results if available would have made it possible to further the knowledge on the mechanisms of hyperglycemia in the present study. Since lipids were not affected in this model, future efforts should be concentrated in explaining the effects of melatonin on glycemic control under IH.

In summary, these results indicate that exposure during 35 days to IH, simulating sleep apnea, promotes high plasma glucose levels, not prevented by N-acetylcysteine. Normal glucose levels were observed in mice receiving melatonin. It remains to be clarified whether the effect of melatonin is due to a direct action on glycemic control or to influences on oxidative stress. Although the mechanisms of the action of melatonin are still unclear, this finding may lead to advances in the care of patients with sleep apnea and diabetes.

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LEGENDS OF FIGURES

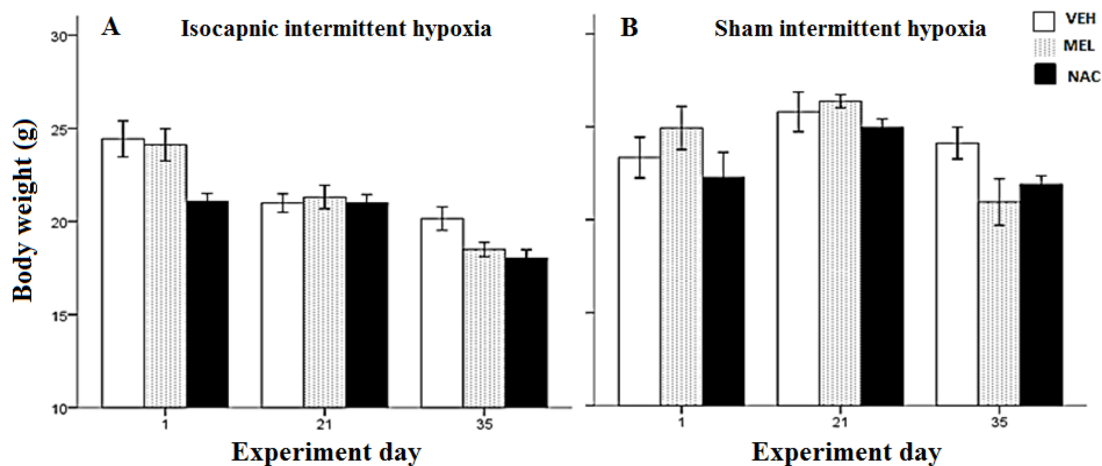

Figure 1: Mean and one standard error of mean of the body weight of the groups of mice during the 35 days experimental period. A. The groups under isocapnic intermittent hypoxia showed weight loss in the first 21 days and in the next 14 days, receiving the intra-peritoneal injections of melatonin and N-acetylcysteine. B. The groups under sham intermittent hypoxia showed weight gain in the first 21 days and weight loss in the next 14 days, receiving the intra-peritoneal injections. VEH: vehicle; MEL: Melatonin; NAC: N-acetylcysteine.

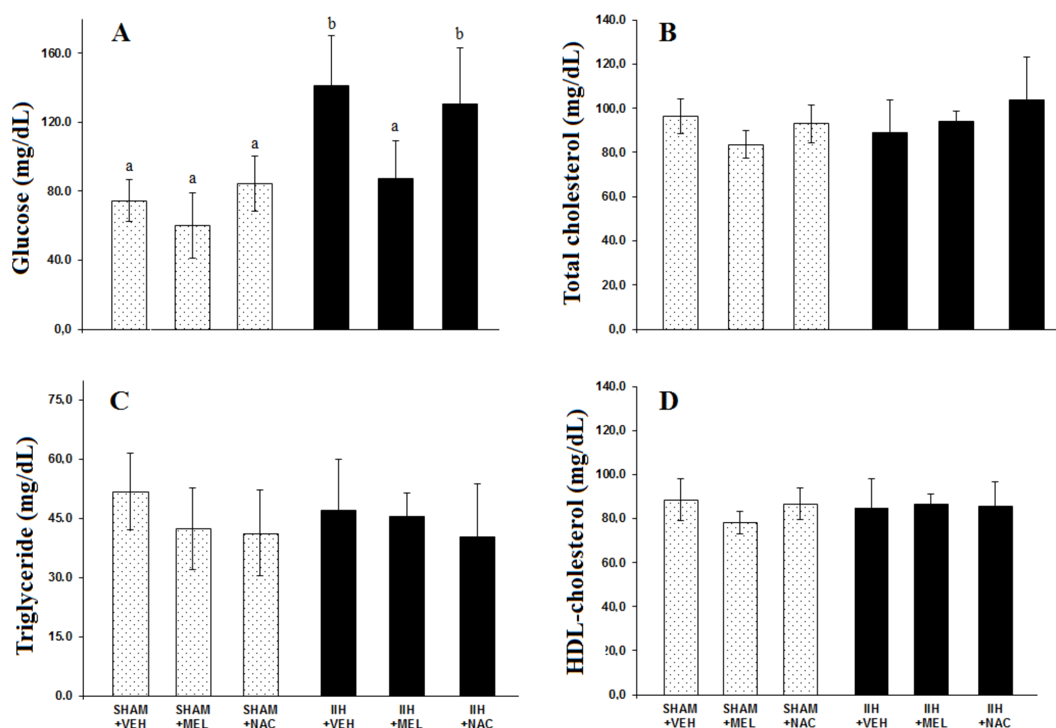

Figure 2: Means and one standard error of the mean of biochemical analyses and statistical significance of differences among the six experimental groups quantified by one-way ANOVA. A: Blood glucose; B: Serum total cholesterol; C: Serum triglycerides; D: Serum HDL-cholesterol. Different letters on top of the bars demonstrate differences between the groups evaluated by Tukey's b test ($p < 0.01$). SIH: Sham intermittent hypoxia; VEH: vehicle; MEL: Melatonin; NAC: N-acetylcysteine; IH: Intermittent hypoxia; HDL: High-density lipoprotein.

8 ANEXOS

CERTIFICADO

Certificamos que

DARLAN PASE DA ROSA

participou do **XXI Congresso Brasileiro de Hepatologia, XIV Simpósio Internacional de Terapêutica em Hepatite Viral, IV Simpósio de Terapia Intensiva em Gastroenterologia e Hepatologia da UGH-HP, I Semana Sul Americana de Fígado** e **III Encontro Amazônico de Hepatites Virais**, realizados no período de 27 de setembro a 01 de outubro de 2011, no Pestana Bahia Hotel

como vencedor do Prêmio Luiz Carlos Gayotto, com o trabalho *"Alterações Hepáticas e Metabólicas Causadas pela Hipóxia Intermitente na Apnéia do Sono e o Tratamento com Antioxidantes"*.

Salvador, 01 de outubro de 2011.

Dr. Raymundo Paraná
Presidente da Sociedade Brasileira de Hepatologia e do Congresso

Dr. Paulo Lisboa Bittencourt
Secretário Geral da Sociedade Brasileira de Hepatologia