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GLUTAMINA PROTEGE DOS DANOS NO INTESTINO E FÍGADO EM MODELO DE ISQUEMIA E REPERFUSÃO INTESTINAL

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Tese apresentada como requisito parcial para obtenção do título de Doutor em Medicina: Ciências Médicas, da Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Medicina: Ciências Médicas.

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RESUMO

Introdução: A lesão de isquemia e reperfusão (I/R) intestinal pode causar danos celular e tecidual local e em órgãos a distância. Alguns fatores podem estar envolvidos nesses processos, tais como: a geração de espécies reativas de oxigênio, mediadores inflamatórios, óxido nítrico (NO) e estresse do retículo endoplasmático (RE). Devido ao envolvimento do estresse oxidativo nas lesões de I/R intestinal, algumas opções terapêuticas com antioxidantes estão sendo estudadas e testadas nas lesões de I/R intestinal. **Objetivo:** Avaliar o efeito local e sistêmico da glutamina no intestino e fígado de animais submetidos à I/R intestinal. **Métodos:** Foram utilizados 20 ratos wistar machos divididos em quatro grupos: Sham operated (SO), Glutamina+Sham operated (G+SO), Isquemia e reperfusão intestinal (I/R); Glutamina+Isquemia e reperfusão intestinal (G+I/R). Os animais foram anestesiados e, após, realizada a laparotomia mediana e identificação da artéria mesentérica superior. A artéria foi clampeada por 30 e após esse tempo, os animais foram mantidos por mais 15 minutos em reperfusão intestinal. A glutamina foi administrada por via intraperitoneal, na dose de 25 mg/Kg diluída em 1 mL de solução fisiológica. O tratamento foi realizado uma vez ao dia, durante 48 horas antes da indução da isquemia. Foram realizadas análises séricas para a função de integridade hepática através das enzimas aspartato aminotransferase (AST), alanina aminotransferase (ALT) e fosfatase alcalina (FA) e danos ao DNA pelo ensaio cometa. Realizamos a análise histológica dos tecidos através da coloração de Hematoxilina-Eosina e imunohistoquímica para avaliar a quantidade de células marcadas com os anticorpos monoclonais IL-1β, IL-6, TNF-α e NF-κB no intestino e fígado. O homogeneizado do intestino e fígado foram utilizados para a avaliação dos níveis de lipoperoxidação (LPO) através das substâncias que reagem ao ácido tiobarbitúrico (TBARS), avaliação da atividade das enzimas antioxidantes catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx), determinação dos níveis de glutationa (GSH), avaliação dos metabólitos do óxido nítrico (nitritos/nitratos) e para as análises moleculares das proteínas iNOS, NF-κB, Nrf2, Keap1, SOD, NQO1, HSP70, GRP78 e ATF-6 por *Western Blot.* **Resultados:** O pré-tratamento com glutamina reduziu os níveis de LPO, óxido nítrico, danos ao DNA, bem como as enzimas de integridade hepática. Observamos que a glutamina foi eficaz na preservação da arquitetura tecidual do intestino e fígado dos animais

submetidos a I/R intestinal, reduzindo parâmetros como infiltrado inflamatório, perda das vilosidades intestinais e necrose. Constatou-se que a glutamina ativou a via do Nrf2 e as enzimas antioxidantes, reduziu o dano celular, e inibiu o estresse do RE, além de reduzir os mediadores do processo inflamatório. **Conclusão**: Neste estudo, sugerimos que o pré-tratamento com a glutamina desempenhou um papel protetor tanto no intestino como no fígado dos animais submetidos a I/R intestinal, demonstrado pelas análises estudadas, possivelmente pela sua ação antioxidante e anti-inflamatória.

