UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE QUÍMICA
DEPARTAMENTO DE FÍSICO-QUÍMICA

Tese de Doutorado

EFEITO DOS S-NITROSOTIÓIS NO BLOQUEIO DA PEROXIDAÇÃO LIPÍDICA

Autora: Fernanda Ibanez Simplicio

Orientador: Prof. Marcelo Ganzarolli de Oliveira

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5. PEDIDOS DE PATENTE ENCAMINHADOS AO INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL (INPI)

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RESUMO

Título: Efeito dos S-nitrosotióis no bloqueio da peroxidação lipídica

Autora: Fernanda Ibanez Simplicio

Orientador: Marcelo Ganzarolli de Oliveira

Palavras-chaves: S-nitrosotióis, nitrosação, peroxidação lipídica, ácido linoleico, LDL

Óxido nítrico ($^{\bullet}$NO) produzido endogenamente em humanos é considerado um antioxidante efetivo na inibição da peroxidação lipídica. Todavia, no plasma e em células mamíferas, $^{\bullet}$NO circula principalmente como S-nitrosotióis primários (RSNOs). Neste trabalho, a peroxidação *in vitro* de comicelas do ácido linoleico-SDS (AL-SDS) e da lipoproteína de baixa densidade (LDL) catalisada por lipoxigenase de soja (SLO), íons Fe (II) e Cu (II), foram monitoradas na presença e na ausência de três RSNOs primários: S-nitrosocisteína (CISNO), S-nitroso-N-acetilcisteína (SNAC) e S-nitrosoglutationa (GSNO) a 37ºC. Medidas cinéticas e espectrofotométricas baseadas na formação de duplas conjugadas, adutos fluorescentes oxidados AL-lisina e na detecção eletroquímica de $^{\bullet}$NO livre, foram utilizadas para mostrar que RSNOs são antioxidantes mais potentes que seus tióis livres correspondentes (RSHs) em codições equimolar. Esses resultados são consistentes com o bloqueio da peroxidação do AL-SDS e LDL por RSNOs através da inativação dos radicais peroxil/alcoxil ($LOO^{\bullet}/LO^{\bullet}$) e pela transnitrosação com hidroperóxidos de AL pré-formado, levando a produtos nitrogenados de AL oxidado, que foram mostrados pela liberação de $^{\bullet}$NO livre por redução com ácido ascórbico. A ação antioxidante de SNAC e GSNO contra a peroxidação da LDL é refletida na quantidade reduzida de $^{\bullet}$NO livre detectado pela decomposição de RSNOs catalisados por Cu (II) na presença da LDL. Esses resultados indicam que RSNOs primários endógenos podem participar no bloqueio da peroxidação lipídica *in vivo*, não somente através da inativação primária dos radicais alcoxil/peroxil mas também através da inativação dos hidroperóxidos lipídicos pré-formados. A administração oral de SNAC previniu o princípio e progressão da doença não alcoólica do fígado gorduroso (NAFLD) em ratos Wistar alimentados com dieta deficiente em colina e reverteu a NAFLD em diferentes dietas com camundongos ob/ob. Esses efeitos foram correlacionados positivamente com um decréscimo na concentração de hidroperóxidos lipídicos no homogenatos de fígado e com habilidade dos RSNOs em previnir a peroxidação lipídica do ácido linoleico e da LDL *in vitro*.

ABSTRACT

Title: Effect of S-nitrosothiols in the blockage of lipid peroxidation

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Keywords: S-nitrosothiols, nitrosation, lipid peroxydation, linoleic acid, LDL

Nitric oxide ($^{\bullet}$NO) produced endogenously in humans is considered an effective chain-breaking antioxidant in the inhibition of lipid peroxidation. However, in the plasma and cells of mammals, $^{\bullet}$NO circulates mainly as primary S-nitrosothiols (RSNOs). In this work, the in vitro peroxidation of linoleic acid-SDS comicelles (LA-SDS) and of low density lipoprotein (LDL) catalyzed by soybean lipoxygenase (SLO), Fe (II) and Cu (II) ions, were monitored in the presence and absence of three primary RSNOs: S-nitrosocysteine (CySNO), S-nitroso-N-acetylcysteyne (SNAC) and S-nitrosoglutathione (GSNO) at 37 ºC. Kinetic and spectrophotometric measurements based on the formation of conjugated double bonds, fluorescent oxidized LA-lysine adducts and the electrochemical detection of free NO, were used to show that RSNOs are more potent antioxidants than their corresponding free thiols (RSHs) in equimolar conditions. These results are consistent with the blockage of LA-SDS and LDL peroxidation by RSNOs through the inactivation of peroxyl/alkoxyl ($LOO^{\bullet}/LO^{\bullet}$) radicals and through the transnitrosation with preformed LA hydroperoxides, leading to nitrogen-containing products of oxidized LA, which were shown to release free $^{\bullet}$NO upon reduction with ascorbic acid. The antioxidant actions of SNAC and GSNO against LDL peroxidation are reflected in a reduced amount of free NO detected upon Cu (II)-catalyzed decomposition of RSNOs in the presence of LDL. These results indicate that endogenous primary RSNOs may play a major role in blocking lipid peroxidation in vivo, not only through the primary inactivation of alkoxyl/peroxyl radicals but also through the inactivation of preformed lipid hydroperoxides. Oral administration of SNAC prevented the onset and progression of nonalcoholic fatty liver disease (NAFLD) in Wistar rats fed a choline-deficient diet and reversed NAFDL induced by different diets in ob/ob mice. These effects were positively correlated with a decrease in the concentration of lipid hydroperoxydes in liver homogenate and with the ability of RSNOs to prevent lipid peroxidation of linoleic acid and LDL in vitro.

Nota explicativa

Esta tese é composta de três manuscritos submetidos à publicação e um artigo publicado, em periódicos arbitrados de circulação internacional. A Doutora Fernanda é a primeira autora em dois destes manuscritos, cujos resultados foram obtidos pela mesma ao longo de seu projeto de doutorado. As participações dos co-autores destes dois manuscritos envolveram a realização e interpretação de resultados complementares e as orientações da Dra. Fernanda pelos orientadores no Brasil e em seu estágio na Universidade de Buenos Aires. Dra. Fernanda é co-autora do terceiro manuscrito, submetido ao *Journal of the American College of Nutrition* e de um artigo já publicado no *World Journal of Gastroenterology*. Estes trabalhos, que envolvem experimentação *in vivo*, contem dados *in vitro* obtidos pela Dra. Fernanda no IQ-UNICAMP e resultam de colaborações científicas do orientador com a Dra. Cláudia PMS de Oliveira, do Departamento de Gastroenterologia da FCM/USP. Deve-se salientar que a Dra. Fernanda não participou de nenhum dos experimentos *in vivo* ou da realização e interpretação das análises histológicas e bioquímicas contidas nestes trabalhos, cujo mérito é exclusivo dos co-autores filiados à Universidade de São Paulo. A Dra. Fernanda, porém, participou da discussão destes dados juntamente com o orientador e a Dra. Cláudia PMS de Oliveira, para a inclusão de seus dados experimentais nestes trabalhos. Estes manuscritos e artigos publicados estão precedidos de uma breve apresentação introdutória. Estão incluídos também na tese, dados e informações complementares não contemplados nos manuscritos e artigos.

ÍNDICE

1. Informações Introdutórias

1.1. Estresse Oxidativo e Peroxidação Lipídica

O estresse oxidativo e a peroxidação lipídica em mamíferos podem levar a inúmeras doenças, como por exemplo, câncer (Bartsch and Nair, 2006), doença não alcoólica do fígado gorduroso (de Oliveira et al., 2006 (A); de Oliveira et al., 2006 (B)) e doença cardiovascular (Libby, 2002; Witztum e Steinberg, 2001). O estresse oxidativo é definido como uma condição na qual o balanço fisiológico entre as espécies oxidantes e antioxidantes é perturbado favorecendo as espécies oxidantes e causando danos ao organismo (Cherubini et al., 2005). A peroxidação lipídica (PL) é um processo degenerativo que afeta a membrana celular, as lipoproteínas e outras estruturas contendo lipídios sob condições de estresse oxidativo (Girotti, 1998).

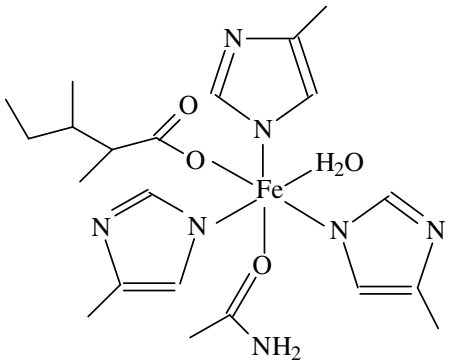
Inicialmente a PL foi estudada devido à deterioração oxidativa dos alimentos (Niki et al., 2005), mas nos últimos 20 anos a chamada "hipótese oxidativa" tem sido o foco central nas investigações da patogênese da aterosclerose e de outras doenças. Esta hipótese considera que a modificação oxidativa das lipoproteínas de baixa densidade (LDL) ou de outras lipoproteínas é central, senão obrigatória, no processo aterogênico (Witztum e Steinberg, 2001). Sabe-se que as partículas de LDL estão envoltas por uma molécula de apolipoproteína B (apo B-100) localizada em sua superfície, em conjunto com fosfolipídios e colesterol não esterificado, e que elas possuem um núcleo hidrofóbico de ésteres de colesterol e triglicérides que contém ácidos graxos poliinsaturados, sendo esta uma característica que influencia a suscetibilidade da LDL no processo de modificação oxidativa (Camejo and Hurt-Camejo, 1999). Além disso, a LDL contém antioxidantes lipofílicos,

incluindo α-tocoferol, carotenóides e ubiquinol-10 na sua superfície, que auxiliam na proteção dos componentes lipídicos no núcleo hidrofóbico (Rubbo et al., 2002). A oxidação da LDL leva ao consumo dos ésteres de ácidos graxos poliinsaturados como os ésteres dos ácidos araquidônico e linoleico e à geração de espécies reativas do derivado lipídico que podem se ligar covalentemente a apo B (Kawai et al., 2004).

A modificação oxidativa das lipoproteínas mediada por células, pode ser prevenida por antioxidantes (Mladenov et al., 2006; de Oliveira et al., 2000; Lisfi et al., 2000 e Rubbo e Odonnel, 2005) e é influenciada por metais (Lynch e Frei, 1995) que podem transitar entre dois estados de oxidação como Cu^{+}/Cu^{2+} e Fe^{2+}/Fe^{3+}. Além disso, o processo oxidativo inicia uma cadeia de reações radicalares de oxidação dos lipídios insaturados da LDL, modificando a apo B e produzindo mais lipoproteínas aniônicas modificadas com maior afinidade pelos macrófagos. O mesmo processo que altera as propriedades da apo B, também gera produtos fluorescentes com emissão em 430 nm quando a excitação ocorre em 360 nm (Cominacini et al., 1991).

Os ácidos graxos poliinsaturados são propícios a sofrerem oxidação, devido ao fato de que em sua cadeia carbônica existem hidrogênios metilênicos bis-alílicos que são mais suscetíveis à abstração por radicais oxidantes do que hidrogênios metilênicos de lipídios saturados, levando essas moléculas a possuírem uma dupla conjugação (após a oxidação) e, portanto uma absorção em 234 nm (Hogg e Kalyanaraman, 1999). Esses ácidos graxos podem ser oxidados por metais (Qian et al., 2000; Ohyashiki et al., 2002; Pinchuk e Lichtenberg, 2002) como Cu(II), Fe(II) e por lipoxigenases (LOX) (Belitz e Grosch, 1987). As LOXs são encontradas em plantas e animais, pertencem as famílias das dioxigenases e são capazes de induzir a peroxidação enzimática em ácidos graxos que contém um sistema 1-cis,4-cis-pentadieno

(Belitz e Grosch, 1987 e Lapenna et al., 2003) catalisando a sua oxidação aos correspondentes derivados de hidroperóxidos. Em plantas, os substratos mais comuns das LOXs são os ácidos linoleico e linolênico que são convertidos em uma variedade de mediadores bioativos envolvidos na defesa da planta, na germinação da semente, no crescimento e no desenvolvimento da planta (Belitz e Grosch, 1987). Em mamíferos os substratos predominantes da LOX são os ácidos araquidônico e linoleico que estão envolvidos em doenças como artrite, câncer e aterosclerose (Belitz e Grosch, 1987; Brash, 1999, Lapenna et al., 2003). Em geral, as LOXs contém um átomo de ferro que está presente como Fe^{2+} na forma de enzima inativa e a ativação enzimática de Fe^{2+} para Fe^{3+} ocorre através da oxidação dirigida, por exemplo pelo hidroperóxido do ácido linoleico. Desta forma, um fato importante da hipótese oxidativa é que a inibição da oxidação de lipídios deve reduzir a progressão da aterosclerose, independentemente da redução de outros fatores de risco, como os níveis elevados de LDL (Libby, 2002 e Witztum e Steinberg, 2001). A figura 1 mostra as estruturas moleculares da Lipoxigenase de Soja (SLO) e do ácido linoleico (AL).



SLO

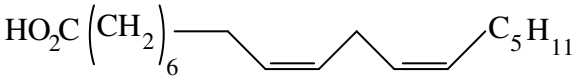


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Figura 1: Estruturas da Lipoxigenase de Soja (SLO) e do ácido linoleico (AL).

1.1.1. **Iniciação, Propagação e Terminação da Peroxidação Lipídica**

A iniciação e a propagação da peroxidação lipídica (PL) são mediadas pelos radicais livres, moléculas muito reativas que têm um elétron desemparelhado (Rubbo et al, 1996). A terminação da PL pode ocorrer com rearranjos de radicais formados durante as etapas de iniciação e propagação e também por antioxidantes como, por exemplo, ascorbato (AH^-) (Mladenov et al., 2006), α-tocoferol (α-TOH) (de Oliveira et al, 2000 e Lisfi et al, 2000), óxido nítrico (NO) (Rubbo et al., 2002 e Rubbo e Odonnel, 2005) e nitrosotióis (RSNO) (de Oliveira et al., 2006 (A); de Oliveira et al, 2006 (B)). As etapas de iniciação, propagação e terminação sem antioxidantes para um ácido graxo poliinsaturado (LH) são mostradas nas equações abaixo (Hummel et al, 2006) (1-5):

$$L\text{-}H + \text{oxidante}^{\bullet} \rightarrow L^{\bullet} + \text{oxidante-}H \quad \text{(iniciação)} \quad (1)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad \text{(propagação)} \quad (2)$$

$$LOO^{\bullet} + L\text{-}H \rightarrow LOOH + L^{\bullet} \quad \text{(propagação)} \quad (3)$$

$$L^{\bullet} + L^{\bullet} \rightarrow \text{produto não radicalar} \quad \text{(terminação)} \quad (4)$$

$$L^{\bullet} + LOO^{\bullet} \rightarrow \text{produto não radicalar} \quad \text{(terminação)} \quad (5)$$

Uma das características dos radicais livres é que reações de terminação em que dois radicais livres reagem para formar uma espécie não radicalar são extremamente rápidas (Rubbo et al., 1996). Abaixo seguem-se alguns exemplos de reações com antioxidantes não radicalares como AH^- e α-TOH

(Equações 6 e 7, respectivamente) e radicalar como $^{\bullet}NO$ (Equação 8). Rubbo e colaboradores em 2005, afirmaram que o $^{\bullet}NO$ é um antioxidante mais eficaz para o bloqueio da PL que os demais antioxidantes não radicalares citados acima. As constantes de velocidade (k) de segunda ordem confirmam a afirmação (Rubbo e Odonnel, 2005). Além disso, tem-se demonstrado *in vitro* que a reação entre ácidos graxos poliinsaturados oxidados (LH) na presença de NO formam produtos contendo nitrogênio, incluindo nitritos de alquila (LONO), nitratos de alquila (LOONO e $LONO_2$), epoxinitrito de alquila ($L(O)NO_2$), nitrohidróxido de alquila (LNO_2OH) e nitrolipídios (LNO_2) (Lima et al., 2002).

$$LOO^{\bullet} + AH^{-} \rightarrow LOOH + A^{\bullet -} \qquad k = 7{,}5.\ 10^{4}\ M^{-1}\ s^{-1} \qquad (6)$$

$$LOO^{\bullet} + \alpha\text{-}TOH \rightarrow LOOH + \alpha\text{-}TO^{\bullet} \qquad k = 2{,}5.\ 10^{6}\ M^{-1}\ s^{-1} \qquad (7)$$

$$LOO^{\bullet} + {}^{\bullet}NO \rightarrow LOONO \qquad k = 3{,}0.\ 10^{9}\ M^{-1}\ s^{-1} \qquad (8)$$

1.2. **Óxido Nítrico (NO)**

A descoberta em 1986 por Ignarro e colaboradores (Ignarro et al., 1987) de que o NO é o fator de relaxamento derivado do endotélio (EDRF), determinou um aumento muito grande nas pesquisas das propriedades químicas e fisiológicas do NO, uma vez que o EDRF ou NO influencia diretamente o relaxamento arterial. Em 1988, foi descoberto que o NO é sintetizado *in vivo* a partir L-arginina e que ele está envolvido em uma série de funções fisiológicas como vasodilatação, inibição da agregação plaquetária e

neurotransmissão e é também um participante ativo no sistema imune (Ignarro et al., 1987). A figura 2 mostra a formação de NO e L-citrulina através da oxidação da L-arginina sendo esta reação catalisada por NO-sintases (Karpuzoglu e Ahmed, 2006). Estas enzimas foram identificadas como: NOS endotelial (eNOS) que gera NO na parede endotelial dos vasos sanguíneos, induzível (iNOS), expressa por macrófagos como uma resposta a infecções bacteriana e viral e neuronal (nNOS), que está presente em neurônios, onde o NO atua como neurotransmissor (Williams, 2003 e Karpuzoglu e Ahmed, 2006).

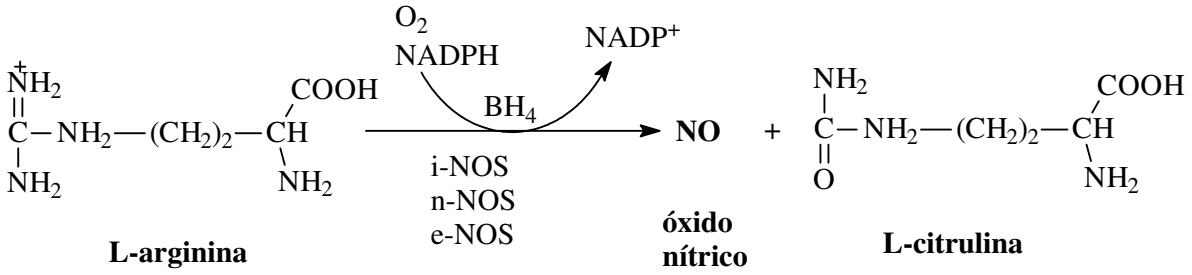


Figura 2: Síntese de óxido nítrico (NO) pela NOS. Óxido nítrico pode ser gerado por três diferentes formas de óxido nítrico sintases (NOS): induzível (iNOS), neuronal (nNOS) e endotelial (eNOS). Essas enzimas catalisam a conversão da L-arginina em L-citrulina e NO na presença de nicotinamida adenina dinucleotídeo fostato (NADPH) e tetrahidrobiopterina (BH_4). Figura modificada de Karpuzoglu e Ahmed, 2006 (Karpuzoglu e Ahmed, 2006).

Cullota e Koshland em 1992 escreveram um artigo para a revista Science, no qual descrevem os efeitos tóxicos e também os possíveis efeitos benéficos do NO, que foi eleito por esta revista como a "Molécula do ano":

uma molécula simples que unifica a neurociência, a fisiologia e a imunologia e revoluciona o conceito dos cientistas sobre a comunicação e a defesa das células (Culotta e Koshland, 1992). Os pesquisadores Louis J. Ignarro (Ignarro, 1999), Robert F. Furchgott (Furchgott, 1999) e Ferid Murad (Murad, 1999) ganharam o Prêmio Nobel em Fisiologia e Medicina em 1998, após terem reunido informações importantes sobre a participação do NO no sistema fisiológico e imunológico dos mamíferos.

Atualmente, sabe-se que o NO exerce papéis reguladores fundamentais como mensageiro intra e intercelular e é uma das principais espécies envolvidas na resposta do sistema imune. Os efeitos biológicos do NO podem ser agrupados em três categorias: regulador, protetor e deletério (Giustarini et al, 2003). A participação do NO tem sido identificada em um grande número de doenças como aterosclerose (Patel et al., 2000), câncer (Napoli e Ignarro, 2001) e doença não alcoólica do fígado gorduroso (NAFLD) (de Oliveira et al., 2006 (A); de Oliveira et al., 2006 (B)). Mais recentemente, o NO foi também identificado como o principal fator envolvido nas propriedades antiateroscleróticas do endotélio (Giustarini et al, 2003). Foi demonstrado que o NO interfere *in vitro* com eventos chave no desenvolvimento da aterosclerose, tais como a adesão de monócitos e leucócitos ao endotélio e as interações de plaquetas com as paredes do vaso (Napoli e Ignarro, 2001 e Cornwell et al., 1994). O NO também diminui a permeabilidade endotelial e reduz o tônus vascular, diminuindo o fluxo de lipoproteínas para o interior da parede vascular e inibe a proliferação e a migração das células musculares lisas *in vitro* e *in vivo* (Sarkar et al., 1995 e Dubey et al., 1995). Resultados expressivos da ação protetora que o NO pode exercer na peroxidação lipídica, foram obtidos em estudos *in vitro* que demonstram que o NO inibe a lipoperoxidação através do bloqueio da propagação das reações radicalares.

Demonstramos mais recentemente que a administração por *via* oral de um doador de NO ou um S-nitrosotiol como fonte exógena pode também reduzir a produção de hidroperóxidos lipídicos no tecido hepático, bloqueando o início da NAFLD em modelos animais (de Oliveira et al., 2006 (A); de Oliveira et al., 2006 (B)).

1.2.1. **S-nitrosotióis (RSNOs)**

Devido ao fato de que a utilização do NO gasoso em sistemas experimentais é muito limitada e que no organismo existem espécies como, por exemplo, ânion superóxido ($O_2^{\bullet}$), que inativa a molécula do NO livre formando peroxinitrito ($ONOO^-$) (Eq. 9), que é um potente oxidante (Violi et al., 1999) (Equação 9), tem-se desenvolvido compostos que prolongam a meia vida ($t_{1/2}$) do NO no organismo, uma vez que a $t_{1/2}$ *in vivo* é muito curta (0,1 – 6 s) (Marcondes et al., 2002).

$$O\text{-}O^{\bullet} + {}^{\bullet}N\text{-}O \rightarrow ONOO^- \qquad (9)$$

Estes compostos que tem a capacidade de liberar NO têm sido amplamente usados como agentes terapêuticos e como ferramentas farmacológicas na investigação do papel do NO na fisiologia e na patofisiologia de doenças. Mais recentemente, o grande interesse na fisiologia do NO tem levado ao desenvolvimento de uma grande variedade de novos doadores de NO (S-nitrosotióis), que apresentam uma série de vantagens sobre os doadores convencionais (nitroglicerina (NTG) e nitroprussiato de sódio (NPS)). A continua exposição a NTG leva a resistência e tolerância a

nitrato que é um problema clinicamente importante que requer um tratamento descontínuo para garantir a eficácia do tratamento. O uso prolongado de NPS em pacientes com função hepática comprometida é associado com o acúmulo de cianeto e tiocianato que são tóxicos ao organismo (Zhang e Hogg, 2004).