Palavras-chave: estresse oxidativo, glutamina, isquemia e reperfusão, processo inflamatório

ABSTRACT

Background: Injury by intestinal ischemia and reperfusion (I/R) can cause local and cellular damage to tissues and organs at distance. Some factors may be involved in those processes, such as the generation of reactive oxygen species, inflammatory mediators, nitric oxide (NO) and endoplasmic reticulum stress. Due to the involvement of oxidative stress in intestinal I/R lesions, some therapeutic options with antioxidants are being studied and tested in order to reduce these damages. **Objective:** To evaluate the local and systemic effect of glutamine in the intestine and liver of animals submitted to intestinal I/R. **Methods:** Twenty male Wistar rats were divided into four groups: Sham operated (SO), Glutamine+Sham operated (G+SO), Intestinal Ischemia and reperfusion (I/R); Glutamine+intestinal ischemia and reperfusion (G+I/R). The animals were anesthetized and after we performed the median laparotomy and identification of the superior mesenteric artery. The artery was clamped for 30 minutes and after that the animals were maintained for another 15 minutes in intestinal reperfusion. Glutamine was administered intraperitoneally at a dose of 25 mg/kg diluted in 1 ml of saline solution. Treatment was performed once daily for 48 hours prior to induction of ischemia. Serum samples for hepatic integrity were collected, and the enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (FA) were accessed. DNA damage was evaluated by the comet assay. We performed the histological analysis of the tissues through the staining of Hematoxylin-Eosin and immunohistochemistry, in order to evaluate the amount of cells labeled with the monoclonal antibodies IL-1β, IL-6, TNF-α and NF-κB in the intestine and liver. The intestinal and liver homogenates were used to evaluate the levels of lipoperoxidation (LPO) through thiobarbituric acid reactive substances (TBARS), evaluation of the activity of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), determination of glutathione levels (GSH) and nitric oxide (nitrites/nitrates), and for the molecular analyzes of the iNOS, NF-κB, Nrf2, Keap1, SOD, NQO1, HSP70, GRP78 and ATF-6 proteins we performed Western blot analysis. **Results:** Pretreatment with glutamine reduced levels of LPO, nitric oxide, DNA damage, as well as liver integrity enzymes. We observed that glutamine was effective in preserving the intestinal and liver tissue architecture of animals submitted

to intestinal I/R, reducing parameters such as inflammatory infiltrate, loss of intestinal villi and necrosis. It was found that glutamine activated the Nrf2 pathway and antioxidant enzymes, reduced cell damage, and inhibited endoplasmic reticulum stress in addition to reducing mediators of the inflammatory process. **Conclusion:** In this study, we suggest that pretreatment with glutamine played a protective role in both intestine and liver of animals submitted to intestinal I/R, demonstrated by the present analyzes, possibly for its antioxidant and anti-inflammatory action.

Key Words: glutamine, oxidative stress, inflammatory process, ischemia and reperfusion

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LISTA DE ABREVIATURAS E SÍMBOLOS

α	*Alfa*
β	*Beta*
ADP	Adenosina difosfato
ALT	Alanina aminotransferase
AMP	Adenosina monofosfato
AMS	Artéria mesentérica superior
ARE	Elemento de resposta antioxidante
AST	Aspartato aminotransferase
ATF-6	do inglês - *activating transcription fator-6*
ATP	Adenosina trifosfato
Ca^{2+}	Cálcio
CAT	Catalase
CHOP	do inglês - *CCAAT/Enhancer-binding protein homologous protein*
eNOS	Óxido nítrico sintase endotelial
EO	Estresse oxidativo
ERSE	Elemento de resposta ao estresse do retículo endoplasmático
ERN	Espécies reativas de nitrogênio
ERO	Espécies reativas de oxigênio
FA	Fosfatase alcalina
Fe	Ferro
Gln	Glutamina
GPx	Glutationa peroxidase
GRP78	Proteina regulada por glicose 78kDa
GSH	Glutationa
GSSG	Glutationa oxidada
H_2O	Água
H_2O_2	Peróxido de Hidrogênio
HSE	Elemento de choque térmico
HSF	Fator de choque térmico
HSP	Proteínas de choque térmico
HSP70	Proteína de choque térmico 70
IL	Interleucina