Desde 1987, a química dos S-nitrosotióis (RSNOs) representa uma parte rica e complexa da química dos óxidos de nitrogênio que auxilia no entendimento da bioquímica do NO (Zhang e Hogg, 2004). Os RSNOs *in vivo* são produtos da reação entre NO produzido endogenamente com os peptídeos que contem o grupo sulfidrila (R–SH), sendo estes denominados tióis. Esta reação de nitrosação ocorre quando o NO está em quantidade suficiente para interagir com oxigênio molecular (O_2) formando a espécie nitrosante (N_2O_3) (Equação 10 e 11), na seqüência N_2O_3 reage em meio aquoso com RSH formando RSNO (Equação 12) (Feelisch et al., 2002 e de Oliveira et al., 2002).

$$NO + O_2 \rightarrow NO_2 \quad (10)$$

$$NO + NO_2 \rightarrow N_2O_3 \quad (11)$$

$$N_2O_3 + RSH \rightarrow RSNO + NO_2^- + H^+ \quad (12)$$

Alternativamente, RSNOs podem ser obtidos pela reação entre RSHs e $ONOO^-$ (Equação 13), sendo $ONOO^-$ proveniente da reação 9.

$$RSH + ONOO^- \rightarrow RSNO + NO_2^- + H^+ \quad (13)$$

A reação de transnitrosação (Giustarini et al., 2003 e Zhang e Hogg, 2004) ocorre quando o ânion tiolato ataca nucleofilicamente o átomo de

nitrogênio de um S-nitrosothiol, resultando na transferência do grupo nitroso para o tiol (Equação 14).

$$RSNO + R'SH \rightarrow RSH + R'SNO \qquad (14)$$

Um exemplo de RSNO é a S-nitrosoglutationa (GSNO) e a figura 3 mostra que a formação da GSNO pode ser obtida por três vias (Zhang e Hogg, 2004):

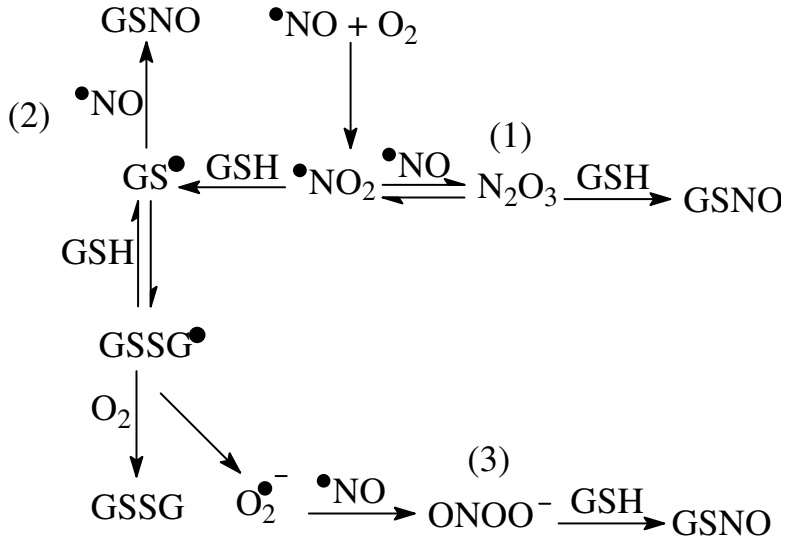
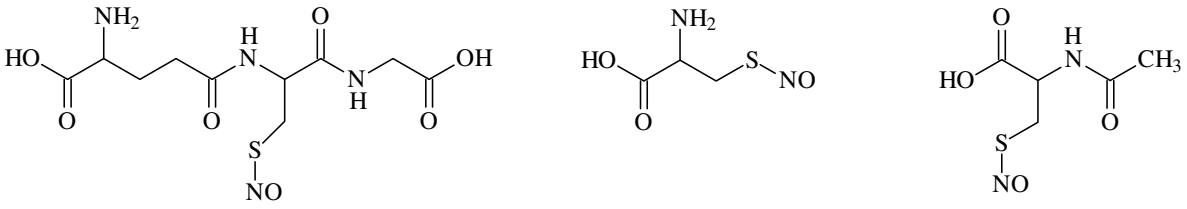


Figura 3: Caminhos para a formação da S-nitrosoglutationa (GSNO) a partir da glutationa (GSH), óxido nítrico (NO) e oxigênio. Modificado segundo Zhang e Hogg, 2004 (Zhang e Hogg, 2004).

Uma vez formado, o RSNO pode liberar o NO livre pela quebra homolítica da ligação S-N (Equação 15) através de uma decomposição térmica. Esta reação pode ser catalisada por metais, principalmente íons Cu(II), sendo acelerada pela irradiação fotoquímica com luz ultravioleta ou visível (de Oliveira et al., 2002).

$$2RSNO \rightarrow NO + RSSR \qquad (15)$$

Entre os RSNOs, encontra-se a S-nitroso-L-cisteína (CISNO), a S-nitroso-N-acetilcisteína (SNAC), a S-nitrosoglutationa (GSNO), a S-nitroso-N-acetilpenicilamina (SNAP), a S-nitrosopenicilamina e a S-nitrosoalbumina. Estes diferentes doadores podem apresentar também diferentes velocidades de liberação de NO em processos espontâneos ou de transferência de NO para outros receptores (transnitrosação) (de Oliveira et al., 2002). A figura 4 mostra a estrutura molecular da GSNO, CISNO e SNAC.



GSNO CISNO SNAC

Figura 4: Estrutura molecular da S-nitrosoglutationa (GSNO), S-nitrosocisteína (CISNO) e S-nitroso-N-acetilcisteína (SNAC).

Os RSNOs são classificados como "primários" quando o átomo de enxofre é ligado a um átomo de carbono primário, diferentemente, por exemplo, da S-nitroso-N-acetilpenicilamina (SNAP), que é amplamente usada em estudos experimentais e onde o átomo de enxofre é ligado a um átomo de carbono terciário, fazendo deste composto um RSNO "terciário".

1.3. Importância do óxido nítrico e S-nitrosotióis no combate a peroxidação lipídica (PL) e às doenças relacionadas ao stress oxidativo

Reações de terminação e supressão da peroxidação lipídica podem ser controladas por óxido nítrico (NO) no ambiente extracelular (Giustarini et al., 2003). O ácido linoleico (AL) possui dois hidrogênios metilênicos bis-alílicos que são suscetíveis à abstração de hidrogênio por radicais oxidantes. Em uma oxidação após a abstração ocorre a formação do radical alquila lipídico ($L^{\bullet}$) que na presença de oxigênio (O_2) forma o radical peroxila lipídico ($LOO^{\bullet}$). *In vivo* o NO pode reagir com o $L^{\bullet}$, mas o O_2 reage preferencialmente com esta espécie com velocidade limitada por difusão, por estar numa concentração substancialmente maior (10 - 100 vezes) em relação ao NO. Sendo assim, o NO reage com $LOO^{\bullet}$ para inibir a peroxidação lipídica levando à formação de produtos de AL oxidado contendo nitrogênio (LOONO). Na ausência de NO a peroxidação lipídica segue até a formação do hidroperóxido (LOOH) que é formado através da abstração de átomos de H de moléculas do AL pelos radicais $LOO^{\bullet}$. O LOOH pode reagir com Fe^{2+} e formar o radical alcoxila lipídico ($LO^{\bullet}$) ou reagir com Fe^{3+} e formar $LOO^{\bullet}$. Este radical $LOO^{\bullet}$ pode

novamente abstrair um átomo H do AL e iniciar uma nova peroxidação numa reação em cadeia onde uma maior quantidade de LOOH é formada (Patel, et al., 2000) .

Como os LOOHs possuem uma forte banda de absorção em 234 nm devido à dupla ligação conjugada (Hogg e Kalyanaraman, 1999), sua formação pode ser analisada por espectrofotometria UV/VIS. O bloqueio da propagação radicalar por RSNOs pode ocorrer através da reação de RSNOs com $LOO^{\bullet}$ ou com $LO^{\bullet}$ (Equações 16 e 17) através de um mecanismo bimolecular. O destino dos radicais tiila ($RS^{\bullet}$) formados nas reações (16) e (17) é a dimerização através da formação de pontes de dissulfeto (RS-SR) (Equações 18 e 19).

$$LOO^{\bullet} + RSNO \rightarrow LOONO + RS^{\bullet} \quad (16)$$

$$LO^{\bullet} + RSNO \rightarrow LONO + RS^{\bullet} \quad (17)$$

$$RS^{\bullet} + RSNO \rightarrow RS\text{-}SR + NO \quad (18)$$

$$RS^{\bullet} + RS^{\bullet} \rightarrow RS\text{-}SR \quad (19)$$

A figura 5 mostra como o S-nitrosotiol inibe a peroxidação lipídica a partir do ácido linoleico e SLO.

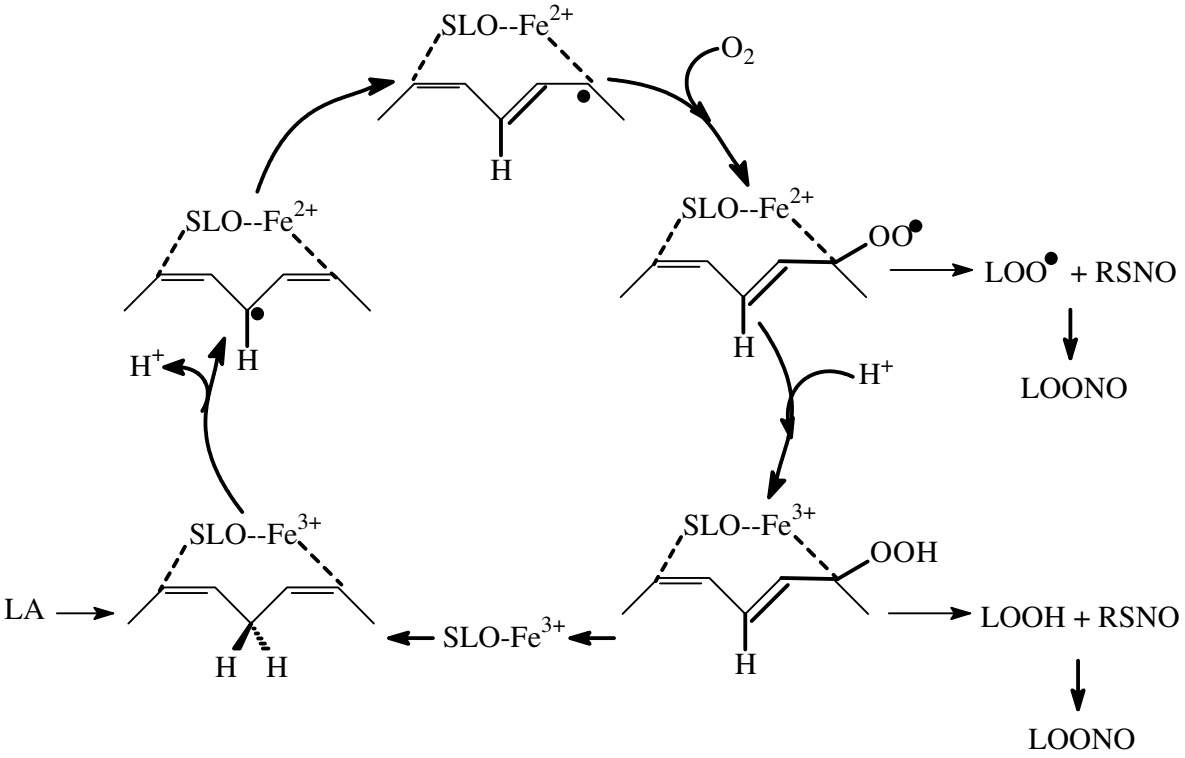


Figura 5: Reações de peroxidação do ácido linoleico (LA) *via* Lipoxigenase de Soja (SLO).

2. Objetivos

1- Avaliar a ação antioxidante de S-nitrosotióis primários nas peroxidações do ácido linoleico e da lipoproteína de baixa densidade (LDL) humana, mediadas por íons Cu (II), Fe(II) e por lipoxigenase de Soja (SLO) *in vitro*.

2- Correlacionar a capacidade antioxidante de S-nitrosotióis *in vitro* com a sua ação biológica no tratamento e reversão da doença não alcoólica do fígado gorduroso (NAFLD) *in vivo* através da administração de RSNOs por via oral em modelos animais.

3. Inibição da peroxidação do ácido linoleico *in vitro* pelos S-nitrosotióis primários

Nesta parte do trabalho, a peroxidação do ácido linoleico (AL) *in vitro* em comicelas de SDS (AL-SDS) catalisada por lipoxigenase de soja (SLO) e íons Fe(II) e Cu(II) foi monitorada na presença e na ausência de três S-nitrosotióis (RSNOs) primários: S-nitrosoglutationa (GSNO), S-nitrosocisteína (CISNO) e S-nitroso-N-acetilcisteína (SNAC), a 37ºC. Medidas cinéticas e espectrofotométricas baseadas na formação da dupla ligação conjugada e da formação de adutos fluorescentes através do AL oxidado com lisina, mostraram que os RSNOs são antioxidantes mais potentes que seus tióis correspondentes (RSHs), em condições equimolares. Os resultados obtidos estão de acordo com o bloqueio da peroxidação lipídica do AL-SDS pelos RSNOs através da inativação dos radicais peroxila/alcoxila ($LOO^{\bullet}/LO^{\bullet}$), levando a produtos nitrogenados do AL oxidado (LOONO/LONO), cuja formação foi demonstrada através da liberação de $^{\bullet}NO$ produzido pela redução destes produtos com ácido ascórbico. Também foi verificado que RSNOs reagem diretamente com hidroperóxidos através da reação de transnitrosação, levando também a produtos nitrogenados do AL oxidado. Esses resultados indicam que RSNOs primários endógenos podem ter um papel importante no bloqueio da peroxidação lipídica *in vivo*, não somente através da inativação primária de radicais alcoxila/peroxila, mas também pela inativação de hidroperóxidos formados. Estes resultados foram submetidos à publicação no periódico *Free radical biology and medicine* e se encontram no manuscrito abaixo.

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In vitro inhibition of linoleic acid peroxidation by primary S-nitrosothiols

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Running title: Inhibition of Linoleic Acid Peroxidation by S-Nitrosothiols

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In vitro inhibition of linoleic acid peroxidation by primary S-nitrosothiols

Abstract

Nitric oxide ($^{\bullet}$NO) produced endogenously in humans is considered as an effective chain-breaking antioxidant in the inhibition of lipid peroxidation. However, in vivo $^{\bullet}$NO circulates mainly as primary S-nitrosothiols (RSNOs). In this work, the in vitro peroxidation of linoleic acid-SDS comicelles (LA-SDS) catalyzed by soybean lipoxygenase (SLO), and Fe (II) and Cu (II) ions was monitored in the presence and absence of three primary RSNOs: S-nitrosocysteine, S-nitroso-N-acetylcysteyne and S-nitrosoglutathione at 37ºC. Kinetic and spectrophotometric measurements based on the formation of conjugated double bond and fluorescent oxidized LA-lysine adducts showed that RSNOs are much more potent antioxidants than their corresponding free thiols (RSHs), in equimolar conditions. The results obtained are consistent with the blocking of LA-SDS peroxidation by RSNOs through the inactivation of peroxyl/alkoxyl (LOO$^{\bullet}$/LO$^{\bullet}$) radicals, leading to nitrogen-containing products of oxidized LA, which were shown to release free $^{\bullet}$NO upon reduction with ascorbic acid. It was also found that RSNOs react with preformed LA hydroperoxides through transnitrosation reactions, leading also to nitrogen-containing products of LA. These results indicate that endogenous primary RSNOs may play a major role in blocking lipid peroxidation in vivo, not only through the primary inactivation of alkoxyl/peroxyl radicals but also through the inactivation of preformed lipid hydroperoxides.

Keywords: Nitric oxide; S-nitrosothiols; Lipid peroxidation; Linoleic acid; Lipoxygenase

Introduction

An increasing amount of evidences have demonstrated that oxidative and nitrosative stresses play a fundamental role in atherosclerosis and in other diseases associated with lipid peroxidation (LPO) [1-4]. In these cases, it is assumed that free radicals, which normally play an essential role in metabolic processes, are released from the active site of enzymes, triggering a cascade of deleterious effects on cells [5]. These effects involve the interaction of free radicals with metal or organic redox centers and the promotion of irreversible oxidation reactions beyond the normal catalytic cycles. Once formed, free radicals are also capable of initiating other radical reactions, which may become self-sustaining through the regeneration of propagating radicals. It is well established that propagating radicals are involved in the oxidation of lipids in humans and that this is a key event in the atherosclerotic process. This conclusion is reinforced by the fact that both primary and secondary lipid oxidation products are found in human atherosclerotic lesions [6,7].

Under normal physiological conditions, endothelium-derived nitric oxide (nitrogen monoxide, $^{\bullet}NO$) has multiple physiological functions in humans, like the regulation of vascular tone in both the systemic and renal circulation [8,9], the prevention of adherence and aggregation of platelets and monocytes in the walls of blood vessels [10] and the regulation of the proliferation and migration of smooth muscle cells [11]. In addition to the actions related to the mediation of signal transduction, via stimulation of guanylate cyclase-mediated cGMP synthesis, NO was also shown to exert several antiatherogenic properties assigned to its ability to react directly with free radicals, blocking the propagation of radical reactions. This protective effect has already been observed in model lipid systems [12,13], low-density lipoproteins (LDL) [14-16] and cells [17,18], and this effect is supported by several in vitro

studies which have demonstrated the formation of nitrogen-containing products of polyunsaturated fatty acids (PUFA), including alkylnitrites (RONO), alkylnitrates (ROONO and $RONO_2$), alkylepoxynitrite ($R(O)NO_2$), alkylnitrohydroxy (RNO_2OH) and nitrolipids (RNO_2), when PUFAs are oxidized in the presence of •NO. Such products have already been characterized in other studies by mass spectrometry and can be taken as markers of the in vivo pro-oxidant and/or antioxidant actions of •NO [12,13,19]. These results stimulate new therapeutic approaches for treating lipid peroxidation-related diseases by enhancing •NO synthesis and/or activity by administration of L-arginine and antioxidants [20]. As an alternative strategy, compounds that act as NO donors could be administrated as exogenous NO sources, as already demonstrated for the treatment of hepatic steatosis via oral administration of the S-nitrosothiol (RSNO) and S-nitroso-N-acetylcysteine (SNAC) [3,4]. RSNOs are peptides or proteins carrying the S-NO moiety and were shown to occur in the plasma and cells of mammals where they have the same physiological functions of free •NO like vasodilation [21,22], inhibition of platelet activation and aggregation [23,24] and post-translational modification of protein function [25,26]. S-nitrosoglutathione (GSNO), S-nitrosoalbumin and S-nitrosohemoglobin, for example, have been considered to be •NO carriers and donors in humans and are the focus of several studies both in vivo and in vitro [27]. Other RSNOs, like S-nitrosocysteine (CySNO) have also been described [3,28] (Fig. 1). What classifies a RSNO as primary is the fact that the sulfur atom of its SNO moiety is bound to a primary carbon atom, differently for example S-nitroso-N-acetylpenicillamine (SNAP), which is widely used in experimental studies and where the sulfur atom is bound to a tertiary carbon atom, making it a “tertiary” RSNO. Evaluating the antioxidant properties of primary RSNOs may have an additional relevance, since the lability of •NO in primary and tertiary RSNOs can be different [28-30]. In any case, one of the important characteristics of having •NO carried as RSNOs is its preservation from inactivation caused by reaction with oxygen,

leading to nitrite (NO_2^-) and further to nitrate (NO_3^-) [31], which are two of the main end products of $^{\bullet}NO$ metabolism. Although several exogenous $^{\bullet}NO$ sources (which are not found endogenously) have been used as antioxidants in LPO studies like organic nitrites [32] and NONOates [32,33], the protective role of primary RSNOs in blocking LPO reactions remains largely unexplored.

In this study, the in vitro peroxidation of linoleic acid - sodium dodecyl sulfate comicelles (LA-SDS) catalyzed by soybean lipoxygenase (SLO) and by Fe (II) and Cu (II) ions was monitored in the presence and absence of three primary RSNOs: CySNO, SNAC and GSNO, and of their corresponding free thiols (RSHs), at 37ºC. Kinetic and spectrophotometric data showed that RSNOs can block LA peroxidation more efficiently than RSHs, by inactivating alkoxyl/peroxyl ($LO^{\bullet}/LOO^{\bullet}$) radicals and LA hydroperoxides, (LOOH = 13-hydroperoxy-octadecadienoic acid, 13-HPODE) through nitration and transnitrosation reactions. These results, suggest that endogenous primary RSNOs may play a major role in blocking lipid peroxidation in vivo.

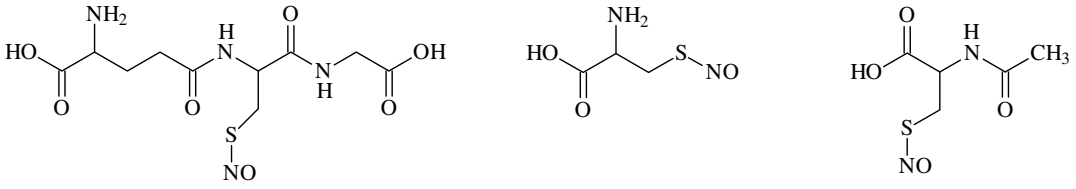


Fig. 1. Molecular structures of S-nitroso-L-cysteine (CySNO), S-nitroso-N-acetylcysteine (SNAC) and S-nitrosoglutathione (GSNO).

Materials and Methods

Ascorbic acid, cysteine (CySH), copper sulfate ($CuSO_4$), ferrous sulfate ($FeSO_4$), Glutathione (γ-Glu-Cys-Glu, GSH), linoleic acid (LA), L-lysine monohydrochloride (Lys), malonaldehyde bis(dimethyl acetal) (MDA), N-acetyl-L-cysteine (NAC), phosphate buffer saline (PBS, pH 7.4), sodium dodecyl sulfate (SDS), sodium nitrite ($NaNO_2$), soybean lipoxygenase (SLO), *tert*-butyl hydroperoxide (tBOOH) sodium hydroxide (NaOH) hydrochoric acid (HCl), mercury chloride ($HgCl_2$) (Sigma/Aldrich, St. Louis, MO) and sulfanilamide (Merck, Germay) were used as received. All the experiments were carried out using analytical grade water from a Millipore Milli-Q gradient filtration system.

Synthesis of GSNO, CySNO and SNAC in aqueous solution

Aqueous GSNO solution was prepared by the reaction of GSH with sodium nitrite in acidic medium as described elsewhere [28,34]. GSNO was obtained as stable reddish crystals in the pure form and was dried by freeze-drying. Solid GSNO was stored at -20°C. Freshly prepared GSNO solutions in PBS were used in the experiments. S-nitroso-N-acetylcysteine (SNAC) and S-nitrosocysteine (CySNO) cannot be precipitated from solution and stored as dry solids because of their high solubility in water. Therefore, aqueous SNAC or CySNO solutions were synthesized through the equimolar reaction of NAC or CyS, respectively, with $NaNO_2$ in acidified aqueous solution freshly prepared. Stock acidic SNAC and CySNO solutions, were diluted in PBS and used immediately.

Spectrophotometric characterization and monitoring of linoleic acid peroxidation

Linoleic acid (LA) oxidation was induced through the addition of SLO to aqueous LA dispersions (final concentration 19 μM) in SDS solution (0.01 M) (LA-SDS comicelles). Each LA dispersion was transferred to a quartz cuvette, blowed with O_2 for 2 min and SLO (final concentration 56 nM) was added to the cuvette with a syringe to start the peroxidation reaction. Peroxidation reactions were monitored in the absence or presence of RSNOs and RSHs (final concentrations 56, 112 and 560 μM) through the increase in absorbance at 234 nm, due to conjugated diene formation. A Hewlett Packard spectrophotometer, model 8453 (Palo Alto, CA, USA) with a temperature-controlled cuvette holder was used to monitor the spectral changes in the range 200 - 600 nm in the dark at 37°C, in time intervals of 2 s. Spectra of the solutions were obtained in 1 cm quartz cuvette referenced against air, under stirring (1,000 r/min). Each point in the kinetic curves of absorbance *vs.* time is the average of two experiments with error bars expressed by the average deviation of the mean.

Characterization of the fluorescent MDA-lysine adduct

To characterize the emission spectrum of the fluorescent adduct formed in the reaction of oxidized linoleic acid (oxLA) and lysine, an MDA-lysine adduct was prepared as a model adduct by reacting MDA with L-lysine in equimolar condition (final concentration 0.25 M) in PBS solution at room temperature and an emission spectrum was obtained in the range 375-600 nm, with excitation wavelength of 360 nm.

Spectrofluorimetric characterization and monitoring of oxidized LA-lysine adduct formation

LA peroxidation was induced through the addition of aqueous $FeSO_4$ solution (final concentration 5.0 μM) to aqueous LA (final concentration 1.2 mM) dispersions in SDS solution (final concentration 0.01 M) in the absence or presence of GSNO (final concentrations 5 and 500 μM) for 2 h in PBS (pH 7.4). After LA oxidation, lysine solution (final concentration 1.0 mM) was transferred to the dispersions followed by incubation for 48 h. The kinetics of formation of fluorescent oxidized LA-lysine adduct (oxLA-Lys) in the reaction between oxLA and Lys during the incubation time was characterized based on the spectral changes in the range 375 to 600 nm and on the emission intensity at 430 nm, under excitation with 360 nm. All the spectrofluorimetric measurements were performed using a Perkin-Elmer LS55 spectrofluorimeter with a temperature-controlled cuvette holder at 37ºC.

Reaction between RSNOs and tert-butyl hydroperoxide

The formation of *tert*-butyl peroxynitrites (tBOONOs) in the reactions between *tert*-butyl hydroperoxide (tBOOH) and RSNOs was characterized by following the decomposition of GSNO, SNAC and CySNO (initial concentrations 1 mM) upon the addiction of tBOOH (initial concentration 25 mM) in basic medium (pH 12). The decomposition of RSNOs in absence or presence of tBOOH was characterized by following the spectral changes of RSNOs solutions in the range 220–1100 nm in the dark, in a 1 cm quartz cuvette referenced against air. Kinetic curves of GSNO, SNAC and CySNO decomposition were obtained from the absorption changes at 336 nm in time intervals of 15 s, at 37ºC for 8 min. The control experiment was performed by incubating RSNOs with pure water at pH 12, adjusted with

NaOH solution. Each point in the kinetic curves of concentration vs. time is the average of two experiments with error bars expressed by the average deviation of the mean.

Detection of $^{\bullet}$NO released from nitrogen-containing products of oxidized LA

The $^{\bullet}$NO released from nitrogen-containing products of oxLA, formed in the peroxidation of LA in the presence of GSNO was detected using a gas-phase chemiluminescence nitric oxide analyzer (NOA, Sievers, Bolder Co, USA). For the analysis of nitrogen-containing products of oxidized LA, aqueous dispersions of LA were incubated in the presence and absence of Cu (II) ions with final concentrations of LA, GSNO and Cu (II) 900 μM. Peroxidation of LA was induced in two different procedures. In the first, the LA-SDS dispersion was previously blowed with O_2 for 2 min, followed by incubation with GSNO for 30 min at room temperature. In the second, peroxidation of LA was induced by heating a sample of pure LA at 80ºC for 1 h under stirring in a glass flask with O_2 atmosphere, obtained by continuously blowing O_2 from a cylinder into the headspace of the flask. After oxidation, oxLA was dispersed in SDS solution (0.01 M) and incubated with GSNO, for 30 min at room temperature. In both cases, after incubation, no reacted excess GSNO was removed from the solution by adding $HgCl_2$ (final concentration 29.4 mM) and allowing GSNO decomposition to GS-SG and free $^{\bullet}$NO to proceed for 15 min. In this condition, $^{\bullet}$NO is quantitatively released from excess GSNO by mercuric catalysis and is rapidly and quantitatively converted to its stable end product, nitrite (NO_2^-). Nitrite formed was removed by adding a 10% v/v solution of sulfanilamide (6.0 mM in HCl 2 M), followed by incubation for 15 min. A volume of 5 mL

of molar excess of aqueous saturated ascorbic acid solution, used as a reducing agent, was added in the reaction vessel of the NOA. Antifoaming agent was used to prevent foaming caused by injection of the samples. Volumes of 100 μL of the final nitrogen-containing products of oxLA suspension were injected into the reaction vessel containing ascorbic acid, through an impermeable septum. Nitrogen gas (Air Liquid, Brazil) was bubbled through the solution into the chemiluminescence meter. Free $^{\bullet}$NO formed in the reaction vessel due to the reduction of nitrogen-containing products of oxLA by ascorbic acid was detected.

Results

Spectrophotometric characterization and monitoring of linoleic acid peroxidation

Figure 2 shows the spectral changes in the range 220–260 nm during LA oxidation by SLO in the absence (Fig. 2A) or presence of RSHs and RSNOs (Figs. 2B to 2J) in the first 80 s of reaction in time intervals of 2 s. The spectral changes show the increase of the absorption band with maximum at 234 nm, assigned to the formation of conjugated double bonds in LA, as a result of peroxidation [35]. It can be seen that the extent of spectral change is reduced in the oxidations performed in the presence of RSHs or of their corresponding RSNOs, showing that both RSHs and RSNOs inhibit LA peroxidation, compared to LA alone. Comparison of the effects of RSHs and RSNOs at the same concentrations (560 μM, Figs. 2B, 2C, 2D and 2H, 2I, 2J) shows that RSNOs exert a much more effective antioxidant action than RSHs in all cases. It can also be seen that there is a concentration-response effect in the antioxidant effects of RSNOs, when the concentration is increased from 56 μM (Figs. 2E, 2F and 2G) to 560 μM (Figs. 2H, 2I and 2J). Moreover, Figs. 2E, 2F and 2G show that RSNOs in

concentrations ten times lower (56 μM, Figs. 2E, 2F and 2G) than their corresponding RSHs (560 μM, Figs. B, C and D) exert similar antioxidant actions.

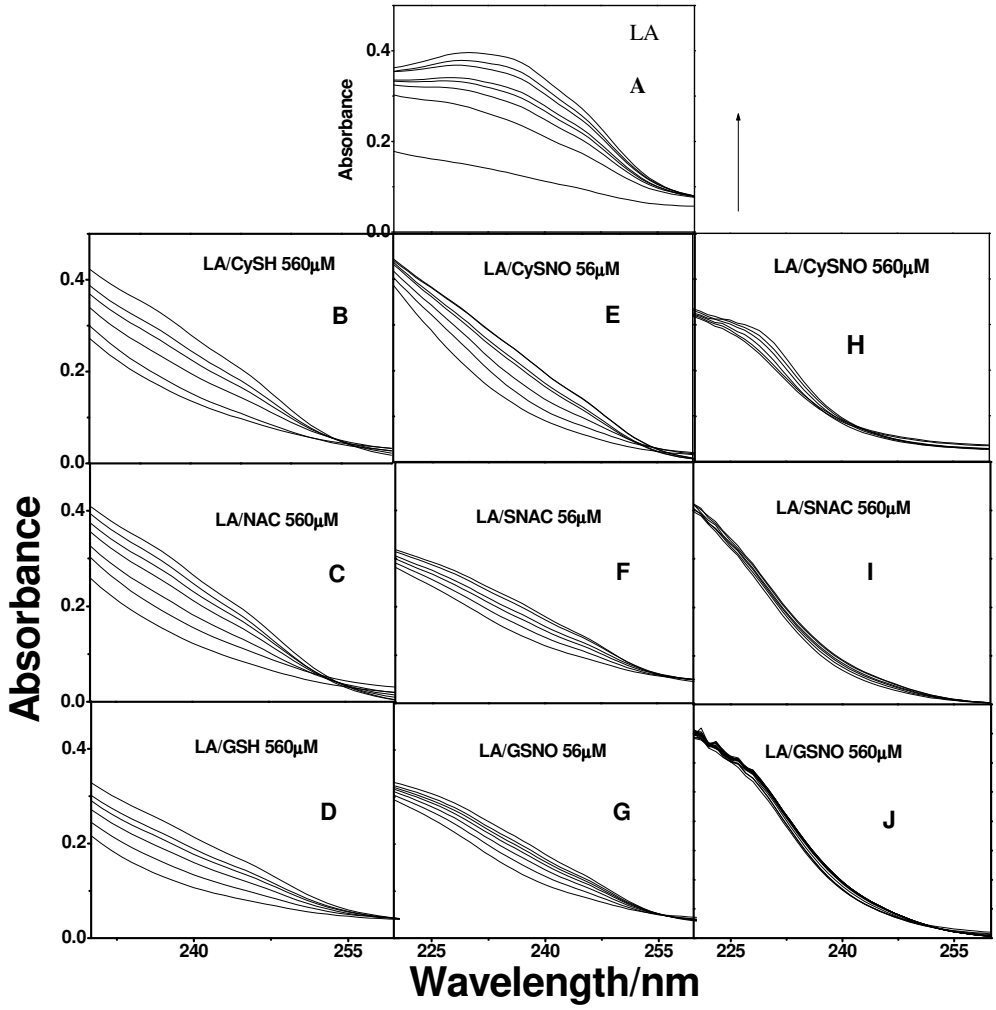


Fig. 2. (A) Spectral changes in the UV/Vis range during LA (19 μM) peroxidation; (B, C, D) LA in the presence of CyS, NAC and GSH (560 μM) respectively; (E, F, G) LA in the presence of CySNO, SNAC and GSNO (56 μM) respectively; (H, I, J) LA in the presence of CYSNO, SNAC and GSNO (560 μM) respectively. Absorbance changes were monitored at 37ºC for 80 s. For the sake of clarity, only six representative spectra from a total 40 s are shown. In all cases, LA peroxidation was catalyzed by SLO (56 nM).

Figure 3 shows the kinetic curves corresponding to the spectral changes of Fig. 2, monitored at 234 nm during the first 80 s of reaction in the presence of RSHs and RSNOs. In this time range, the curves reach an apparent plateau after ca. 20 s in all cases. The initial rates of reaction (I_r), as well as the height of the plateaus (H), are significantly decreased in the presence of RSHs and RSNOs (curves b, c and d), compared to the peroxidation of LA alone (curve a). It must be noted that the presence of RSHs at concentration 560 μM leads to a decrease in the height of the plateaus of about 1/2 of their values for LA alone, while in the presence of RSNOs at a concentration 10 times lower (56 μM), the height of the plateaus are decreased to ca ¼ of their values for LA alone. As the height of the plateaus can be taken as a measurement of the extent of the peroxidation reaction in its initial phase, this result indicates that RSNOs exert a much more extensive blockage of the peroxidation reaction than the corresponding RSHs in this time range. However, this blockage does not increase proportionally with the increase in RSNOs concentration from 56 to 560 μM, as can be seen when comparing curves c and d.

The kinetic parameters I_r and H were extracted from the curves of Fig. 3 at 560 μM (where peroxidation inhibition is higher) and are shown in the bar graphs of Figs. 4A and 4B, for comparison. It can be seen in Fig. 4A that the I_r of LA peroxidation (19 μM) is decreased to about ½ of the control value in the presence RSHs (560 μM), i.e. at a molar ratio RSH/LA = 29.5. This result is practically the same for the three RSHs used, indicating that there is no significant difference among the antioxidant actions of CySH, NAC and GSH when this kinetic parameter is analyzed in this condition. In contrast, the actions of RSNOs at the same concentration reduced I_r values to ca. 1/5 of the control value, but also without significant differences among the three RSNOs.

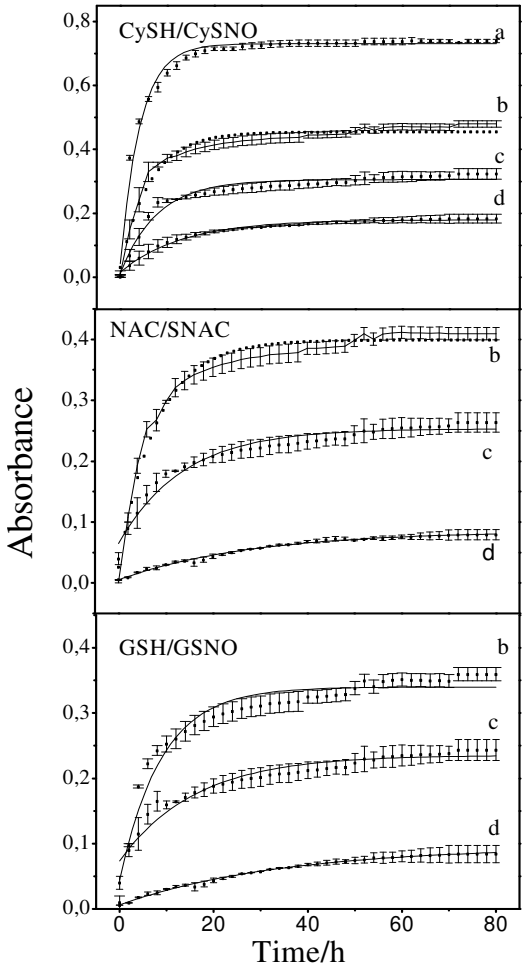


Fig. 3. Kinetic curves of LA (19 μM) peroxidation catalyzed by (SLO) (56 nM). (a) in the absence of RSH or RSNO; (b) in the presence of CySH, NAC or GSH 560 μM; (c), in the presence of CySNO, SNAC and GSNO 56 μM and (d) in the presence of and CySNO, SNAC and GSNO 560 μM. Absorbance changes were monitored at 234 nm at 37ºC.

An apparent trend in the antioxidant actions of the three RSHs is reflected in the comparison among the heights of the plateaus in Fig. 4 B, indicating that the antioxidant action of RSHs follows the order GSH > NAC ≈ CySH. Similarly, the heights of the plateaus for the three RSNOs indicate that SNAC and GSNO are more effective as antioxidants than CySNO.

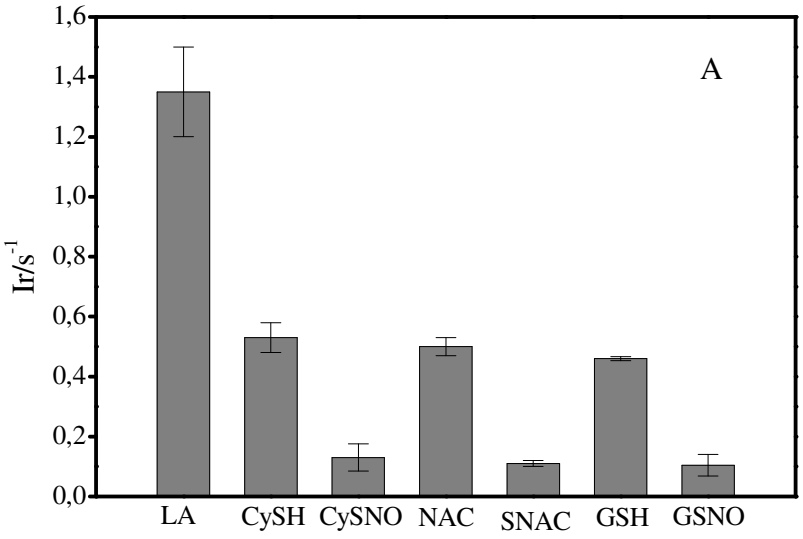


Fig. 4A.

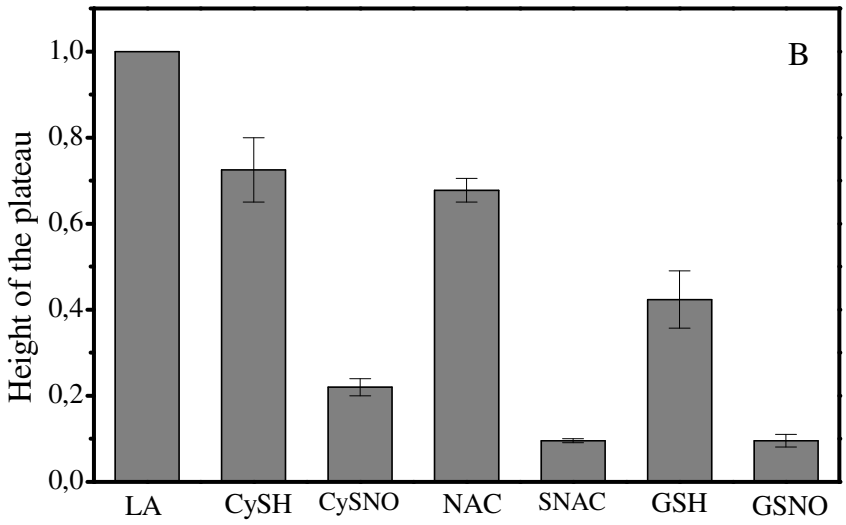


Fig. 4B.

Fig. 4. Initial rates (I_r) (A) and heights of the plateaus (H) (B) achieved after ca. 20s of LA peroxidation, catalyzed by SLO, in the absence and presence of RSHs and their corresponding RSNOs. [LA] = 19 μM; [SLO] = (56 nM); [RSHs] and [RSNOs] = 560 μM. The scale of Fig. (B) was normalized considering the maximum absorbance of LA oxidation in the absence of RSNOs = 1. Data extracted from the curves of Fig. 3.

Fluorimetric characterization and monitoring of oxidized LA-lysine adduct formation

Figure 5A shows the emission spectra obtained after LA oxidation catalyzed by Fe (II) ions for 2 h, followed by incubation of the solution with lysine for 48 h at 37ºC. The peroxidation reactions were performed in the absence (a) and presence of GSNO 5.0 μM (b) and 500.0 μM (c). The inset in Fig. 5A shows the emission spectrum obtained after the incubation of MDA with lysine in equimolar concentrations of 0.25 M, as a control experiment. Fig. 5B shows the kinetic curves of the fluorescent oxLA-lysine adduct formation in the reaction between oxidized LA and lysine in conditions (a), (b) and (c) of Fig. 5A. The curves were based on the spectral changes monitored at 430 nm, during 48 h after lysine addition. It can be observed that the formation of the oxLA-Lys adduct follows a sigmoid pattern with an apparent induction or "lag" phase, which is evident in curves (a) and (b). In curve (c), the reaction presents a lag phase until 48 h, although a small rate of fluorophore formation can be detected since the beginning of the reaction.

Detection of $^{\bullet}$NO released from nitrogen-containing products of oxidized LA

Figure 6 shows the of light emission peaks obtained in the chemiluminescence reaction of free $^{\bullet}$NO, released from nitrogen-containing products of oxLA, formed in the reaction between oxLA and GSNO. The two peaks shown were obtained after reduction of nitrogen-containing products of oxLA by ascorbate, according to the procedures described above. Peak (a) was obtained in the reduction of a sample of LA-SDS dispersion oxidized in the presence of Cu (II) ions and GSNO.

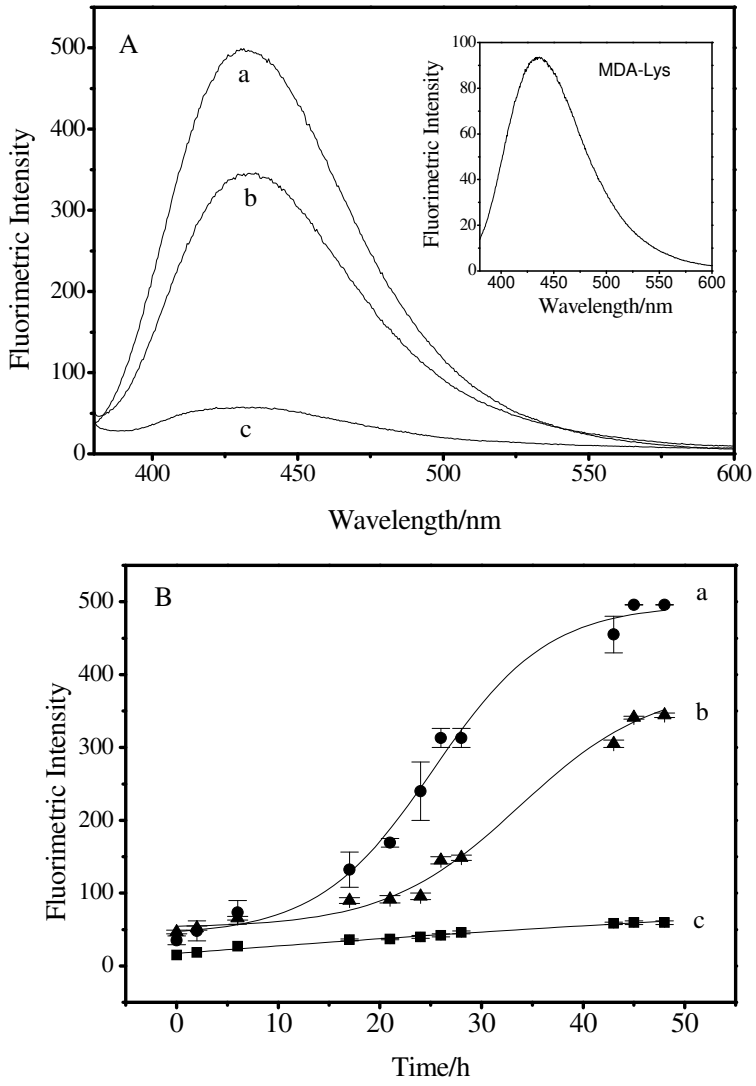


Fig. 5. A- Final emission spectra obtained after linoleic acid (LA) oxidation (final concentration 1.2 mM) catalyzed by Fe^{2+} ions ($FeSO_4$, final concentration 5.0 μM) for 2 h, followed by incubation of the solution with lysine (Lys) (final concentration 1.0 mM) for 48 h at 37ºC in the absence (a) and presence of GSNO 5.0 μM (b) and 500.0 μM (c). Excitation/emission wavelengths 360/430 nm. Inset: Emission spectra of MDA incubated with Lys in equimolar concentrations of 0.25M, used as a control. B- Kinetic curves of fluorescent oxidized LA- Lys adduct formation during the reaction between oxidized LA and Lys in the conditions (a), (b) and (c) of Fig. 5A, based on the spectral changes monitored at 430 nm during 48 h, after Lys addition.

The same results were observed when SNAC or CySNO were used in the place of GSNO (data not shown). Peak (b) was obtained in the reduction of a sample of LA-SDS dispersion incubated with GSNO, where pure LA had been previously oxidized by heating under O_2 at 80°C. The detection of free $^{\bullet}NO$ in these cases shows that nitrogen-containing products of oxLA are formed both when LA is oxidized in aqueous dispersion in the presence of GSNO and when a dispersion of oxLA-SDS is subsequently incubated with GSNO. Control curves are for the measurements of samples of water incubated with GSNO without LA-SDS and Cu (II) (control 1), and of water incubated with GSNO and Cu (II) without LA-SDS (control 2), which show that GSNO was completely eliminated through decomposition by Hg (II) ions, followed by NO_2^- trapping by sulfanilamide, before injection in the ascorbic acid solution. These results suggest that RSNOs may react with both $LO^{\bullet}$ and $LOO^{\bullet}$ radicals during radical propagation reactions and with LOOH previously formed in LA oxidation. Formation of LOOH in the LA oxidized by heating under O_2 was proven by observing the appearance of an IR absorption band with maximum around 1178 cm^{-1}, assigned to the C-O-O vibration of hydroperoxides (LOOH) [36] (see supplementary data).

Reaction between RSNOs and tert-butyl hydroperoxide

Figure 7 shows the kinetic curves corresponding to the spectral changes due the disappearance of the RSNOs CySNO, SNAC and GSNO during their reaction with *tert*-butyl hydroperoxide (tBOOH) with formation of *tert-butyl* peroxynitrites (tBOONOs).

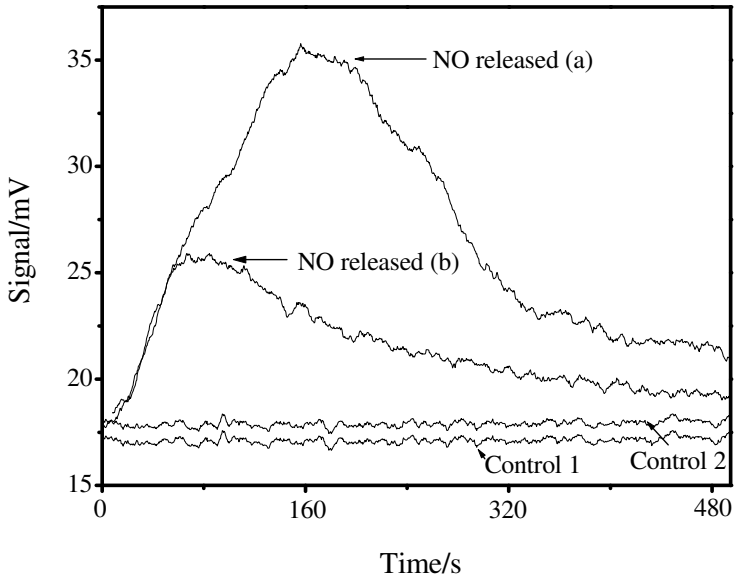


Fig. 6. Light emission peaks obtained in the chemiluminescence reaction of free NO, released from nitrogen-containing products of oxLA formed in the peroxidation reaction of LA in the presence of GSNO, with ozone. The two peaks shown were obtained after reduction of nitrogen-containing products of oxLA by ascorbate. For details see experimental part. Peak (a) was obtained in the incubation of LA with Cu (II) ions in the presence of GSNO. Peak (b) was obtained after the incubation of LA with GSNO without the addition of Cu (II) ions. Final concentrations of LA, GSNO and Cu (II) were 900 μM.

Control curves correspond to the monitoring of RSNOs solutions at the same temperature and pH conditions, but in the absence of tBOOH. It can be seen that the RSNOs solutions are quite stable in the absence of tBOOH and that their thermal decompositions are negligible in this time range. On the other hand, the presence of tBOOH leads to the fast disappearance of the absorption bands of the three RSNOs, indicating that they react with tBOOH. The rates of reaction of SNAC and GSNO are very similar and follow pseudo-first order kinetics. However, CySNO shows a different kinetic pattern, with an apparent bimodal

behavior. In this case, the rate of reaction is lower and approximately constant up to ca. 3 min and increases after this time and become similar to the rates observed in the last 4 min for SNAC and GSNO.

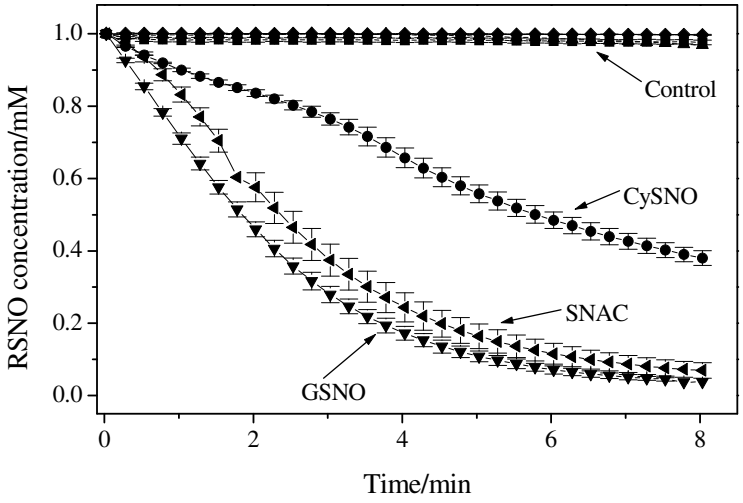


Fig. 7. Kinetic curves corresponding to the spectral changes of CySNO, SNAC and GSNO (initial concentration 1 mM) solutions in the presence and absence of tBOOH (final concentration 25 mM), monitored at 336 nm for 8 min, at 37ºC. Control curves correspond to the thermal decomposition of RSNOs solutions in the same temperature and pH conditions but in the absence of tBOOH.

Discussion

In the lipid pool of plasma and cells, polyunsaturated fatty acids (PUFAs) have higher propensity to oxidation due to the fact that bis-allylic methylene hydrogens are more susceptible to hydrogen abstraction by oxidizing radicals than are the methylene hydrogens from fully saturated lipids [35]. After such initiation process, the rapid reaction between the formed carbon-centered radical and dioxygen (O_2) forms a lipid peroxyl radical ($LOO^{\bullet}$). Propagation occurs via the reaction between $LOO^{\bullet}$ and intact fatty acid (LH) molecules forming lipid hydroperoxides (LOOH), leading to the formation of more $LOO^{\bullet}$ species through the decomposition of LOOH catalyzed by Cu (II) or Fe (III) ions either free or in the form of heme proteins [37-39].

Linoleic acid (LA), one of the components of LDL particles, is a major unsatured fatty acid in the American diet and is considered to be atherogenic because of its pro-oxidative and pro-inflammatory response by activation of endothelial cells [40,41]. An increase in LA levels has been reported in the phospholipid fractions of human coronary arteries in cases of sudden cardiac death due to ischemic heart disease [42]. Additionally, concentrations of LA in adipose tissue were positively correlated with the degree of coronary disease [43]. Linoleic acid has the double bond configuration with bis-allylic methylene hydrogens. For this reason, and for the reasons mentioned above, LA is an appropriate model compound for LPO studies. The monitoring of conjugated double bond formation in LA-SDS comicelles catalyzed by SLO (Fig. 2) shows that LA is effectively oxidized in aqueous dispersion by dissolved O_2. It must be considered that, in this particular condition, SLO is also an appropriate catalyzer as a member of a well known group of enzymes able to induce enzymatic peroxidation of polyunsaturated fatty acids in biological membranes and lipoproteins [44,45]. In general, such

enzymes contain an essential iron atom, which is present as Fe^{2+} in the inactive enzyme form; enzymatic activation occurs through hydroperoxide-driven oxidation of Fe^{2+} to Fe^{3+}. From the analysis of the LPO model used in the present work, the antioxidant actions of the RSHs and primary RSNOs used emerge clearly from the summary of kinetic parameters shown in Fig. 4. The RSNOs used here cannot be considered classical antioxidants like α-tocopherol (α-TOH) or ascorbic acid. To understand the main difference between RSNOs and classical antioxidants, it must be remembered that α-TOH react with $LOO^{\bullet}$ forming a tocopheroxyl radical (α-$TO^{\bullet}$) and also that α-$TO^{\bullet}$ can scavenge another $LOO^{\bullet}$, allowing two $LOO^{\bullet}$ to be scavenged by one α-TOH. The primary product of this reaction is LOOH, accumulation of which exposes lipids to subsequent oxidation mediated by metal ions [39]. A similar process can be described for other classical antioxidants found in cells like ascorbic acid and glutathione (GSH). Both are highly susceptible to hydrogen abstraction, generating other radicals (ascorbyl and thyil) and both lead to LOOH formation in their primary reactions. In the case of GSH, the fate of the glutathionyl radical ($GS^{\bullet}$) formed is dimerization, forming oxidized glutathione (GS-SG), the ration GSH/GS-SG being a well-known marker of oxidative stress [46]. The situation becomes different when RSNOs are the antioxidant species. As $^{\bullet}NO$ donors, their actions are primarily linked to the well-known antioxidant action of $^{\bullet}NO$ as a radical-chain terminator, which arises from the fact that $^{\bullet}NO$ is itself a free radical. Like its reaction with superoxide ($O_2^{\bullet-}$) generating peroxynitrite ($OONO^-$, $k = 6.7 \times 10^9\ M^{-1}s^{-1}$) [47] free $^{\bullet}NO$ may reacts extremely rapid with $LOO^{\bullet}$ ($k = 2 \times 10^9\ M^{-1}s^{-1}$) [48], removing this chain carrying radical from the reaction scene. The radical chain terminating products of this reaction may include nitrogen-containing products of oxLA such as LONO and LOONO which can rearrange to form $L(O)NO_2$ species [47]. Although formation of $OONO^-$ is usually associated with a pro-oxidant response reflected in the nitration of tyrosine residues [48,39], the deleterious actions of $OONO^-$ have been shown to depend strongly on

the balance between $^{\bullet}NO$ and $O_2^{\bullet-}$ [39]. More generally, the balance between oxidant species and $^{\bullet}NO$ seems to be fundamental in allowing a protective action of $^{\bullet}NO$ against LPO. Hummel et al. [18] for example, have shown that quite low levels of $^{\bullet}NO$ (> 50 nM) are enough to suppress Fe^{2+}-O_2 lipid oxidation (Fe^{2+} = 20 μM) in in vitro cell models. However, either in the extracellular environment or inside the cell membranes, O_2 is found in much higher concentration (10 – 100 X more) than $^{\bullet}NO$ [39] and in aqueous media the reaction between $^{\bullet}NO$ and O_2 leads to NO_2^-:

$$4\,^{\bullet}NO + O_2 + 2\,H_2O \rightarrow 4\,H^+ + 4\,NO_2^- \quad (1)$$

which cannot be considered an efficient radical scavenger at physiological pH. It is thus unlikely that free $^{\bullet}NO$ can efficiently compete with O_2 for the reaction with alkyl radicals ($R^{\bullet}$) to avoid the formation of peroxyl radicals ($LOO^{\bullet}$):

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (2)$$

Similarly, it is unlikely that free $^{\bullet}NO$ is the only $^{\bullet}NO$ species which reacts directly with $LOO^{\bullet}$ radicals in vivo, leading to their inactivation as LOONO:

$$^{\bullet}NO + LOO^{\bullet} \rightarrow LOONO \quad (3)$$

Although reaction 3 cannot be ruled out and is probably operative to some extent in vivo, RSNOs are more likely to be involved in the antioxidant actions of endogenous $^{\bullet}NO$, through their direct reaction with $LOO^{\bullet}/LO^{\bullet}$ species leading to the same nitrated products (Eq. 4), than free $^{\bullet}NO$.

$$2RSNO + LO^{\bullet}/LOO^{\bullet} \rightarrow LONO/LOONO + RS^{\bullet} \quad (4)$$

The fate of the thiyl radicals ($RS^{\bullet}$) formed in reaction 4 is dimerization through the formation of a thermodynamically stable disulphide (S-S) bond. This bimolecular dimerization reaction can arise from the encounter between two thiyl radicals or between one thiyl radical and an intact RSNO molecule [28,49] (Eqs. 5 and 6).

$$RS^{\bullet} + RS^{\bullet} \rightarrow RS\text{-}SR \quad (5)$$

$$RS^{\bullet} + RSNO \rightarrow RS\text{-}SR + {}^{\bullet}NO \quad (6)$$

Although in vitro aqueous RSNOs solutions may spontaneous release free $^{\bullet}NO$ through the homolytic cleavage of the S-N bond, the chemical stability of RSNOs solutions at low concentrations is high enough to allow these compounds to react directly with other substrates in a bimolecular mechanism. One of the most important reactions of this type is the transnitrosation reaction with the thiol group of proteins, leading to post-tranlational modification of protein function [50]:

$$R\text{-}SNO + R'\text{-}SH \rightarrow R'\text{-}SNO + RSH \quad (7)$$

Similarly, RSNOs may react directly with alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals formed after H abstraction in LPO reactions. In the case of LA peroxidation, the primary reaction of RSNOs with $LO^{\bullet}$ or $LOO^{\bullet}$ species will lead to the formation of LOONO or LONO species and not to LOOH or LOH species, as in the case of hydrogen abstraction from classical antioxidants. Although LOONO species may be able to further release $^{\bullet}NO$, they are not susceptible to subsequent oxidations regenerating $LOO^{\bullet}$ or $LO^{\bullet}$, which propagate LPO highlighting the particular radical propagating blockage obtained with RSNOs.

As a complementary analysis of the protective action of RSNOs in LPO, one may also consider that linoleate hydroperoxide (LOOH) formed as a primary oxidation product of LA

is expected to undergo β-scission generating free aldehydes. It is generally assumed that adducts formed in the conjugation of free aldehydes generated during peroxidation of PUFAs with amino groups on LDL particles are proteins with Schiff bases (containing the -C=N- group). Formation of such adducts is central in the atherosclerotic process once it neutralizes the cluster of positive charges on the surface of LDL particles, conferring to them a higher anionic electrophoretic mobility and a reduced recognition by the LDL receptor on fibroblasts, while increasing their recognition by macrophages [51]. As Schiff bases have fluorescence properties [52,53], it was assumed in this work that such adducts could be used to characterize the formation of aldehydes from hydroperoxides in the peroxidation of LA. It was found here that the fluorescence emission spectra obtained after LA oxidation catalyzed by Fe^{2+} ions followed by incubation of the solution with Lys has exactly the same shape and position as the spectrum obtained in the incubation of MDA with Lys. This result shows that products of LA peroxidation are also reactive toward lysine, forming the same fluorescent Schiff base adduct formed in oxidized LDL (like the MDA-lysine adduct). The fluorescent adduct identified in this work was assigned to the reaction between the aldehydes formed from the reduction and β-scission of LOOH, with lysine (Scheme 1). More specifically, the reaction involves the nucleophilic addition between the amino group of lysine and the carbonyl group of the aldehydes, forming hemiaminals, followed by dehydration to generate stable imines. In Scheme 1, these reactions are represented for the two possible aldehydic fragments of LOOH β-scission: 12-oxododecanoic acid and hexanal. Of course, in different oxidative situations several other aldehydic products may form after LA peroxidation, which may also generate fluorescent adducts with lysine. In addition to the aldehyde-type lysine adducts, amide-type-lysine adducts have also been described by Kawai et al. [54,55] as a new class of protein adducts derived from lipid peroxidation.

The kinetic curves of Fig. 5 B show that when the peroxidation reactions are performed in presence of GSNO 5.0 μM (b) and 500.0 μM (c) the rate of formation of the aldehyde-type lysine adducts is greatly reduced, with a substantial increase in the observed apparent lag phase of this reaction. This result is in accordance with a reduced amount of aldehydes formed in the presence of GSNO, confirming the concentration-dependent protective action of GSNO on LA peroxidation, described above.

The formation of nitrogen-containing products of oxLA during the peroxidation of LA in the presence of RSNOs was demonstrated in the present work by reducing these products to free $^{\bullet}NO$ and hydroperoxides with ascorbic acid, according to procedures already described in other works [12]. The reaction involved can be written as:

$$2LOONO/LONO + AscH^{-} + H^{+} \rightarrow 2LOOH/LOH + DHA + 2^{\bullet}NO \quad (8)$$

where $AscH^{-}$ is the ascorbate anion and DHA is dehydroascorbic acid formed in the oxidation (hydrogen abstraction) of $AscH^{-}$. Free $^{\bullet}NO$ released in this reaction was unequivocally detected by chemiluminescence, after its removal from the solution by bubbling with N_2 and its reaction with ozone (O_3). It must be emphasized here that to avoid any possible interference of $^{\bullet}NO$ released from excess GSNO, instead of $^{\bullet}NO$ released from nitrated LA, excess GSNO (that did not reacted with $LOO^{\bullet}$) was completely eliminated from the solution by addition of Hg (II) ions and sulfanilamide. It is know that $^{\bullet}NO$ is quantitatively liberated from $GSNO^{\bullet}$ by mercuric catalysis, and it is rapidly and quantitatively converted to its stable solution end-product, nitrite (NO_2^{-}) in aerated medium. By adding sulfanilamide to the solution, NO_2^{-} formed is completely removed, and therefore the $^{\bullet}NO$ signal detected in this analysis can be attributed solely to $^{\bullet}NO$ released from nitrogen-containing products of oxLA. This conclusion is supported by the control experiments, which confirmed that GSNO is

completely eliminated by the mercuric catalysis/sulfanilamide procedure. In addition, it was observed that RSNOs react with LA previously oxidized through heating, once incubation of oxLA-SDC comicelles with GSNOs, led also to the formation of nitrogen-containing products of oxLA, detected by their reduction to •NO with ascorbate. This result points to the ability of RSNOs to inactivate preformed LA hydroperoxides (LOOH). In this case, the bimolecular reaction is expected to proceed via a transnitrosation mechanism similar to reaction 7, and can be written as:

$$LOOH + RSNO \rightarrow LOONO + RSH \quad (9)$$

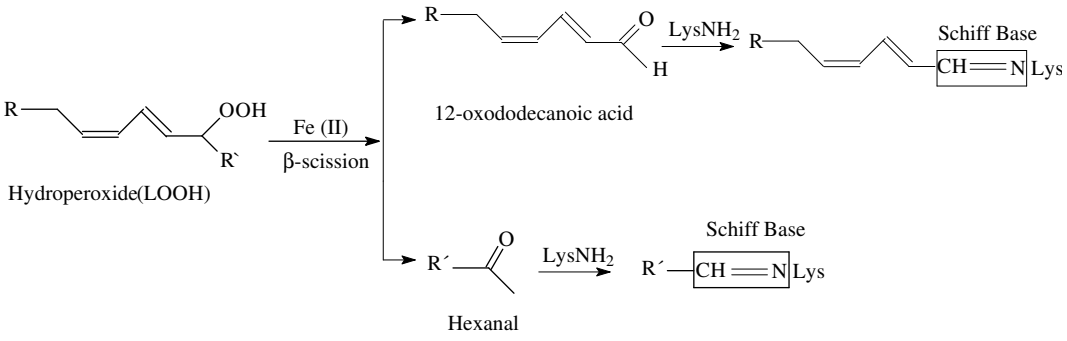
Additional evidence for the occurrence of this kind of reaction was obtained in the incubation of CySNO, SNAC and GSNO with tBOOH, used here as a model hydroperoxide. This reaction, which must lead to the formation of alkyl-peroxynitrite, can be written for *tert*-butyl-peroxinitrite (tBOONO) as:

$$tBOOH + RSNO \rightarrow tBOONO + RSH \quad (10)$$

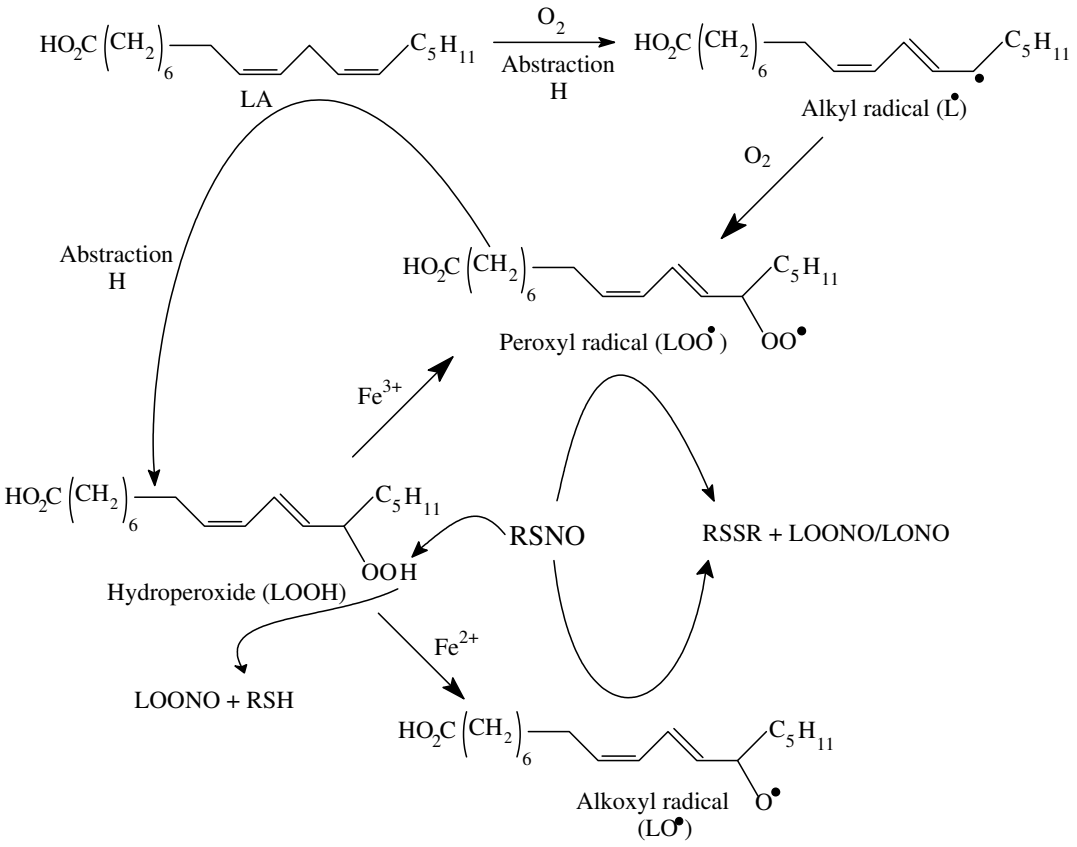
The fast reaction of these three primary RSNOs with tBOOH (Fig. 7) along with the detection of free •NO released in the reduction of oxLA incubated with RSNOs, shows that RSNOs can also effectively inactivate preformed alkyl hydroperoxides.

This ability of primary RSNOs to block the propagation of lipid peroxidation reaction not only by inactivating LO• or LOO• radicals but also by inactivating preformed LOOH, reinforces the potential role of endogenous RSNOs as modulators of peroxidation reactions in vivo. The relevance of this evidence can be better appreciated by considering that the reduction of LOOH to LO• radical by metal ions as Fe (II) or Cu (II) represents an important via for the propagation of radical reactions in the lipid peroxidation process. Thus, if the role

of endogenous •NO as an antioxidant species is linked, in vivo, to its presence in primary RSNOs, this role must be extended beyond its classical radical chain termination action, to encompass the inactivation of deleterious hydroperoxides also present in the cellular milieu. These multiple protective actions of RSNOs are summarized in Scheme 2 for the case of LA peroxidation and can be generalized for other lipids. These results raise the possibility that primary RSNOs are involved in the formation of nitrogen-containing lipids (and perhaps of nitroalkanes), which may all be natural membrane components and may have biological activities intimately linked with the biological activities of RSNOs.



Scheme 1. Formation of lipid fluorescent oxidized LA-lysine adducts.



Scheme 2. Key sites of primary S-nitrosothiols action on pathways of linoleic acid peroxidation.

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List of Abbreviations

Alkoxyl radical ($LO^{\bullet}$), ascorbate anion ($AscH^{-}$), cysteine (CyS), cyclic guanosine monophosphate (cGMP), dehydroascorbic acid (DHA), glutathione (GSH), hydrochloric acid (HCl), LA hydroperoxides (LOOH), 13-hydroperoxy-octadecadienoic acid (13-HPODE), linoleic acid (LA), linoleic acid-SDS comicelles (LA-SDS), lipid peroxidation (LPO), L-lysine monohydrochloride (Lys), malonaldehyde bis(dimethyl acetal) (MDA), N-acetyl-L-cysteine (NAC), nitrate lipids (LOONO/LONO), nitrite (NO_2^{-}), oxidized linoleic acid (oxLA), oxidized linoleic acid-SDS comicelles (oxLA-SDC), oxidized LA- lysine adduct (oxLA-Lys), peroxyl radical ($LOO^{\bullet}$), phosphate buffer saline (PBS), polyunsaturated fatty acids (PUFAs), S-nitrosocysteine (CySNO), S-nitroso-N-acetylcysteine (SNAC), S-nitrosoglutathione (GSNO), S-nitrosothiols (RSNOs), sodium dodecil sulfate (SDS), soybean lipoxygenase (SLO), superoxide ($O_2^{\bullet-}$), peroxynitrite ($OONO^{-}$), *tert*-butyl hydroperoxide (tBOOH), *tert-butyl* peroxynitrites (tBOONOs), thiyl radicals ($RS^{\bullet}$), thiol (RSH).

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Supplementary Material

Experimental Procedure

Infrared characterization of linoleic acid peroxidation

Linoleic acid peroxidation was induced by heating a sample of pure LA at 80ºC for 4 h under stirring in a glass flask with O_2 atmosphere, obtained by continuously blowing O_2 from a cylinder into the headspace of the flask. Aliquots of LA were removed from the reaction flask 2 and 4 h after the beginning of the peroxidation reaction. Capillary films of non-oxidized and peroxidized LA were obtained between two calcium fluoride (CaF_2) windows, which were mounted in sample holder. IR spectra were obtained in the range 4000 – 1000 cm^{-1} using an FTIR Bomem MB-series, model B-100. An IR spectrum of non-oxidized LA was obtained as a control.

Results and discussion

Figure 1 shows the spectral change associated with the heating of pure LA at 80ºC under O_2 atmosphere. The appearance of the absorption band with maximum at ca. 1180 cm^{-1} can be assigned to the C-O-O vibration of hydroperoxides (LOOH) [36]. This result reinforces the proposal that oxidized LA reacts with RSNOs through the transnitrosation between -COOH and –SNO moieties, leading to the formation of LOONO products, and that these are the products that are reduced to free NO (detected by chemiluminescence) with ascorbic acid.

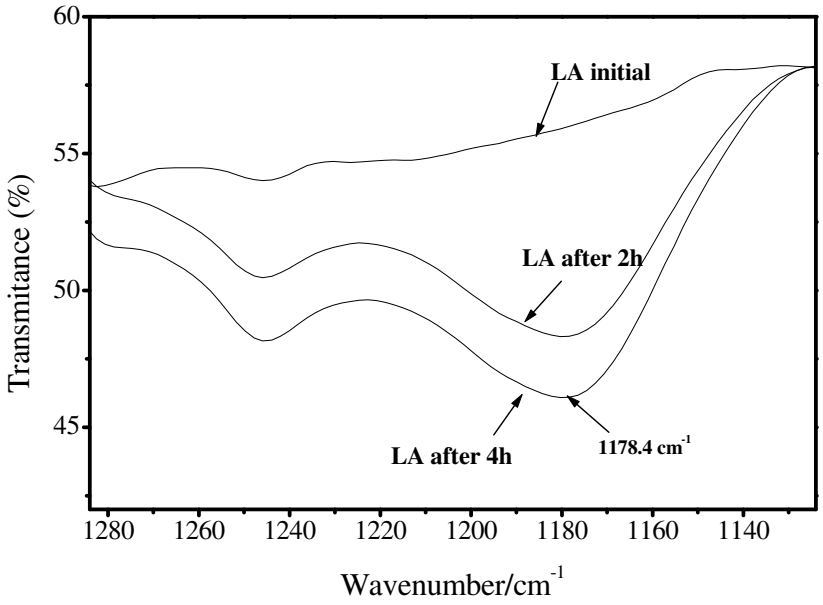

Fig 1. Spectral changes obtained in the infrared spectrum of linoleic acid after its oxidation at 80ºC under O_2 atmosphere for 2 and 4 h.

3.2. Material suplementar

Nos resultados suplementares abaixo são mostradas as variações espectrais (Fig. 1A) e as curvas cinéticas correspondentes (Fig. 1B), representativas, para a oxidação do ácido linoleico (AL) catalisada por íons Cu(II). Para obter estes dados a oxidação do AL foi induzida através da adição de $CuSO_4$. As dispersões aquosas de AL (concentração final 75 μM) foram preparadas em solução de SDS (0,01 M), sendo que a dispersão foi transferida para uma cubeta de quartzo submetida a um fluxo com O_2 por 2 min e a solução de $CuSO_4$ (concentração final 15 μM) foi adicionada à cubeta com uma seringa para começar a reação de peroxidação. Um espectrofotômetro Hewlett Packard, modelo 8453 (Palo Alto, CA, USA) foi utilizado para monitorar a variação espectral na faixa de 200-600 nm no escuro a 37ºC, em intervalos de tempo de 3 s. Os espectros das soluções foram obtidos em cubeta de quartzo de 1 cm contra o ar, sob agitação de 1000r/min. Cada ponto nas curvas cinéticas de absorbância *vs.* Tempo é a média de dois experimentos com barras de erros expressadas pelos seus erros padrões da média (SEM).

Pode-se observar nestes resultados que a peroxidação do AL pode ser catalisada também por íons Cu(II), levando a formação de hidroperóxidos (LOOH) que são caracterizados pela absorção em 234 nm, conforme discutido no manuscrito submetido ao periódico *Free radical biology and medicine*. O esquema 2 deste manuscrito mostra a formação dos hidroperóxidos em questão.

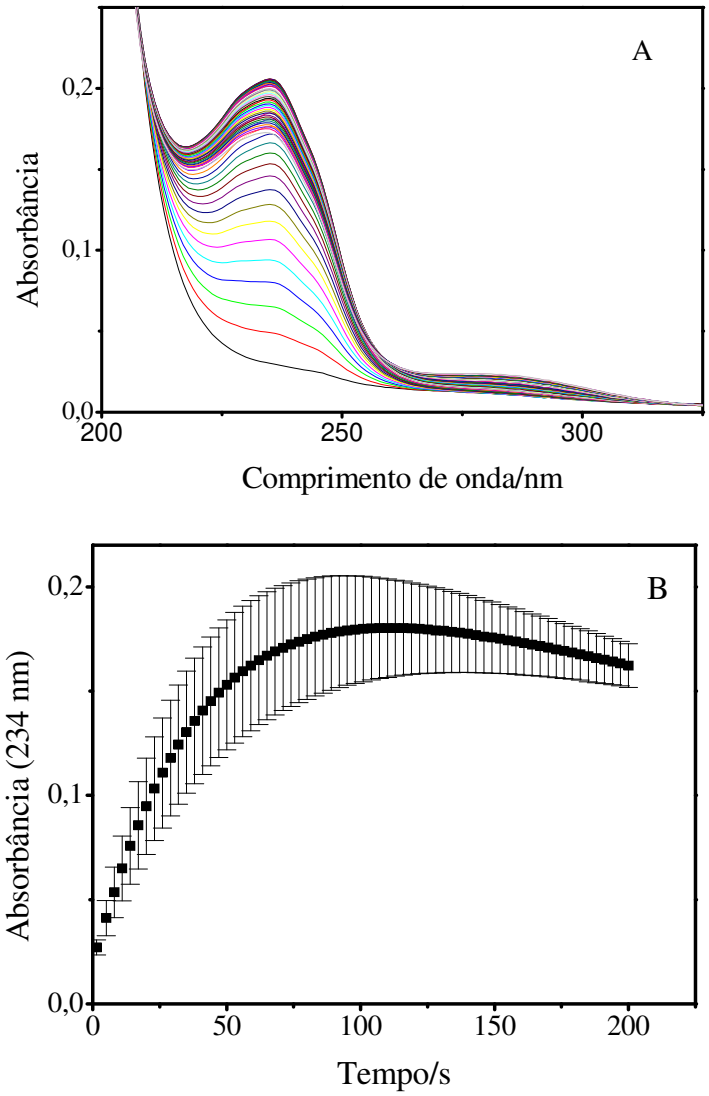

Fig. 1. (A) Variação espectral no UV/Vis durante a oxidação de comicelas de LA em SDS (LA, concentração final 75 μM: SDS, concentração final 0,01 M) catalisada por íons Cu(II) ($CuSO_4$, concentração final 15 μM). (B) Curva cinética referente à absorção em 234 nm pela Fig. 1A.

Os resultados suplementares mostrados nas Figs. 2 e 3 mostram as variações espectrais referentes à Fig. 5A do manuscrito submetido ao periódico *Free radical biology and medicine*. Nesta figura, consta somente o espectro final de cada experimento, razão pela qual não será descrita detalhadamente a parte experimental deste material suplementar.

A variação espectral na peroxidação do AL catalisada por íons Fe(II) por 2h, seguida pela incubação com solução de lisina por 48 h a 37ºC, na ausência da solução de S-nitrosoglutationa (GSNO) é mostrada na Fig. 2. Além disso, são mostradas as variações espectrais da peroxidação do AL na presença de GSNO 5,0 μM (Fig. 3A) e na presença de GSNO 500,0 μM (Fig. 3B).

A discussão destes resultados se encontra no manuscrito submetido ao periódico *Free radical biology and medicine*.

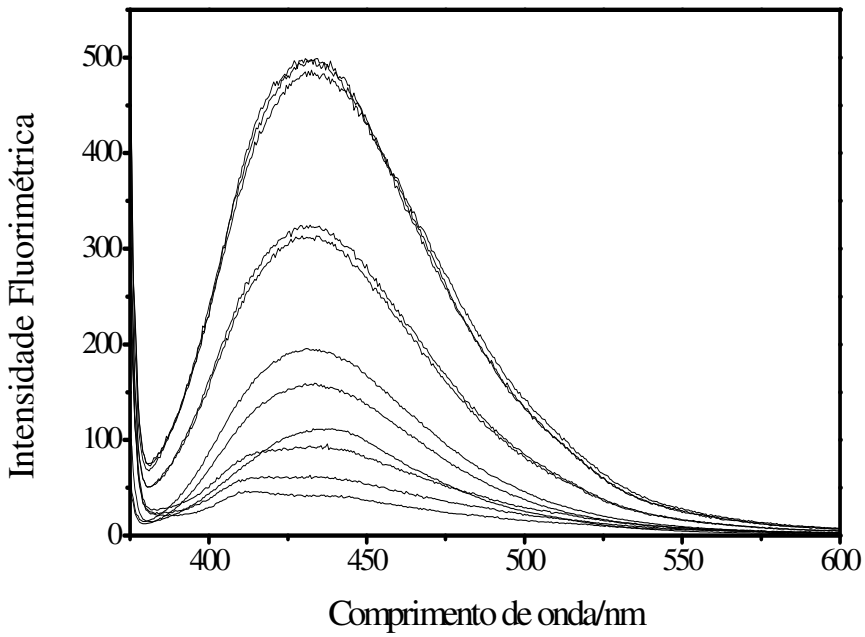

Fig. 2. Espectros de emissão obtidos depois da oxidação do AL catalisado por íons Fe (II) ($FeSO_4$, concentração final 5,0 μM) por 2h, seguida pela incubação com solução de lisina (concentração final 1.0 mM) por 48h a 37ºC. Comprimentos de onda de excitação/emissão 360/430 nm.

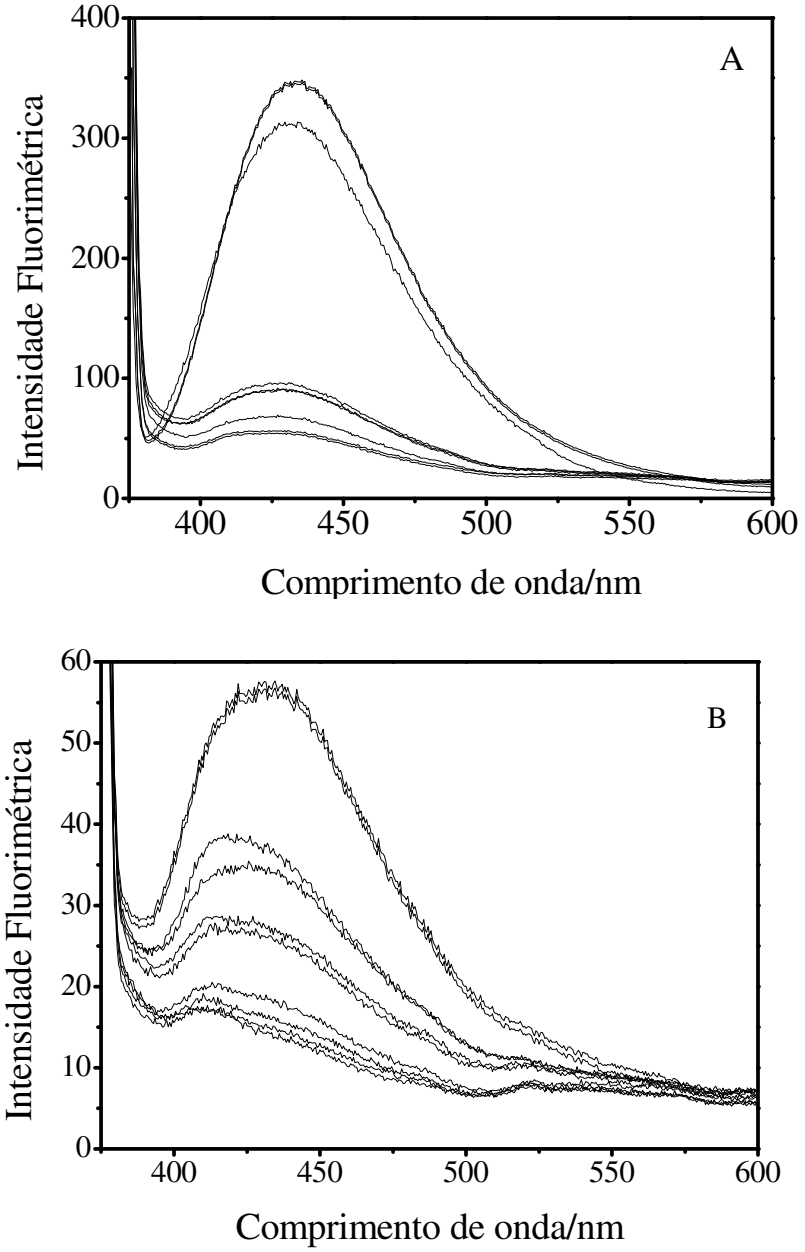

Fig. 3. Espectros de emissão obtidos após a oxidação do ácido linoleico (AL) catalisada por íons Fé (II) ($FeSO_4$, concentração final 5,0 μM) por 2h, seguida pela incubação com solução de lisina (concentração final 1,0 mM) por 48 h a 37ºC. (A) oxidação na presença de GSNO 5,0 μM. (B) oxidação na presença de GSNO 500,0 μM. Comprimentos de onda de excitação/emissão 360/430 nm.

Os resultados suplementares mostrados nas Figs. 4A e 4B mostram resultados semelhantes aos da Fig. 6 do manuscrito submetido ao periódico *Free radical biology and medicine*. A figura do maunuscrito se refere à utilização da S-nitrosoglutationa (GSNO) como antioxidante. As Figs. 4A e 4B mostram resultados semelhantes obtidos com a S-nitrosocisteína (CISNO) e a S-nitrosoacetilcisteína (SNAC) no lugar da GSNO, respectivamente. A parte experimental destes experimentos é análoga à descrita no manuscrito.

Pode-se observar nestes resultados que CISNO e SNAC também levam à formação de produtos nitrogenados do ácido linoleico (AL) oxidado. A discussão detalhada destes dados se encontra no manuscrito acima.

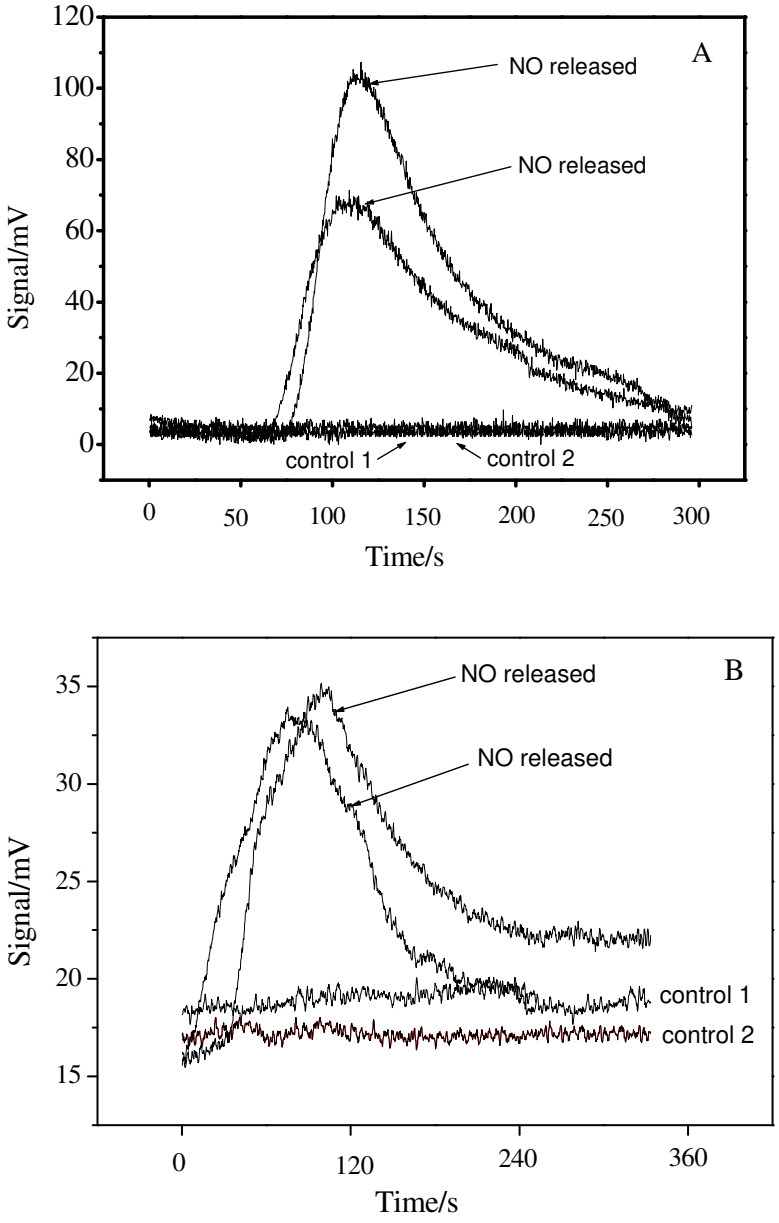

Fig. 4. Picos de emissão de luz obtidos na reação quimiluminescente de NO livre, liberado pelos produtos nitrogenados do ácido linoleico (AL) oxidado na presença de CISNO (A) e SNAC (B), com ozônio. Os dois picos mostrados em cada figura foram obtidos depois da redução de produtos nitrogenados do AL oxidado pelo ascorbato. Sinais de NO obtidos na incubação de AL com íons Cu(II) na presença de CISNO (A) e SNAC (B).

4. Inibição da peroxidação da lipoproteína de baixa densidade (LDL) *in vitro* pelos S-nitrosotióis primários

Durante o período de doutorado foi realizado um estágio por dois meses na Faculdade de Ciências Exatas e Naturais pela Universidade de Buenos Aires (UBA) sob coordenação do Professor Roberto Etchenique. Neste período foram realizados experimentos para detecção eletroquímica de óxido nítrico (NO) liberado depois da peroxidação da lipoproteína de baixa densidade (LDL) utilizando o sensor amiNO-700 para a detecção e S-nitrosoglutationa (GSNO) e S-nitroso-N-acetilcisteína (SNAC) para a inibição da peroxidação lipídica. Estes dados foram importantes para compreender o mecanismo de inibição da peroxidação da LDL. No manuscrito que está em fase de finalização para ser submetido ao periódico "*Chemistry and Physics of Lipids"* descreve detalhadamente os experimentos e a discussão sobre a peroxidação da LDL catalisada por íons Cu (II) e a inibição da mesma por S-nitrosotióis primários.

Simplicio, F. I.; Etchenique, R.; de Oliveira, M. G. **In vitro inhibition of low density lipoprotein peroxidation by primary S-nitrosothiols**. Manuscrito em preparação para ser enviado para *Chemistry and Physics of Lipids*.

In vitro inhibition of low density lipoprotein peroxidation by primary S-nitrosothiols

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Running title: S-nitrosothiols inhibition of LDL peroxidation

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Abstract

S-nitrosothiols (RSNOs) can act as nitric oxide (NO) donors exerting effective action as chain-breaking antioxidants in free radical-mediated lipid peroxidation. The aim of this work was to evaluate the consumption of NO from the primary RSNOs *S*-nitroso-*N*-acetylcysteine (SNAC) and S-nitrosoglutathione (GSNO) during the peroxidation of low density lipoprotein (LDL) *in vitro*. Lipid peroxidation of LDL emulsions was induced by cooper (II) ions in the absence and presence of SNAC and GSNO in solution. The amount of free NO released in the Cu(II)-mediated RSNOs decomposition was used as a measure of the RSNOs consumed in the peroxidation reaction. Free NO was quantified by using a selective NO electrode immersed in the reaction medium. It was observed that the amount of free NO released from GSNO and SNAC is reduced to c.a. 0.6 and 0.25, respectively, in the presence of LDL with two different conditions, compared to the NO release under the same conditions in the absence of LDL. These results indicate that RSNOs are consumed by free radicals generated in LDL peroxidation. Thus, primary RSNOs might act directly as antioxidants protecting LDL from oxidative damage *in vitro.*

Key Words: Nitric oxide S-nitrosothiols, LDL, lipid peroxidation, atherosclerosis

Introduction

Atherosclerosis may be viewed as an inflammatory disease linked to an abnormality in oxidant-mediated signals in the vasculature (Kunsch and Medford, 1999) and oxidation of low-density lipoprotein (LDL) has been implicated in the early stages of atherosclerotic lesion formation (Hogg, 1993). The LDL particle is surrounded by a molecule of apolipoprotein B (apo B-100) with a monolayer of phospholipids and unesterified cholesterol. The hydrophobic core of the particle contains cholesteryl esters and triglycerides with polyunsaturated fatty acids, a feature that influences the susceptibility of LDL to oxidative modification processes. In addiction, LDL contains lipophilic antioxidants, including α-tocopherol and ubiquinol-10 that help in the protection of the lipids contained in the hydrophobic core (Rubbo et al, 2005 and Rubbo et al, 2002, Esterbauer et al, 1992). Oxidation of LDL leads to the consumption of polyunsaturated fatty acid esters such as arachidonic acid and linoleic acid esters, and to the generation of lipid-derived reactive species that can covalently bind to apolipoprotein B (apo B) altering its properties what includes hydrolysis of phosphatidylcholine and loss of esterified cholesterol (Kawai et al, 2004). Hydroperoxides and aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) have been extensively investigated as lipid peroxidation end-products. These species are highly reactive electrophiles which form protein adducts with free amino groups of the lysine residues neutralizing the cluster of positive charges that are recognized by ionic interaction by the classical LDL receptor . The loss of these positive charges confers to the LDL particle a higher anionic electrophoretic mobility and a reduced recognition by the LDL receptor on fibroblasts, while increasing its recognition by macrophages, contributing to the atherosclerotic process (Esterbauer et al, 1992).

Lipid peroxyl or alkoxyl radicals ($LOO^{\bullet}/LO^{\bullet}$) can be formed through the decomposition of LOOH catalyzed by Cu^{2+}/Cu^{+} or Fe^{3+}/Fe^{2+} ions, either free or in the form of heme proteins (Ohyashiki et al., 2002, Pinchuk and Lichtenberg, 2002). Inhibition of lipid peroxidation through the inactivation of these radicals can be performed by classical antioxidants like ascorbic acid and alfa-tocopherol. Nitric oxide endogenously produced can also be envolved in this action as shown in Eqs. 1 to 3 (Rubbo et al., 2005, Cominacini et al, 1991).

$$LOO^{\bullet} + AH^{-} \rightarrow LOOH + A^{\bullet -} \quad (1)$$

$$LOO^{\bullet} + \alpha\text{-}TOH \rightarrow LOOH + \alpha\text{-}TO^{\bullet} \quad (2)$$

$$LOO^{\bullet} + {}^{\bullet}NO \rightarrow LOONO \quad (3)$$

In addition to regulating the vascular tone in both the systemic and renal circulation in humans (Broere et al, 1998 and Haynes et al, 1997), under normal physiological conditions, endothelium-derived NO exerts other actions which are considered antiatherogenic. These include the prevention of adherence and aggregation of platelets and monocytes on the wall of vases (Napoli and Ignarro, 2001) and the regulation of cell shape, adhesion and migration of smooth muscle cells (Hassid et al, 1999). On the bases of these actions, different experimental and clinical studies have related the reduction in NO synthesis and/or activity with the contribution to the initiation and progression of atherosclerosis in humans (Drexler et al, 1999). Therefore, novel therapeutic strategies have been oriented to enhance NO synthesis and/or activity by administration of L-arginine and antioxidants. Recently, it has been reported recently that S-nitroso-N-acetylcysteine (SNAC) attenuates plaque development by 55%

in LDLr -/- mice fed a hypercholesterolemic diet for 15 days, but does not prevent endothelial–dependent vascular alterations (Krieger et al, 2006). The mechanism underlying these effects may involve direct donation of NO and/or decrease in superoxide levels due to NO scavenging action. This result suggests that administration of primary RSNOs may be a novel therapeutic strategy for treating cardiovascular diseases and other diseases associated with lipid peroxidation like NAFLD (de Oliveira et al, 2006 and de Oliveira et al, 2006).

In this work, the *in vitro* peroxidation of LDL catalyzed by Cu (II) ions was monitored in the presence and absence of two primary RSNOs: SNAC and GSNO at 37ºC. Electrochemical data showed that the amount of free NO released from GSNO and SNAC is reduced to c.a. 0.6 and 0.25, respectively, in the presence of LDL at two different conditions, compared to the NO release under the same conditions in the absence of LDL. These results indicate that RSNOs are consumed by free radicals generated in LDL peroxidation. Thus primary RSNOs may act directly as antioxidants and protect LDL from oxidative damage *in vivo.*

Materials and Methods

Materials

Low density lipoprotein (LDL), glutathione (γ-Glu-Cys-Glu, GSH), N-acethyl-L-cysteine (NAC), potassium iodide (KI), sulfuric acid (H_2SO_4), cooper sulfate ($CuSO_4$), sodium nitrite ($NaNO_2$) and phosphate buffer saline (PBS, pH 7.4), (Sigma/Aldrich, St. Louis, MO) were used as received. All the experiments were

carried out using analytical grade water from a Millipore Milli-Q Gradient filtration system.

Methods

Synthesis of GSNO and SNAC in aqueous solution

Aqueous GSNO solution was prepared by the reaction of GSH with sodium nitrite in acidic medium as described elsewhere [28,34]. GSNO was obtained as stable reddish crystals in the pure form and was dried by freeze-drying. Solid GSNO was stored at -20°C. Freshly prepared GSNO solutions in PBS were used in the experiments. S-nitroso-N-acetylcysteine (SNAC) cannot be precipitated from solution and stored as dry solids because of their high solubility in water. Therefore, aqueous SNAC solutions were synthesized through the equimolar reaction of NAC or CyS, with $NaNO_2$ in acidified aqueous solution. Freshly prepared, stock acidic SNAC solutions, were diluted in PBS and used immediately.

Calibration of the NO electrode

The electrochemical detection of NO was performed using an amperometric amiNO-700 electrode (Innovative Instruments Inc., FL, USA). This sensor measures

NO concentration in aqueous solutions by oxidizing NO at the working electrode. NO diffuses through the gas permeable membrane of the sensor and is oxidized in the platinum electrode, resulting in an electrical current. The redox current is proportional to the NO concentration outside the membrane which is continuously monitored. For calibration, different volumes of a sodium nitrite ($NaNO_2$) standard solution 25 μM were used to generate free NO. A chemical titration calibration was performed by using an acidic reducing solution (0.1 M KI, 0.1 M H_2SO_4) to which increasing volumes of the $NaNO_2$ solution were added in bolus under constant stirring at 37ºC. NO is formed stoichometrically and is constantly measured by the electrode immersed in the solution. The production of free NO through the reduction of $NaNO_2$ is represented in the following equation (Hummel et al., 2006 and Zhang, 2004):

$$2NaNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + Na_2SO_4 + K_2SO_4 \quad (1)$$

The measurements of the currents generated after each additions of $NaNO_2$ solution are shown in Fig. 1A. The relationship between the NO concentration and the output current of the amiNO-700 electrode was always linear. The detection limit of the electrode is 2 nM in aqueous solutions. This limit allowed adding volumes of 0.1mL of 25 μM $NaNO_2$, which were diluted in the 10 mL reaction flask, resulting in concentrations ranging from 0 – 1000 nM (Fig. 1B). The reducing solution was always replaced by a fresh one before starting the experiments with LDL and RSNOs. The data recording system was set for measurements at 0.2 s intervals. Only the peak currents were used to quantify the NO released after each addition. Data from these instruments were imported into a PC using a software developed using Quick Basic 4.5. The reaction flask was thermostatized at 37ºC.

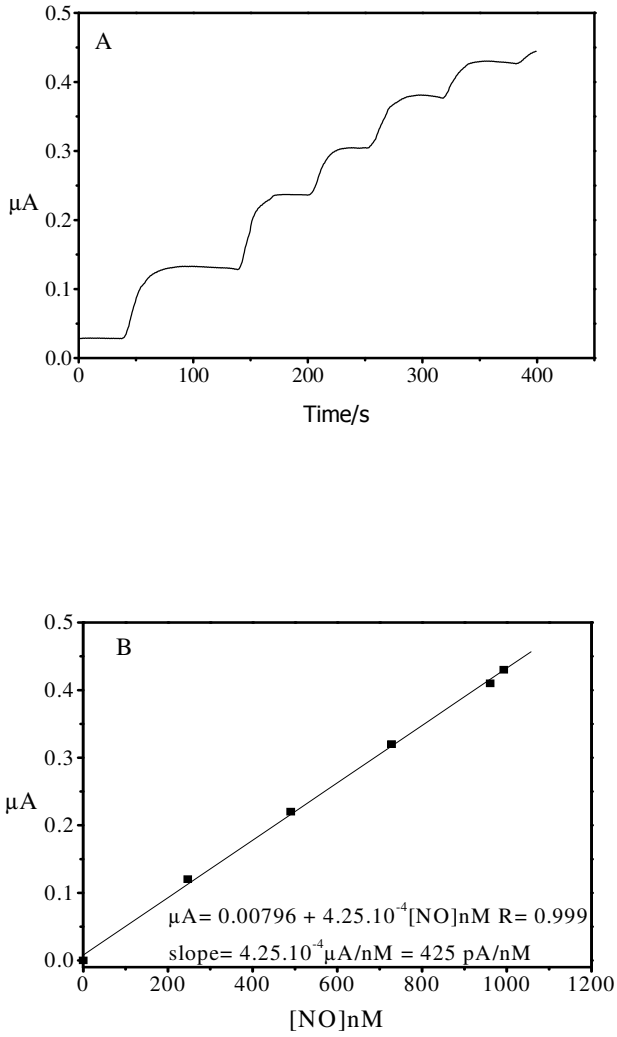

Fig. 1: (A) Representative recording of a calibration of the amiNO-700 sensor with increasing additions of 0.1mL of 25 μM $NaNO_2$ to a 10 mL reaction flask containing a reducing acidic KI solution with constant stirring at 37ºC. (B) Representative calibration curve obtained from data of Fig 1 (A). Linear regression showing a well-correlated straight line.

Electrochemical Detection of NO released from GSNO and SNAC

For the evaluation of the protective action of RSNOs against LDL peroxidation, NO released from the Cu(II) catalyzed RSNOs decompositions was electrochemically measured in the presence and absence of LDL emulsion. Cu(II) ions catalyze not only the RSNOs decomposition but also the LDL oxidation in aerated medium. These two reaction were promoted simultaneously in order to measure the consumption of free NO and/or RSNOs by free radicals generated during LDL peroxidation.

In each experiment, the NO sensor was immersed vertically in LDL suspension (final concentration ranging from 0.48 - 0.50 μg/mL) in the absence and presence of GSNO or SNAC (final concentrations 3.10 and 3.30 μM, respectively) in the reaction flask at 37ºC. After the homogenization of the LDL emulsions in the absence or presence of GSNO or SNAC, appropriate volumes of $CuSO_4$ solution were added in bolus, in order to obtain two different final concentrations in the reaction flask: 0.10 and 0.48 μM. GSNO and SNAC decompose to free NO and disulfide by-products according to the following general equation for RSNOs:

$$RSNO + RSNO \rightarrow RS\text{-}SR + 2NO \qquad (2)$$

NO released in solution was continuously and quantitatively detected by the NO electrode.

RESULTS AND DISCUSSION

Calibration of the NO electrode

Fig 1A shows that the currents generated by NO production due to the additions of $NaNO_2$ solution reach a maximum 30 s after each addition which is in accordance with the fast answer previously reported for this kind of electrode (Berkels et al, 2001). Figure 1B shows the linear response of the electrode from 100 to 1000 nM of which the slope of the line provides the current-to-concentration ratio for the electrode. In this range, the linear response of the electrode usually provided a correlation coefficient R of 0.99. The concentration and the amount of NO released from the $NaNO_2$ in the calibration procedure can be calculated by an equation similar to the equation in the inset of Fig. 1B. It must be remembered that KI and H_2SO_4 are present in excess concentration, thus the limiting reagent in the calibration is $NaNO_2$. Equation 1 shows that molar ratio between $NaNO_2$ e NO is 1:1. Therefore, the amount of NO released can be calculated from the amount of $NaNO_2$ added.

Electrochemical Detection of NO released from GSNO and SNAC

Figure 2 shows the recordings of the amiNO-700 electrode obtained in aqueous LDL suspensions oxidized by Cu^{2+} ions in the presence of LDL (curves a and c) and absence of GSNO (curves b and d).

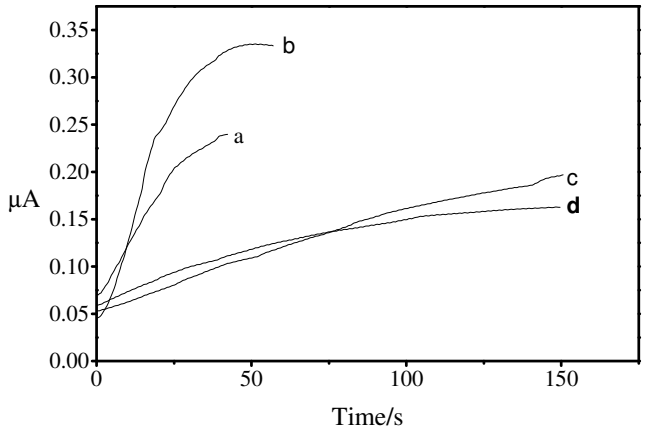

Fig. 2: Representative recordings of the amiNO-700 sensor obtained in aqueous LDL suspensions oxidized by Cu^{2+} ions in the presence of GSNO. (a) [LDL] = 0.50 μg/mL; [GSNO] = 3.3 μM; [$CuSO_4$] = 0.10 μM, b) [GSNO] = 3.3 μM; [$CuSO_4$] = 0.10 μM, (c) [LDL] = 0.48 μg/mL; [GSNO] = 3.10 μM; [$CuSO_4$] = 0.48 μM, and (d) [GSNO] = 3.1 μM; [$CuSO_4$] = 0.48 μM.

The data indicate that the addition of Cu^{2+} ions catalyzes GSNO decomposition with NO released in all cases. However, in the presence of LDL emulsion, the amount of NO released is significantly decreased, indicating that part of NO released by the catalytic action of Cu^{2+} ions was consumed in the peroxidation of LDL which is simultaneously taking place. A similar result was obtained for SNAC in the place of GSNO (data not shown). These results are summarized in the bar graph of Fig. 3, which show that the amount of free NO released from GSNO and SNAC is reduced to c.a. 0.6 and 0.25, respectively, in the presence of LDL under two different conditions ([$CuSO_4$] = 0.1 and 0.48 μM), compared with the NO release under the same conditions in the absence of LDL. These results can be interpreted by considering that NO is consumed in the reaction with peroxyl or oxyl ($LO^{\bullet}$/$LOO^{\bullet}$) radicals generated in the oxidation of LDL in aerated medium.

Two kinds of reaction can be considered to explain the reduced amouts for free NO in the presence of LDL. RSNOs can react primarily with $LO^{\bullet}/LOO^{\bullet}$ radicals, before their decomposition to free NO according to the equation:

$$2RSNO + LO^{\bullet}/LOO^{\bullet} \rightarrow LONO/LOONO + RS^{\bullet} \quad (3)$$

Where LONO/LOONO are possible nitrogen-containing products of oxidized LDL. At the same time, RSNOs previously decomposed by Cu(II) ions can have their free NO trapped by the same $LO^{\bullet}/LOO^{\bullet}$ radicals formed in LDL oxidation according to:

$$2NO + LO^{\bullet}/LOO^{\bullet} \rightarrow LONO/LOONO \quad (4)$$

In both cases, the final result will be a lower amount of free NO detected by the electrode. Although further studies must be performed to elucidate the specific reactions involved and also to identify the nitrogen-containing products of LDL oxidation, these results show a potential protective effect of primary RSNOs against the peroxidation of LDL suggesting that primary RSNOs may act directly as antioxidants protecting LDL from oxidative damage *in vivo.*

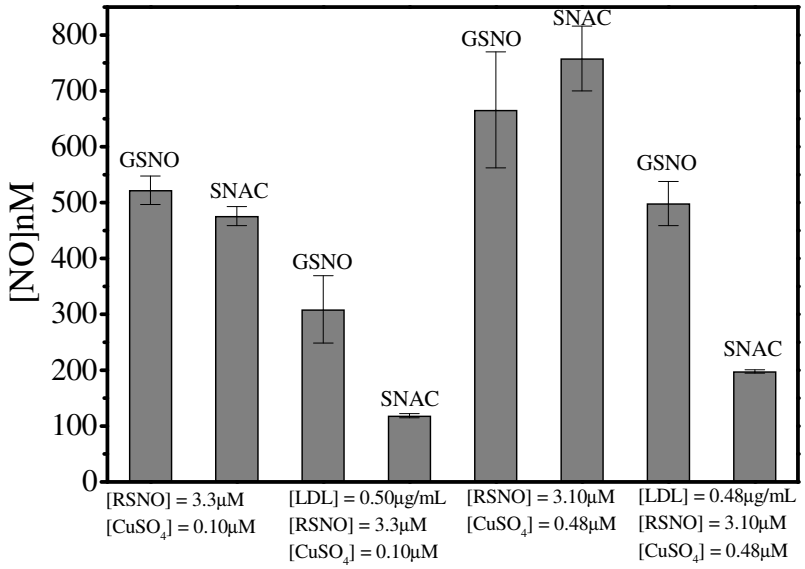

Fig. 3: Concentration of NO released in GSNO and SNAC (final concentrations, 3.3 μM and 3.10 μM) solutions by the action of Cu(II) ions (final concentrations, 0.10 μM and 0.48 μM), in the absence and in the presence of LDL suspension.

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5. Participação em outros trabalhos de colaboração

Ao longo do curso de doutorado foram realizados trabalhos em colaboração com a Profa. Cláudia PMS de Oliveira do Departamento de Gastroenterologia da FCM/USP, SP. Esta colaboração foi importante para correlacionar os dados *in vitro* de inibição da peroxidação lipídica pela S-nitroso-N-acetilcisteína (SNAC) com a capacidade desta droga em inibir o desenvolvimento da doença não alcoólica do fígado gordo (NAFLD) por administração *via* oral da SNAC em 2 modelo animais com 3 diferentes dietas. Os resultados desta colaboração se encontram em manuscritos mostrados a seguir.

NAFLD inclui esteatose não alcoólica, esteatohepatites (NASH) e eventualmente cirroses. Fatores como obesidade e diabetes tem sido relatada para NAFLD e uma das hipóteses para a NASH está associada ao estresse oxidativo e peroxidação lipídica.

Os modelos animais foram ratos Wistar e camundongos ob/ob tratados com diferentes dietas na presença e na ausência de S-nitrosocisteína (SNAC). Estes resultados estão presentes nos três manuscritos a seguir e a conclusão exposta resumidamente é que a administração oral de SNAC previne o princípio da NAFLD e que este efeito está correlacionado com a habilidade da SNAC bloquear a propagação da peroxidação lipídica *in vitro* e *in vivo*. Previne também porque há uma anulação dos efeitos citotóxicos de espécies de oxigênio reativo e peroxidação lipídica. Além disso, administrando SNAC via oral em camundongos ob/ob a SNAC mostrou um ótimo resultado frente a NASH.

de Oliveira, C. P. M. S.; Simplicio, F. I.; de Lima, V. M. R.; Yuahasi, K.; Lopasso, F. P.; Alves, V. A. F.; Abdalla, D. S. P.; Carrilho, F. J.; Laurindo, F. R. M.; de Oliveira, M. G. Oral administration of S-nitroso-N-acetylcysteine prevents the onset of non alcoholic fatty liver disease in Rats, *World Journal of Gastroenterology*, **2006**, 12 (12):1905-1911.

Oral administration of *S*-nitroso-*N*-acetylcysteine prevents the onset of non alcoholic fatty liver disease in Rats

Running title: SNAC prevents the onset of NAFLD in Rats

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Abstract

AIM: Oxidative stress is implicated in the pathogenesis of Nonalcoholic Fatty Liver Disease (NAFLD). The aims of this work were to evaluate the potential of *S*-nitroso-N-acetylcysteine (SNAC) in the inhibition of lipid peroxidation and the effect of oral SNAC administration in the prevention of NAFLD in an animal model.

METHODS: NAFLD was induced in Wistar male rats by choline-deficient diet for 4 wk. SNAC treated animals (n = 6) (1.4 mg/kg/day of SNAC, orally) were compared to 2 control groups: one (n = 6), which received PBS solution and another, (n = 6) which received NAC solution (7 mg/kg/day). Histological variables were semiquantitated with respect to: macro and microvacuolar fat changes, its zonal distribution, foci of necrosis, portal and perivenular fibrosis and inflammatory infiltrate with zonal distribution. LOOHs from samples of liver homogenates were quantified by HPLC. Nitrate levels in plasma of portal vein were assessed by chemiluminescence. Aqueous LDL suspensions (200 μg protein/mL) were incubated with $CuCl_2$ (300 μmol/L) in the absence and presence of SNAC (300 μmol/L) for 15 h at 37 ºC. Extent of low-density lipoprotein (LDL) oxidation was assessed by fluorimetry. Linoleic acid (LA)(18.8 μmol/L) oxidation was induced by soybean lipoxygenase (SLO) (0.056 μmol/L) at 37 ºC in the presence and absence of N-acetylcysteine (NAC) and SNAC (56 and 560 μmol/L) and monitored at 234 nm.

RESULTS: Animals in the control group developed moderate macro and microvesicular fatty changes in periportal area. SNAC-treated animals displayed only discrete histological alterations with absence of fatty changes and did not develop liver steatosis. The absence of NAFLD in the SNAC-treated group was positively correlated with a decrease in the concentration of LOOH in liver homogenate, compared to the control group (0.7±0.2 vs 3.2±0.4 nmol/mg protein, respectively, $P<0.05$), while serum levels of aminotransferases were unaltered. The ability of SNAC in preventing lipid peroxidation was confirmed in the *in vitro* experiments, using LA and LDL as model substrates.

CONCLUSION: Oral administration of SNAC prevents the onset of NAFLD in choline-deficient fed Wistar rats. This effect is correlated with the ability of SNAC in blocking the propagation of lipid peroxidation *in vitro*, and *in vivo.*

Key words: Nitric Oxide, S-nitroso-*N*-acetylcysteine, oxidative stress, nonalcoholic fatty liver disease

INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is considered a particular type of a large spectrum of nonalcoholic fatty liver disease (NAFLD), which includes fat alone and fat with nonspecific inflammation[1,2]. Although several predisposing factors have been related to NAFLD, such as obesity and diabetes, the pathogenesis of NAFLD and its progression to fibrosis and chronic liver disease are still unclear[3,4,5]. One of the main hypotheses is that the mechanism of hepatocyte injury in NASH is associated with oxidative stress and lipid peroxidation resulting from the imbalance between pro-oxidant and antioxidant chemical species[6]. Such imbalance is associated with increased β-oxidation of fatty acids by means of by mitochondria, peroxisomes, and cytochrome P450 2E1 (CYP2E1) pathways. These oxidative processes produce free electrons, H_2O_2, and reactive oxygen species (ROS) while depleting the potent antioxidants glutathione, and vitamin E[1]. The increased levels of free fatty acids present in the fatty liver provide a perpetuating and propagating mechanism for oxidative stress via lipid peroxidation, with secondary damage to cellular membranes and key organelles such as the mitochondria[6]. Lipid peroxidation usually leads to the formation of peroxyl radicals, which are central species in the peroxidation chain reaction. Enzymatic lipid peroxidizing systems include lipoxygenases (LOXs), which are a family of nonheme iron-containing dioxygenases, able to induce enzymatic peroxidation of polyunsaturated fatty acids using atmospheric oxygen (O_2) as a second substrate. In contrast to lipid monooxigenases like cytochrome P-450, whose main catalytic activity is the hydroxylation of substrates, LOXs are able to introduce peroxides in lipid substrates, forming reactive fatty acid hydroperoxides (LOOH). In general, LOXs contain an essential iron atom, which is present as Fe^{2+} in the inactive enzyme form. Enzymatic activation occurs through hydroperoxide-driven oxidation of Fe^{2+} to Fe^{3+}. Among LOXs of particular interest is 15-LOX, which can also oxidize esterified fatty

acids in biological membranes and lipoproteins and has been implicated in the pathogenesis of atherosclerosis[7,8,9]. Site-specific oxidation of lipidic substrates can also be performed in model systems when metal ions (Cu(I)/Cu(II)) or Fe(II)/Fe(III)) are used to generate radicals in the absence of chelant species [10].

Nitric oxide can act as a potent inhibitor of the lipid peroxidation chain reaction by scavenging propagatory lipid peroxyl radicals, and by inhibiting many potential initiators of lipid peroxidation, such as peroxidase enzymes[11]. However, in the presence of superoxide ($O_2^{\bullet-}$), NO forms peroxynitrite ($OONO^-$), a powerful oxidant, which is able to initiate lipid peroxidation[12]. An excess of NO is expected to exert a protective effect against lipid peroxidation, while an excess of $O_2^{\bullet-}$, or equimolar concentrations of NO and $O_2^{\bullet-}$ are expected to induce lipid peroxidation[13]. Thus, the balance between NO and $O_2^{\bullet-}$ may have important implications in NAFLD, where oxidative stress seems to have a pivotal role in the onset and/or progression of the disease[12,13]. NO is believed to coexist in cells with S-nitrosothiols (RSNOs) which are considered to be endogenous NO carriers and donors in mammals[14]. NO covalently bound to the sulfur atom in RSNOs resists oxidant inactivation by oxyhemoglobin and has the same physiological properties of free NO, including its protective action in oxidative stress[15]. RSNOs have been considered potential therapeutic agents in a variety of pathologies in which NO may be involved[16] and S-nitroso-N-acetylcysteine (SNAC) is a relatively stable RSNO and a potent vasodilator[17]. SNAC is among the RSNOs, which can be synthesized through the S-nitrosation of the corresponding free thiol, (in this case, N-acetylcysteine, NAC). Free thiols (R-SH) play also an important role *in vivo* as antioxidants. Hydrogen abstraction from thiol group is particularly fast compared to hydrogen abstraction from carbon atoms or alkoxyl radicals [18,19,20,21]. At physiological pH values, thiyl radicals ($R\text{-}S^{\bullet}$) formed, can react with excess thiol anions ($R\text{-}S^-$) to give disulphide radical anions ($R\text{-}SS\text{-}R^{\bullet-}$), or can dimerize, giving rise to inter or

intramolecular RS-SR cross-links in a termination process. Compared to free thiols, RSNOs can be more powerful terminators of radical chain-propagation reactions, by reacting directly with $ROO^{\bullet}$ radicals, yielding nitro derivatives (ROONO) as end products, as well as dimmers RS-SR.

The aim of this study was to evaluate the role of SNAC as an NO donor, in the prevention of NAFLD in an animal model where NAFLD was induced by a choline deficient diet. Our results show, for the first time, that SNAC is able to block the onset of NAFLD in this animal model. This result was correlated with *in vitro* experiments which have confirmed the ability of SNAC in preventing the oxidation of low-density lipoprotein (LDL) and linoleic acid (LA), as model substrates, by Cu(II) ions and soybean lipoxygenase (SLO), respectively.

MATERIALS AND METHODS

Materials

N-acetyl-L-cysteine (NAC), linoleic acid, sodium nitrite, hydrochloric acid, human lyophilized low-density lipoprotein (LDL), soybean lipoxygenase, sodium dodecil sulfate (SDS), phosphate buffer saline (PBS, pH 7.4) and copper (II) chloride (Sigma, St. Louis, MO) were used as received. All the experiments were carried out using analytical grade water from a Millipore Milli-Q Gradient filtration system.

SNAC Synthesis

SNAC was synthesized through the S-nitrosation of N-acetyl-L-cysteine (Sigma Chemical, St. Louis, MO) in an acidified sodium nitrite solution[17]. Stock SNAC solutions were further diluted in PBS. Solutions were diluted to 2.4×10^{-4} mol/L in PBS (pH 7.4) before administration.

Nitrate quantification

Nitrate (NO_3^-, a stable metabolite of NO) levels in plasma of portal vein of the animals were assessed by chemiluminescence using a Sievers Nitric Oxide Analyzer (NOA-280, Boulder, CO) according to a method described elsewhere[22]. Higher nitrate concentrations were found in the plasma of animals that received SNAC orally (10.8 μmol/L) than intraperitoneally (4.2 μmol/L). This result was used as criteria to chose oral administration as a protocol to achieve greater SNAC absorption.

Effect of NAC and SNAC on the in vitro LDL oxidation

Oxidation of LDL was induced through the addition of $CuCl_2$ (300 μmol/L) to oxygenated aqueous LDL suspensions (200 μg/mL) in the absence and presence of SNAC (300 μmol/L). Aqueous LDL suspensions were prepared by diluting solid LDL to 200 μg protein/mL with EDTA-free PBS and incubated with $CuCl_2$ (300 μmol/L) for 15 h at 37 ºC. The extent of LDL oxidation was assessed by measuring the fluorescence intensity of LDL suspensions. Oxidation of LDL results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products, leading to fluorescent free and protein-bound Schiff base conjugates[23,24]. In all cases, fluorescence spectra of such conjugates were firstly recorded in the range 430 to 600 nm, in order to characterize the shape and position of the emission peak. All the spectrofluorimetric measurements were performed using a Perkin-Elmer LS-55 luminescence spectrometer with a temperature-controlled cuvette holder thermostatized at 37 ºC. Spectra of the solutions were obtained in 1 cm quartz cuvette. The excitation and emission wavelengths were 360 and 433 nm, respectively. Native LDL (200 μg/mL) served as the control.

Effect of NAC and SNAC on the in vitro LA oxidation

Oxidation of LA was induced through the addition of SLO to aqueous LA dispersions. LA was dispersed in SDS solution (0.01 mol/L). The final LA concentration was 18.8 μmol/L. LA was aliquoted into a quartz cuvette, flushed with O_2 for 1 min and SLO (0.056 μmol/L), was added with a syringe to start the oxidation. The oxidation reactions were monitored in the absence or presence of NAC and SNAC (56 and 560 μmol/L) at 37 ºC through the increase in absorbance at 234 nm, due to conjugated diene formation. A Hewlett Packard spectrophotometer, model 8453 (Palo Alto, CA, USA) with a temperature-controlled cuvette holder was used to monitor the spectral changes in the range 200 - 600 nm in the dark and at 37 °C. Spectra of the solutions were obtained in 1 cm quartz cuvette referenced against air, under stirring (1 000 r/min). Each point in the kinetic curves of absorbance vs. time is the average of two experiments with the error bars expressed by their standard deviations (SD).

Animals

Male Wistar rats, weighing 300 to 350 g, were housed in cages with controlled light/dark cycle, receiving free water. Fatty liver was induced in the animals by choline deficient diet for four weeks. The animals were randomly divided into three groups: 1 - Control group (n = 6) fed with choline deficient diet plus oral administration of vehicle (0.5 mL of PBS); 2 - SNAC group (n = 6) fed with choline-deficient diet plus oral administration of SNAC solution (0.5 mL of SNAC solution, to reach 1.4 mg/kg/day); 3 – NAC group (n = 6) fed with choline-deficient diet plus oral administration of NAC solution (0.5 mL of NAC solution, to reach 7 mg/kg/day). After four weeks of treatment, plasma samples were collected, animals were sacrificed, and their livers were collected for histological examination and lipid peroxidation analysis. All procedures for animal experimentation were in accordance to the Helsinki

Declaration of 1975, and the Guidelines of Animal Experimentation from the School of Medicine of the University of São Paulo.

Biochemical analysis

Serum alanine amininotransferase (AST), aspartate aminotransferase (ALT), cholesterol and triglycerides were analyzed by standard methods[25].

Histological analysis

Fragments of liver tissues previously fixed by immersion in formaldehyde saline (10%) solution were processed and submitted to hematoxylin-eosin (HE) and Masson Trichrome stains for histological analysis. Scharlach red (O-tolylazo-o-tolylazo-β-naphthol) fat stain[26] was used for more accurate evaluation of fatty change. Histological variables were blindly semiquantitated from 0 to 4+ with respect to: both macro and microvacuolar fatty change, its zonal distribution, foci of necrosis, portal and perivenular fibrosis as well as the inflammatory infiltrate with zonal distribution.

Lipid peroxidation

Samples of liver homogenates were extracted with a mixture of acetonitrile:hexane (4:10, v/v). The contents were vortexed for 2 min and centrifuged at 2 500 r/min for 10 min for phase separation. The hexane phase, containing cholesteryl ester derived hydroperoxides (LOOH), was collected and evaporated under nitrogen. The residue was dissolved in methanol:butanol (2:1, v/v), filtered through a 22 μm Millex filter (Millipore, São Paulo, Brazil) and analyzed by HPLC (Perkin-Elmer series 200, Beaconsfield, Buckinghamshire, England) using a LC18DB column (Supelco, Bellefonte, PA, USA). LOOHs were eluted in methanol:butanol 2:1 (v/v) at a flow rate of 1.0 mL/min through a pump (Perkin-Elmer series

200) and a LC-240 fluorescence detector (Perkin-Elmer) with the excitation source switched off. A solution of 100 mM borate buffer pH 10/methanol 3:7 (v/v) containing microperoxidase (25 mg/L) was used as the reaction solution for the postcolunm reaction[27]. Peaks were identified using external standards prepared from their respective oxidation products as previously described[27] and quantified using the package Turbochrom Navigator software (Perkin-Elmer). Results are expressed as nmol of lipid hydroperoxides/mg of protein.

Statistical analysis

All data are expressed as mean ± SE or mean ± SD. Statistical significance was evaluated using the one-way ANOVA test for comparisons among three groups (Control X NAC X SNAC – LOOH quantification) and t-test for the comparison between two means (Control X SNAC - biochemical analysis). A value of $P<0.05$ was considered statistically significant.

RESULTS

Figure 1 shows the micrographs of liver tissues extracted from animals treated with choline-deficient diet, which received vehicle or SNAC solutions during four weeks. A moderate macro and microvacuolar steatosis in periportal zone can be seen in the control group (Figure 1A) while in the SNAC-treated group the animals did not develop liver steatosis (Figure 1B). Scharlach staining has shown a fatty change (positive staining) in the control group (Figure 1C), whereas in the SNAC-treated group no fat change was detected (negative staining) (Figure 1D). In both animal groups, necroinflammatory activity was minimal and no fibrosis was detected. In the NAC-treated group there was macro and microvacuolar steatosis in periportal zone (data not shown).

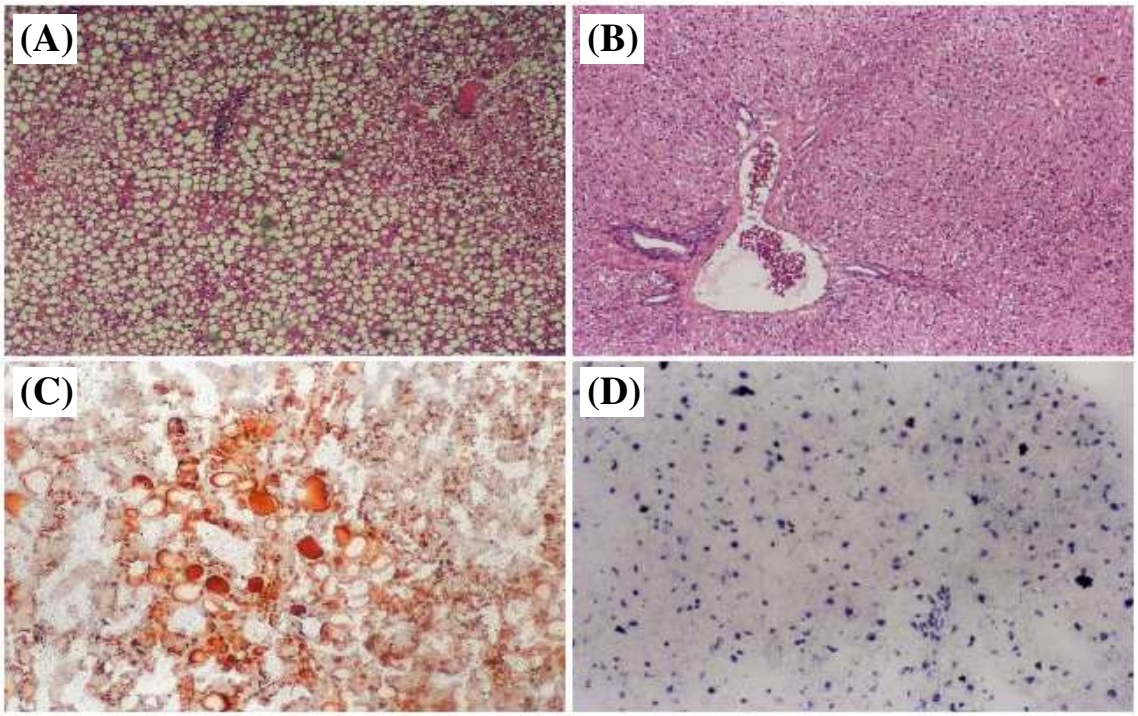

Fig. 1. Histological features of liver tissues of rats fed with choline-deficient diet. (A) Control, showing moderate macro and microvacuolar steatosis in periportal zone; (B) SNAC-treated animals showing normal liver in periportal zone (hematoxylin-eosin stain-HE); (C) Control group showing positive Scharlach stain; (D) SNAC-treated animals showing negative Scharlach stain.

Figure 2 shows that SNAC prevented the rise of LOOH concentration in the liver of the SNAC-treated group, compared to the control group (0.3 ± 0.1 vs 3.2 ± 0.4 nmol/mg protein, respectively). The protective effect of NAC is also expressed in a reduction of hydroperoxides formation that can be seen in the *ca.* 4.6-fold reduction in LOOH formation (0.7 ± 0.2 vs 3.2 ± 0.4 nmol/mg protein, respectively).

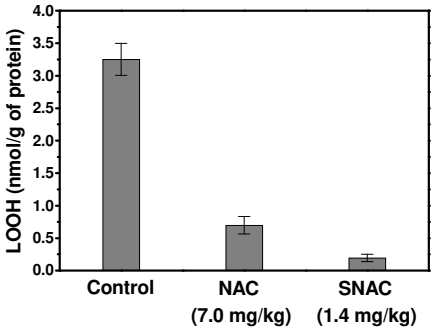

Fig. 2. Concentration of hydroperoxides (LOOH) in the liver homogenates of the control group (Control), NAC and SNAC-treated animals.

On the other hand, table 1 shows that the levels of AST and triglycerides were increased to a similar extent in the control and SNAC-treated groups and that SNAC treatment in the choline-deficient fed rats did not lead to changes in ALT and cholesterol levels.

Table 1 Levels of alanine amininotransferase (AST), aspartate aminotransferase (ALT), cholesterol and triglycerides in the serum of rats fed with choline-deficient diet.

Group	Number of animals	AST (U/L)	ALT (U/L)	Cholesterol (U/L)	Triglyceride (U/L)
Control [1]	6	108±3	40±1	36±1	88±3
SNAC [2]	6	95±4	37±8	35±1	70±1

Date expressed as mean ± SD

Normal values in U/L for AST:10-34; ALT:10-44; mg/dl: cholesterol and triglyceride: 45-89

[1] Control - animals fed with choline-deficient diet

[2] SNAC – animals fed with choline-deficient diet and treated daily with oral SNAC administration

Figure 3 shows the emission spectra of human LDL suspension (200 μg/mL) in PBS. The two emission peaks at *ca*. 410 and 440 nm (Figure 3A) can be assigned to the partial

oxidation of the freshly prepared LDL suspension. It can be seen that these two peaks increase after the incubation of LDL with $CuCl_2$ (300 μmol/L) (Figure 3B) reflecting the oxidation of LDL catalyzed by Cu (II) ions. However, incubation of LDL with $CuCl_2$ in the same condition, but in the presence of SNAC (300 μmol/L) completely blocked the growth of the 410 and 440 nm peaks (Figure 3C). In fact, the peak at 440 nm was extinguished in this case.

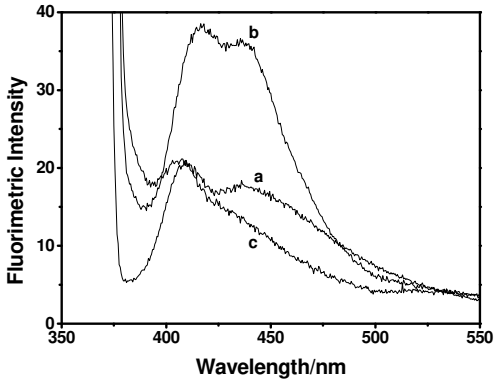

Fig 3. Emission spectra of human LDL (200 μg/mL) suspended in aerated PBS. (a) Freshly prepared suspension; (b) after incubation with $CuCl_2$ (300 μmol/L) for 15 h; (c) after co-incubation with $CuCl_2$ (300 μmol/L) and SNAC (300 μmol/L). The excitation and emission wavelengths were 360 and 433 nm, respectively.

Figure 4 shows the effect of SNAC on the kinetics of LA oxidation by SLO. This effect can be evaluated through the analysis of two kinetic parameters: initial rate and extent of the peroxidation reaction until the achievement of the chemical equilibrium. Kinetic curves were obtained from the corresponding spectral changes in the UV, monitored through the band with maximum at 234 nm. This band is characteristic of conjugated dienes and can thus

be taken as a marker of LA peroxidation. While initial rates of reaction correspond to the inclination of the initial sections of the curves (*ca*. 10 s), extents of the reactions correspond to the absorbance values at the plateaus. It can be seen that both parameters are maximum when LA (18.76 μmol/L) is incubated with SLO (0.056 μmol/L) (Figure 4A). Co-incubation with NAC (560 μmol/L) reduced the extent and rate of oxidation (Figure 4B), but this reduction is much more pronounced in the co-incubation with SNAC at a concentration ten times lower than NAC (56 μmol/L) (Figure 4C). The reduction is further increased in the presence of SNAC (560 μmol/L) (Figure 4D). These effects can also be evaluated in the bar graph of Fig. 5, where the initial rates of reaction and the extents of reaction were extracted from the kinetic curves of Fig. 4. It can be seen in Fig. 5 that both the rates and the extents of reaction in the presence of SNAC were reduced to about half of those obtained in the presence of NAC at a concentration ten times higher.

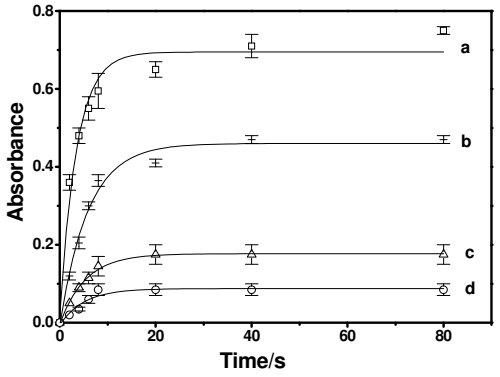

Fig. 4. Kinetic curves of linoleic acid (18.76 μmol/L) peroxidation catalyzed by (a) soybean lipoxygenase (SLO) (0.056 μmol/L); (b) SLO co-incubated with NAC (560 μmol/L); (c) SLO co-incubated with SNAC (56 μmol/L) and (d) SLO co-incubated with SNAC (560 μmol/L). Absorbance changes monitored at 234 nm at 37 ºC.

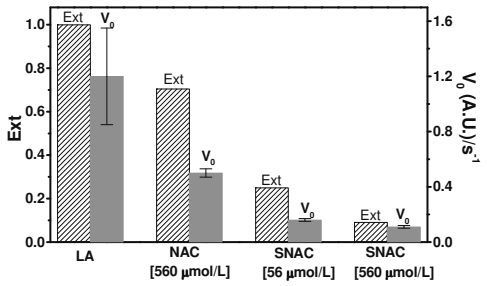

Fig. 5. Barr graph showing the extent (Ext) and initial rates (V_0), of the peroxidation reaction of linoleic acid (LA) by SLO. Data extracted form the curves of Fig. 4.

DISCUSSION

Choline-deficient diet is a classical general model of NAFLD, where Cyp2E1 is up regulated and the animals develop steatosis, steatohepatitis and hepatic fibrosis[28]. The results obtained in this animal model show a strong inhibitory effect of SNAC on fatty change, which is the initial step of NAFLD. The protective effect of SNAC observed here can be analyzed according to the suggested role of oxidative stress in the pathology of NAFLD[29,30,31]. Although the exact role of antioxidants in the prevention of NAFLD is not well established yet, a number of studies have shown that markers of oxidative stress are increased, while levels of endogenous antioxidants (e.g. vitamin E and glutathione, GSH) are decreased in NAFLD[29,30]. The microsomal enzymes CYPs 2E1 and 4A are believed to be involved in the fatty acid oxidation in the liver of humans with NASH, contributing to the pathogenesis of this disease[31]. In the present case, formation of lipid hydroperoxides (LOOH), which are one of the main products of the lipid peroxidation process, was observed to be expressively reduced in the liver tissue of the SNAC-treated animals, indicating that SNAC acted as a potent inhibitor of lipid/lipoproteins oxidation. This result is in accordance with the reactivity

of NO from SNAC and the ability of NO in blocking the propagation of radical chain reactions by forming nitrated lipid derivatives as end products[32,33,34,35,36].

SNAC-induced inhibition of LDL oxidation by Cu(II) as a model system, was confirmed in the *in vitro* experiments as can be seen in Fig. 3. The emission peaks at 410 and 440 nm in the fluorescence spectra of LDL suspensions are assigned to adduct formation (Schiff bases) between oxidation products of the lipid content of LDL particles (mainly malondialdehyde, MDA) and amino groups of the apolipoprotein (mainly Apo-B-100) and are well known markers of LDL oxidation[37,38]. The inhibition of their formation in the co-incubation of LDL with Cu (II) and SNAC, shows that SNAC blocks LDL oxidation in this condition. The protective effect of SNAC was also confirmed *in vitro* using LA as a second model compound in which peroxidation was catalyzed by SLO (Figs. 4 and 5). The co-incubation of LA with SNAC (56 μmol/L) and with its correspondent reduced thiol, NAC (560 μmol/L) highlights the much more potent effect of SNAC in the inhibition of LA peroxidation, once SNAC at a concentration ten times lower than NAC exerted a much more important antioxidant effect. The fact that an increase in SNAC concentration to 560 μmol/L did not lead to a proportional reduction in the kinetic parameters associated with LA peroxidation, is probably due to the relatively fast initial steps of LA peroxidation.

As SNAC does not react directly with aldehydes or ketones, the protective effect observed here must be associated with the termination of lipid radical chain propagation reactions, through the inactivation of alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) intermediates, which were already demonstrated to be converted into inactive ROONO products by NO[32,33,34,35,36]. *in vivo*. A general equation for these reactions can be written as:

$$2RS\text{-}NO + LO^{\bullet} / LOO^{\bullet} \rightarrow LONO / LOONO + RS\text{-}SR \quad (1)$$

where RSNO can be any primary S-nitrosothiol and RS-SR is the corresponding oxidized thiol, yielded as a dimmer. The same RS-SR dimmers are formed if the RSNOs release NO primarily according to[39]:

$$2\ RSNO \rightarrow RS\text{-}SR + 2NO \qquad (2)$$

Free NO released in Eq. 2 is also capable of reacting with $LO^{\bullet}/LOO^{\bullet}$ species[35], leading to the same termination products of Eq. 1.

Although NAC (the precursor of SNAC) has also an important antioxidant action due to the easy of hydrogen abstraction from its thiol group (data not shown) the protective action of SNAC cannot be assigned to its conversion into NAC. Such reaction doesn't take place in an oxidative environment. In such conditions, the anti-oxidant effect of SNAC can be assigned mainly to the lability and reactivity of NO, according to Eqs. 1 and 2. This statement is supported not only by the greater antioxidant action of SNAC, compared to NAC, in the *in vitro* experiments with LDL and LA, but also by the *in vivo* results showing that the daily oral administration of NAC at a concentration five times higher than SNAC, did not prevent the development of liver steatosis in the present animal model, and led to a lower reduction in the LOOH level in the liver tissue. The protective action of NAC in this animal model is not entirely dissimilar than those obtained with other more classical anti-oxidants. However, ascorbic acid, which reduces liver steatosis in rats under choline-deficient diet, is not able to inhibit the onset of this pathology, and α-tocopherol (vitamin E), does not even reduce fat accumulation in the hepatic tissue in the same animal model[40].

The important protective action of an NO donor in this model allow to suggest that NAFLD can be associated with an impairment of endogenous NO production in the liver. Since the production of endothelium-derived NO was already demonstrated to be impaired in

other diseases related to oxidative stress, like atherosclerosis[41,42] the effects of NO in NAFLSD can involve other mechanisms in addition to those associated solely to oxidative stress. NO is also known to be a signal transduction mediator and accumulating data suggests that S-nitrosation and nitrosilation reactions performed by NO may be a ubiquitous posttranslational modification involved in signal transduction regulation[43]. The absence of correlation between the reduction of LOOH concentration and the occurrence of macro and microvacuolar steatosis in the NAC-treated group, is an evidence that protective mechanisms, other than the inhibition of lipid peroxidation, are operative when SNAC is administered to choline deficient animals. Such mechanisms are probably associated to the biochemical/signaling actions of NO and can be specifically linked to the biochemistry of RSNOs. In contrast to other NO donors which are already in widespread clinical use, like organic nitrates and nitrites and sodium nitroprusside, few clinical studies have been reported for RSNOs. Therefore, the use of RSNOs as exogenous NO sources in the treatment of NAFLD can bring new perspectives for understanding the pathogenesis of this disease.

In conclusion, our results show that oral administration of SNAC as an exogenous NO source, can block the onset of NAFLD and that the reduction of LOOH production in liver tissue as a result of this treatment can be associated to the ability of SNAC in blocking the lipid peroxidation. These results can have clinical implications, regarding novel therapeutic strategies for the treatment of NAFLD.

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Prevention and reversion of nonalcoholic steatohepatitis in ob/ob mice by S-nitroso-N-acetylcysteine treatment

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Short running: Prevention and reversion of NASH in ob/ob mice

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ABSTRACT

Objective: To evaluate the role oral administration of *S*-nitroso-*N*-acetylcysteine (SNAC), a NO donor drug, in the prevention and reversion of NASH in two different animal models.

Methods: NASH was induced in male ob/ob mice by methionine-choline deficient (MCD) and high-fat (H) diets. Two animal groups received or not SNAC orally for four weeks since the beginning of the treatment. Two other groups were submitted to MCD and H diets for 60 days receiving SNAC only from the 31st to the 60th day.

Results: SNAC administration inhibited the development of NASH in all groups, leading to a marked decrease in macro and microvacuolar steatosis and in hepatic lipid peroxidation in the MCD group. SNAC treatment reversed the development of NASH in animals treated for 60 days with MCD or H diets, which received SNAC only from the 31st to the 60th day.

Conclusions: Oral administration of SNAC markedly inhibited and reversed NASH induced by MCD and H diets in ob/ob mice.

Key words: NASH, Oxidative stress, S-nitroso-N-acetylcysteine, Nitric oxide

INTRODUCTION

Nonalcoholic Fatty Liver Disease (NAFLD) includes the whole spectrum of fatty liver, including nonalcoholic steatosis, steatohepatitis (NASH) and eventually cirrhosis [1]. Although several predisposing factors have been related to NAFLD, such as obesity and diabetes, the pathogenesis of liver cell injury, inflammation and the progression to hepatic fibrosis are still unclear [2,3]. One of the main hypotheses is that the mechanism of hepatocyte injury in NASH is associated to oxidative stress and lipid peroxidation, resulting from the imbalance between prooxidant and antioxidant chemical species. Such imbalance is associated with increased β-oxidation of fatty acids by mitochondria, peroxisomes, cytochrome P450, CYP2E1, and the CYP4A system. These oxidative processes produce free electrons, H_2O_2, and reactive oxygen species (ROS), while depleting the potent antioxidants glutathione, and vitamin E [4-6]. The increased levels of free fatty acids present in the fatty liver provide a perpetuating and propagating mechanism for oxidative stress via lipid peroxidation, with secondary damage to cellular membranes and key organelles such as the mitochondria [6]. Nitric oxide (NO) was already shown to act as a potent inhibitor of lipid peroxidation chain reactions by scavenging propagatory lipid peroxyl radicals and by inhibiting many potential initiators of lipid peroxidation, such as peroxidase enzymes [7-9]. On the other hand, in an oxidative stress setting with the formation of superoxide anion ($O_2^{\bullet-}$), NO forms peroxynitrite ($OONO^-$), a strong oxidant agent which is able to promote tyrosine nitration forming nitrotyrosine (NTY) [10,11]. An abnormal intrahepatic accumulation of NTY in chronic virus hepatitis has already been reported [12] suggesting that the balance between NO and $O_2^{\bullet-}$ may have important implications in NASH, in which oxidative stress seems to have a pivotal role in the onset and/or progression of the disease [8,9]. In addition to its role in the balance between pro and anti oxidant species in the cellular milieu, NO may

affect lipid synthesis in the liver through the inactivation of coenzymeA, which is central to the pathway of fatty acid and cholesterol synthesis [13,14].

NO is believed to coexist in cells with S-nitrosothiols (RSNOs) which are considered to be endogenous NO carriers and donors in mammals [15]. NO covalently bound to the sulfur atom in RSNOs resists oxidant inactivation by oxyhemoglobin and has the same physiological properties of free NO, including its protective action in oxidative stress [16]. RSNOs are compounds, which spontaneously release NO at different rates [17] and can be considered as potential therapeutic agents in a variety of pathologies in which NO may be involved [18]. S-nitroso-N-acetylcysteine (SNAC) is a relatively stable RSNO and a potent vasodilator [19]. RSNOs can be powerful terminators of radical chain-propagation reactions, by reacting directly with $ROO^{\bullet}$ radicals, yielding nitro derivatives (ROONO) as end products, as well as dimmers RS-SR [7,8].

The aim of this study was to evaluate the role of SNAC as a NO donor, in the prevention and treatment of NASH in ob/ob mice fed with methionine-choline deficient or high-fat diets. It was shown for the first time that SNAC can inhibit and revert NASH in these animal models. These data suggest a novel therapeutic potential for the treatment of NASH with NO donors.

MATERIALS AND METHODS

Materials

N-acetyl-L-cysteine (NAC), phosphate buffer saline (PBS, pH 7.4), Low Density Lipoprotein (LDL) from human plasma, copper sulfate and sodium nitrite (Sigma, St. Louis, MO, USA), were used as received. All experiments were carried out using analytical grade water from a Millipore Milli-Q Gradient filtration system.

SNAC Synthesis

SNAC was synthesized from the S-nitrosation of NAC as described elsewhere [17,19]. Fresh stock solutions of SNAC were diluted to 2.4 x 10^{-4} mol/L in phosphate buffer (pH 7.4), before oral administration.

Animals

Male ob/ob mice (Jackson Laboratories, Bar Harbor, Maine, USA), weighing 20-30 g, were housed in temperature and humidity controlled rooms, under 12-h light/dark cycles and were allowed food and water *ad libidum*. All procedures for animal experimentation were in accordance to the Helsinki Declaration of 1975, *(NIH Publication No. 85-23, revised 1996)* and the Guidelines of Animal Experimentation from the University of São Paulo, School of Medicine. NASH was induced in male ob/ob mice by a methionine-choline deficient (MCD) diet or by a high-fat (H) diet. Animals were divided into five groups that received SNAC solution or vehicle by gavage daily for four weeks: 1- MCD group (n = 6): MCD diet plus vehicle; 2 - H group (n = 6): H diet plus vehicle; 3 - MCD/SNAC group (n = 6): MCD diet plus SNAC solution (1.4 mg/kg/day); 4 - H/SNAC group (n=6): H diet plus SNAC solution (1.4 mg/kg/day); 5 - C group (n = 6): Control animals fed a standard diet. Two additional animal groups were submitted to a MCD or H diet for 60 days and started receiving SNAC from the 31st to the 60th day: 6 - MCD/SNAC 31 group (n = 6): MCD diet plus SNAC solution (1.4 mg/kg/day); 7 - H/SNAC 31 group (n = 6): H diet plus SNAC solution (1.4 mg/kg/day). After the treatments, the animals were sacrificed and samples of plasma and liver tissue were collected for biochemical and histological examination.

Laboratory evaluation

Laboratory analysis included the measurements of levels of alanine amininotransferase (ALT), aspartate aminotransferase (AST), cholesterol and triglycerides in the serums of the animals.

Histological analysis

Fragments of liver tissues previously fixed by immersion in formaldehyde saline (10%) solution, were processed and submitted to hematoxylin-eosin (HE) and Masson Trichrome stains for histological analysis. Histological variables were blindly semiquantitated from 0 to 4+, by an experienced pathologist, with respect to: macro and microvacuolar fatty change, zonal distribution, foci of necrosis, pericellular and perivenular fibrosis as well as inflammatory infiltrate.

Oxidative Stress Analysis

Malondialdehyde (MDA) formation, measured as thiobarbituric acid-reactive material, was used to quantify lipid peroxidation in tissues. Tissues (100 mg/mL) were homogenized in 1.15% KCl buffer, and centrifuged at 14,000 × g for 20 min. The supernatant was stored at –70 ºC until the assay. An aliquot of supernatant was added to a reaction mixture of 1.5 mL of thiobarbituric acid (0.8% vol/vol), 200 µL of SDS (8.1% vol/vol), 1.5 mL of acetic acid (20% vol/vol, pH 3.5), and 600 µL of distilled water and heated to 90 °C for 45 min. After cooling to room temperature, the samples were cleared by centrifugation (10,000 × g for 10 min), and their absorbances were measured at 532 nm using 1,1,3,3-tetramethoxypropane as an external standard. The quantity of lipid peroxides was expressed as nanomols of MDA per milligram of protein.

Glutathione (GSH) assay

Tissue samples (100mg/mL) were homogenized in sulfosalacylic acid (5% vol/vol). The homogenates were centrifuged at 10,000 x g for 20 min, and an aliquot of the clear supernatant (20 mL) was combined with 160 mL of Na_2HPO_4 0.3 mol/L and 20 mL of 5-5-9-dithiobis-(2-nitrobenzoic acid) (0.04%) in sodium citrate (1%). After 10 min incubation at room temperature, absorbance was read at 405 nm in a Spectramax microplate reader. Concentrations of GSH were calculated from a standard curve obtained with known concentrations of reduced GSH and expressed as µg GSH per mg protein.

Effect of SNAC on the in vitro LDL oxidation

Oxidation of LDL was induced through the addition of $CuSO_4$ (5 µmol/L) to oxygenated aqueous LDL suspensions (200 µg/mL) in the absence and presence of SNAC (5 and 500 µmol/L). Aqueous LDL suspensions were prepared by diluting LDL 6.3 mg protein/mL to 200 µg protein/mL with EDTA-free PBS and incubated with $CuSO_4$ (5 µmol/L) for 22 h at 37 ºC. Oxidation of LDL results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products, leading to fluorescent free and protein-bound Schiff base conjugates [21,22]. In all cases, fluorescence spectra of such conjugates were firstly recorded in the range 430 to 600 nm in order to characterize the shape and position of the emission peak. All the spectrofluorimetric measurements were performed using a Perkin-Elmer LS-55 luminescence spectrometer with a temperature-controlled cuvette holder thermostatized at 37 ºC. Spectra of the solutions were obtained in 1 cm quartz cuvette. The excitation and emission wavelengths were 360 and 433 nm, respectively. Native LDL (200 µg/mL) served as the control. Fluorescence intensities were used to evaluate the extent of LDL oxidation.

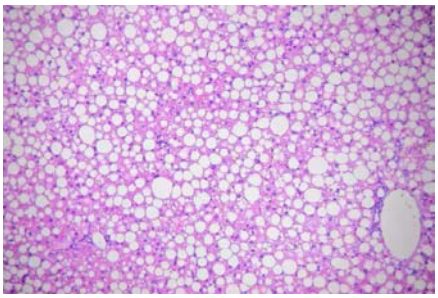
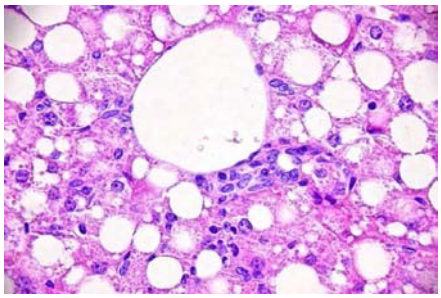
Statistical analysis

Data were expressed as means ± standard deviation (SD). Groups were compared using univariate analysis (ANOVA); *p* values under 0.05 were considered significant.

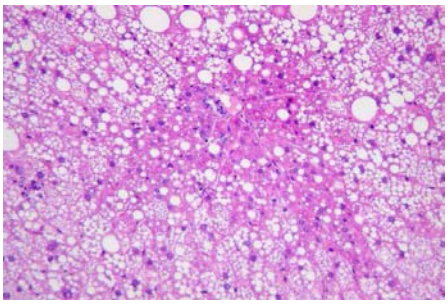
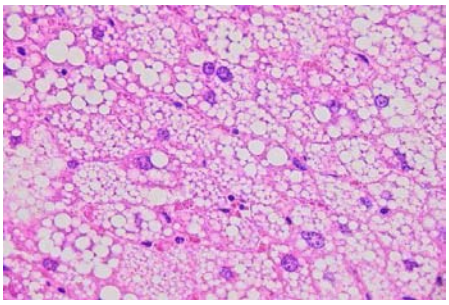
RESULTS

Biochemical and histological analysis

Figure 1 shows the histological features of ob/ob livers of mice fed with MCD and H diets, which received or not SNAC by gavage for four weeks. It can be seen that the MCD group developed diffuse moderate macro and microvacuolar steatosis, hepatocellular ballooning and inflammatory infiltrate (Figure 1a). In the H group, diffuse microvacuolar steatosis was observed, hepatocellular ballooning was not seen and inflammatory infiltrate was smaller than in the MCD group (Figure 1b). SNAC administration led to a marked decrease in histological alterations in both groups. These results show that SNAC treatment can abolish the development of NASH in these animal models (Figures 1c and 1d). Moreover, mice fed with MCD or H diets for 30 days, which started to receive SNAC only from the 31st day and continued receiving SNAC up to the 60th day, did not show histological alterations after the 60th day of treatment (Figures 1e and 1f). These results show that SNAC treatment, started after the onset of the disease, was able to completely reverse NASH both in the MCD and H groups.

(a)

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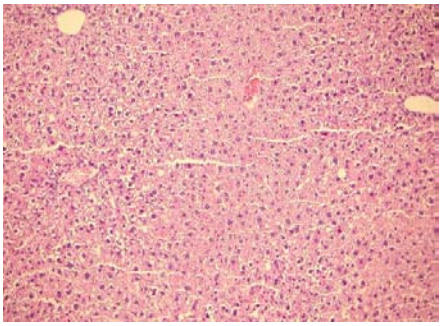
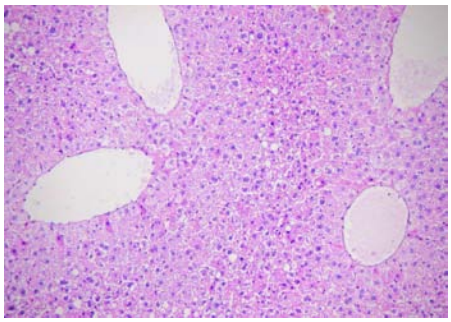
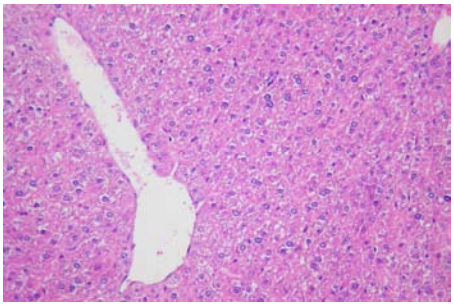
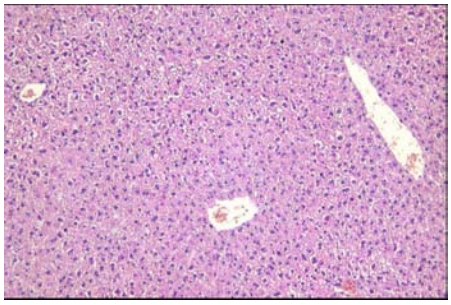



(c) (d)

(e) (f)

Figure 1. Histological features of ob/ob livers of mice fed with methionine-choline deficient diet (MCD group) showing diffuse moderate macro and microvacuolar steatosis, hepatocellular ballooning and inflammatory infiltrate **(a)**. Histological features of livers of mice fed with high-fat diet (H group) showing diffuse microvacuolar steatosis and slight inflammatory infiltrate **(b)**. Histological features of livers of mice fed with MCD or H diets, which received SNAC orally (MCD/SNAC group **(c)** and H/SNAC group **(d),** respectively) showing no histological alterations in both cases. Histological features of livers of mice fed with MCD or H diets for 30 days which received SNAC from the 31^{st} to the 60^{th} days (MCD/SNAC 31 group **(c)** and H/SNAC 31 group **(d),** respectively), showing no histological alterations in both cases. Magnifications: (a) and (b) left and (c), (d), (e) and (f) = 100X; (a) and (b) right = 400X.

Serum AST and ALT levels were highly elevated in the MCD and H groups (specially in the H group). Cholesterol level was slightly above the normal value in both groups. Triglycerides levels were unaltered in both groups. SNAC treatment led to a marked decrease in the levels of ALT and AST and to a small decrease in the levels of cholesterol in the two groups. The triglycerides level was also decreased in the MCD/SNAC group relative to the MCD group but increased in the H/SNAC group, relative to the H group (Table 1).

Lipid peroxidation

Figure 2 shows the MDA levels in liver samples of animals fed with C, MCD and H diets, which received or not SNAC by gavage for four weeks. It can be seen that basal hepatic lipoperoxide concentrations were significantly increased in the MCD and H groups, relative to the C group. The MDA level was significantly reduced in the MCD/SNAC group relative

to the MCD group. However there was no significant change in the MDA level when comparing H and H/SNAC groups.

Table 1. Levels of alanine amininotransferase (ALT), aspartate aminotransferase (AST), cholesterol and triglycerides in the serum of ob/ob mice fed with methionine-choline deficient diet (MCD) or high-fat diet (H), which received or not SNAC by gavage for four weeks (MCD/SNAC and H/SNAC, respectively).

Group	N	AST (U/L)	ALT (U/L)	Cholesterol (U/L)	Triglycerides (U/L)
MCD	6	623±6	230±1	105±1	93±3
MCD/SNAC	6	192±6*	21±6*	89±1	62±1
H	6	3405±5	527±6	123±2	46±3
H/SNAC	6	146±3*	24±8*	89±1	75±3

Normal values in U/L for: AST = 10-34 mg/dl; ALT = 10-44 mg/dl: cholesterol and triglycerides = 45-89.

*p<0.05; MCD x MCD/SNAC; H x H/SNAC
Data expressed as mean ± SD.

Figure 3 shows the reduced glutathione levels in liver samples of animals fed with C, MCD and H diets, which received or not SNAC by gavage for four weeks. It can be seen that basal hepatic reduced glutathione levels were significantly lowered in the MCD and H groups relative to the C group. In the MCD/SNAC group the GSH level was significantly increased, however there was no significant change in the reduced glutathione levels when comparing H and H/SNAC groups.

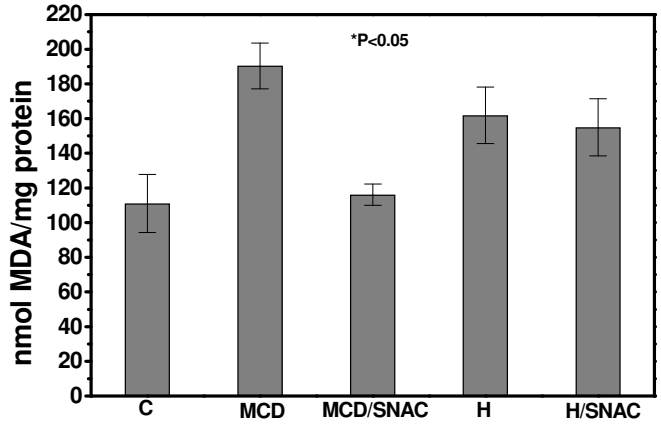


Figure 2. Malondialdehyde (MDA) level in liver samples of animals fed with control (C), methionine-choline deficient (MCD) and high-fat (H) diets, which received or not SNAC by gavage for four weeks. Data expressed as mean ± SD; $^{*}p < 0.05$ MCD x MCD/SNAC.

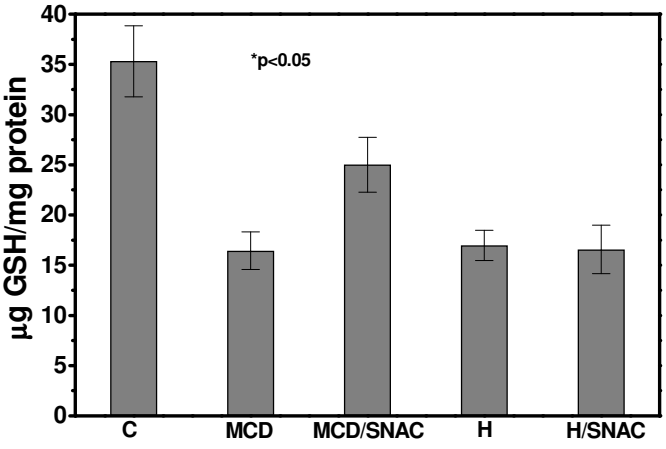


Figure 3. Glutathione (GSH) level in liver samples of animals fed with control (C), methionine-choline deficient (MCD) and high-fat (H) diets, which received or not SNAC by gavage for four weeks. Data expressed mean ± SD; $^{*}p < 0.05$ MCD x MCD/SNAC.

% of Mass Change

Figure 4 shows the percentages of mass change of the animals fed with C, MCD and H diets, which received or not SNAC by gavage for four weeks. Control animals showed an increase of *ca*. 13% in body mass after four weeks. While animals fed with MCD diet showed a slight increase in body mass, animals fed with H diet had an average increase of ca 28% in their body mass. Treatment with SNAC practically abrogated mass increase in the MCD group and led to a significant reduction in the mass increase of the H group.

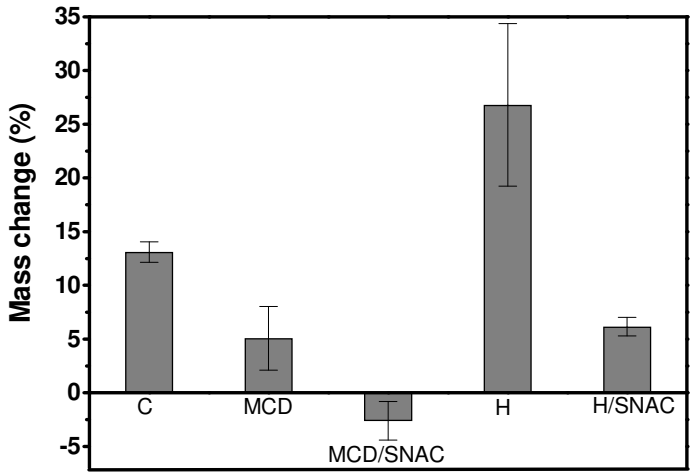


Figure 4. Percentage of mass change in animals fed with control (C), methionine-choline deficient (MCD) and high-fat (H) diets which received or not SNAC by gavage for four weeks. Data expressed as mean ± SD

Effect of SNAC on the LDL oxidation

Figure 5 shows the emission intensity changes in the *in vitro* oxidation of LDL (200 μg/mL) by $CuSO_4$ (5 μmol/L) in the absence (a) and presence of the SNAC (5 and 500 μmol/L) (b and c, respectively) based on the emission peak with maximum at 433 nm (inset). It can be seen that incubation of LDL with Cu (II) ions in the presence of SNAC 5 μmol/L led to a decrease in the extent of LDL oxidation, reflected in the lower emission intensity obtained. Incubation with SNAC 500 μmol/L completely inhibited LDL oxidation.

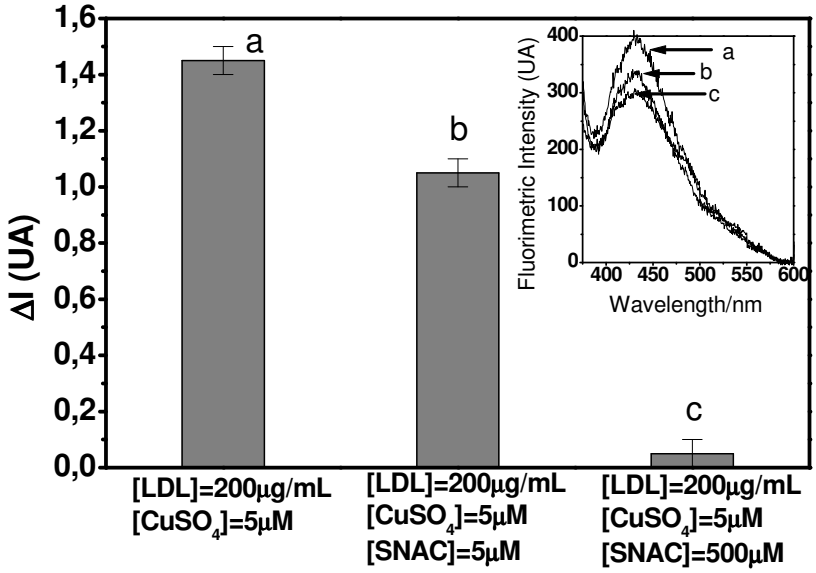


Figure 5. Emission intensity changes in the oxidation of low density lipoprotein (LDL) (200 μg/mL) by $CuSO_4$ (5 μmol/L) in the absence (a) and presence of SNAC (5 and 500 μmol/L) (b and c respectively), measured at 433nm with excitation at 360 nm. Inset: Emission spectra of LDL (200 μg/mL) incubated with $CuSO_4$ (5 μmol/L) in the absence (a) and presence of SNAC (5 and 500 μmol/L) (b and c respectively).

DISCUSSION

Nonalcoholic steatohepatitis is ascribed to an imbalance between the excessive uptake of free fatty acids by the liver with subsequent increase in triglycerides (TG) synthesis, and the reduction of fatty acid oxidation and TG secretion (VLDLs) in the liver. Several predisposing factors have been related to NASH especially obesity, insulin resistance and diabetes mellitus, along with other components of the metabolic syndrome (arterial hypertension, hypertriglyceridemia and visceral distribution of adipose tissue). Leptin-deficient ob/ob mice show many characteristics of obesity, including excess peripheral adiposity as well as severe hepatic steatosis, at least in part, due to increased hepatic lipogenesis [23]. Lipogenesis in hepatocytes depends upon diet, substrate availability and hormone status and is stimulated by carbohydrate diet and an overeating diet with reasonable high fat content (35%) enriched with saturated fatty acid [23,24]. Methionine and choline deficient diet is a classical model of NAFLD, where Cyp2E1 is up regulated and the animals develop steatosis, steatohepatitis and hepatic fibrosis [25]. Although, ob/ob mice develop liver steatosis sponteanously these animals do not develop NASH spontaneously, requiring a second hit with MCD or H diets. In the present study, ob/ob mice received high-fat diet enriched with lard and egg yolk (saturated fatty acid) or MCD diet and developed, in both diet models, classical patterns of NASH. The main differences between the two diet models were reflected in the histological patterns: It was observed that the MCD diet caused predominantly macrovacuolar steatosis, and more inflammation and ballooning than the H diet, which caused predominantly microvacuolar liver steatosis, without hepatocellular ballooning and slight inflammation. Thus, these two models can be used to study nonalcoholic steatohepatitis.

It was shown in this work that SNAC exerts a strong inhibitory effect in NASH induced in ob/ob mice by both diets. Also, NASH was reversed by SNAC after 30 days of treatment of these animals with MCD or H diets, even with the continuity of these diets until the 60th day. The observation that SNAC inhibits LDL oxidation in vitro in a dose-dependent manner, reinforce the proposal that SNAC acts in vivo by blocking lipid peroxidation and that this is an important mechanism in the onset and progression of NASH. This result is correlated with the detection of a decrease in the MDA level (and an increase in the GSH level) of the MCD-deficient animals, which received SNAC by gavage, once MDA and GSH are two well-known markers of oxidative stress. Several works have already shown that markers of oxidative stress are increased, while levels of endogenous antioxidants (eg. vitamin E, GSH) are decreased in NAFLD [26-28]. In such situations the microsomal enzymes CYPs 2E1 and 4A are believed to be involved in the fatty acid oxidation in the liver of humans contributing to the pathogenesis of this disease [6]. The observed reduction in the MDA concentration, concomitantly with the increase in GSH concentration in the liver tissue of the SNAC-treated animals, indicates that SNAC acted as an inhibitor of lipid/lipoproteins oxidation in the present models. This result is in accordance with the known fact that NO can play a potent oxidant-protective role *in vivo* by inhibiting oxygenase-dependent lipid and lipoprotein oxidation [8] and suggests that NASH may be associated with an impairment of the endogenous NO production in the liver.

The protective action of SNAC is in accordance with the study reported by Laurent et al [31] where these authors observed that the concentrations of nitrite and nitrate were increased, while the nitrosothiol concentration was decreased in ob/ob mice. This result indicates that in these animals, the oxidative stress situation led to a consumption of the endogenous nitrosothiol pool. As a result the nitrite and nitrate concentration is expected to increase as observed. Conversely, the observed increase in nitrosothiol concentration,

simultaneously with the decrease in nitrite and nitrate concentrations with the administration of NAC, in this work, can be understood as a preservation of the nitrosothiol pool, due to the antioxidant action of NAC. Although NAC can also act as an antioxidant *in vivo* it is not expected that NAC will be formed as a result of the antioxidant action of SNAC. Treatment of ob/ob mice submitted to MCD diet, with NAC in the place of SNAC, in the same conditions described in the experimental part, did not inhibit NASH (data not shown). On the other hand, accumulation of oxidized lipid/lipoproteins in the liver may not be the primary cause of NAFLD. It has been demonstrated that endogenously produced NO may affect lipid synthesis in the liver by reacting with the active cysteine thiol group of coenzimeA to form inactive S-nitrosoCoA [13]. This S-nitrosation reaction may be the fundamental mechanism underlying the experimental fact that endogenous NO impairs protein synthesis and that exogenous NO donors such as S-nitrosoglutathione (GSNO) can modulate lipogenesis and ketogenesis in isolated cultures of hepatocytes [14]. More recently it has been demonstrated that oral administration of SNAC can prevents the onset of NAFLD in Wistar rats fed with choline-deficient diet [29] and in ob/ob mice fed with MCD diet [30]. In the last case, SNAC treatment led to the downregulation of several genes belonging to oxidative phosphorylation, fatty acid biosynthesis, fatty acid metabolism and glutathione metabolism pathways. Therefore, the protective action of SNAC may involve both gene regulation and post-translational enzyme modification.

CONCLUSIONS

Our results suggest that NASH may be associated with an impaired NO production in hepatocytes and that the oral treatment with SNAC as an exogenous NO source may block

and reverse the development of NASH. These results can have clinical implications, regarding novel therapeutic strategies for NASH.

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5.3. Material suplementar

Nos resultados suplementares abaixo são mostrados os espectros de emissão após a oxidação da lipoproteína de baixa densidade (LDL) catalisada por íons Cu(II) na ausência (Fig. 1) e na presença de S-nitrosoacetilcisteína (SNAC) (Fig. 2). Pode-se observar nestes resultados, que a presença de SNAC em maior concentração (500 μM) leva a uma inibição completa da peroxidação lipídica. A parte experimental, assim como a discussão detalhada destes experimentos se encontram no manuscrito submetido ao *Journal of the American College of Nutrition*.

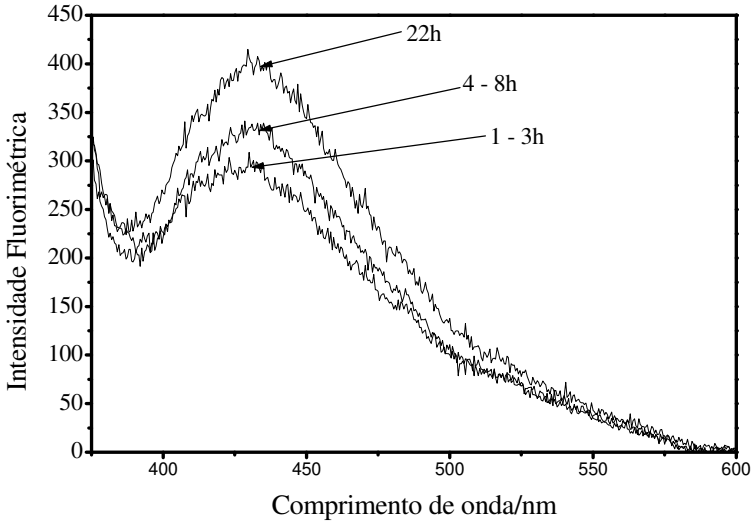

Fig. 1. Espectros de fluorescência após a oxidação da lipoproteína de baixa densidade (LDL) (concentração final 200 μg/mL) catalisada por íons Cu(II)

(concentração final 5 μM) a T = 37ºC. Comprimentos de onda ex/em = 360/430 nm.

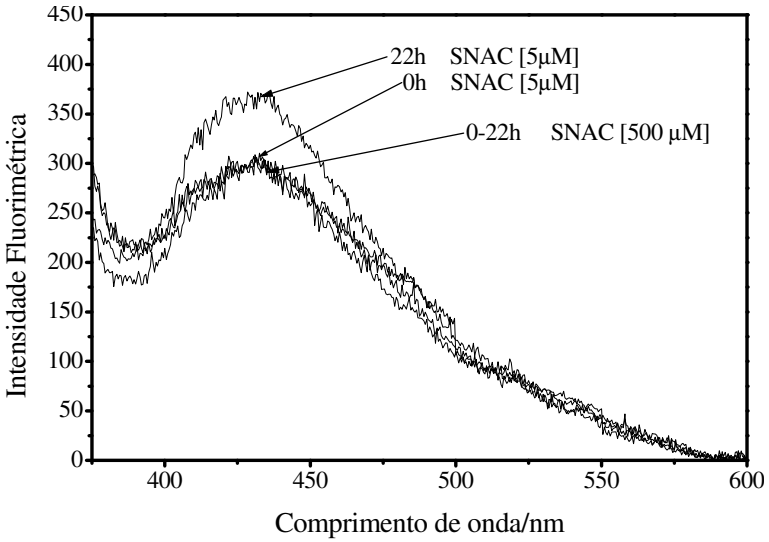

Fig. 2. Espectros de emissão após a oxidação da lipoproteína de baixa densidade (LDL) (concentração final 200 μg/mL) catalisada por íons Cu (II) (concentração final 5 μM) na presença de S-nitrosoacetilcisteína (SNAC) (concentrações finais de 5 e 500 μM). T = 37ºC; Comprimentos de onda ex/em = 360/430 nm.

Deve-se observar que, tanto na Fig. 1 como na Fig. 2, a LDL já apresenta uma banda de emissão associada com a presença de hidroperóxidos, antes mesmo da peroxidação catalizada pela adição de íons Cu(II). Isto é, trata-se de LDL já parcialmente oxidada.

6. Conclusões

Os S-nitrosotióis primários (RSNOs) utilizados neste trabalho, S-nitroso-N-acetilcisteína (SNAC), S-nitrosoglutationa (GSNO) e S-nitrosocisteína (CISNO) exercem ações antioxidantes que podem ser associadas com o bloqueio da peroxidação do ácido linoleico (AL) e da lipoproteína de baixa densidade (LDL) invitro. Estas ações antioxidantes são significativamente maiores que as obtidas pelos seus tióis correspondentes em condições equimolares.

As reações entre os RSNOs e radicais peroxila ($LOO^{\bullet}$)/alcoxila ($LO^{\bullet}$) ou hidroperóxido (LOOH) bloqueiam a propagação da peroxidação lipídica levando à formação de produtos nitrogenados do AL oxidado, detectados pela redução posterior destes produtos a NO livre e de produtos nitrogenados do LDL oxidado, evidenciados pelo consumo de NO livre detectado eletroquimicamente.

A formação posterior de adutos de lisina com produtos da oxidação do AL *in vitro* pode ser usada para a avaliação da extensão das reações de peroxidação lipídica do LA.

A administração por via oral de RSNOs oferece perspectivas para o tratamento da esteatose hepática não alcoólica.

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