IL-1β	Interleucina-1β
IL-6	Interleucina-6
iNOS	Óxido nítrico sintase induzível
I/R	Isquemia e reperfusão
IRE-1	do inglês - *inositol-requiring enzyme-1*
Keap1	do inglês - *Kelch-like ECH associated protein 1*
LPO	Lipoperoxidação
LPS	Lipopolissacarídeos
MDA	Malondialdeído
NADH	Nicotinamida adenina dinucleotídeo
NADPH	Fosfato nicotinamida adenina dinucleotídeo
NF-κB	Fator de transcrição nuclear *kappa* B
NO	Óxido nítrico
NOS	Óxido nítrico sintase
nNOS	Óxido nítrico sintase neuronal
NQO1	NADPH quinona oxidorredutase 1
Nrf2	Fator de transcrição nuclear eritróide 2
$^1O_2$	Oxigênio *singlet*
O_2	Oxigênio
$O_2^{\cdot-}$	Ânion superóxido
$^{\cdot}OH$	Radical hidroxila
$ONOO^-$	Peroxinitrito
PERK	do inglês - *protein kinase RNA-like ER-kinase*
RE	Retículo endoplasmático
RL	Radicais livres
SDMO	Síndrome de disfunção de múltiplos órgãos
SRIS	Síndrome de resposta inflamatória sistêmica
SOD	Superóxido dismutase
TBARS	Substâncias que reagem ao ácido tiobarbitúrico
TNF-α	Fator de necrose tumoral *alfa*
UPR	do inglês - *Unfolded Protein Response*
UTI	Unidade de terapia intensiva
XD	Xantina desidrogenase
XO	Xantina oxidase

SUMÁRIO

1 INTRODUÇÃO

A isquemia é um evento caracterizado pela restrição parcial ou total do fluxo sanguíneo arterial ou venoso a determinados tecidos e órgãos. Nas situações onde o fluxo sanguíneo é reduzido, comprometem a função celular e integridade do organismo (Cerqueira *et al.*, 2005; Camara-Lemarroy, 2014).

A reperfusão ocorre para restabelecer o suprimento sanguíneo; porém, nesta etapa ocorrem alterações que podem causar dano celular, tecidual aos órgãos isquêmicos denominado lesão de isquemia e reperfusão (I/R). Muitos agentes agressores estão envolvidos nas lesões de I/R como: geração de espécies reativas de oxigênio (ERO), óxido nítrico (NO), liberação de citocinas pró-inflamatórias, fator de transcrição nuclear *kappa* B (NF-κB) e estresse de RE (Bilecová-Rabajdová *et al.*, 2010; Medling *et al.*, 2010; Granger e Kvietys, 2015).

O intestino é suscetível às lesões de I/R sendo que alguns processos estão envolvidos na I/R intestinal tais como: obstrução de vasos por aterosclerose, trombose, hérnias, enterocolites necrosantes e pós-transplante (Arumugam *et al.*, 2004; Cerqueira *et al.*, 2005; Lenaerts *et al.*, 2013).

Na I/R intestinal, além do dano tecidual, existe o risco de originar lesões teciduais e processo inflamatório em órgãos à distância como: fígado, pulmão e coração, podendo ocasionar a síndrome de resposta inflamatória sistêmica (SRIS) e, consequentemente, a síndrome de disfunção de múltiplos órgãos (SDMO) levando à falência de diferentes sistemas e órgãos (Yasuhara *et al.*, 2005; Fan *et al.*, 2014; Zabot *et al.*, 2014).

O entendimento dos mecanismos envolvidos na I/R intestinal é extremamente importante para a descoberta de estratégias terapêuticas para minimizar seus danos. Devido ao envolvimento das ERO nas lesões de I/R intestinal, alguns antioxidantes estão sendo estudados (Sakrak *et al.*, 2008; Sato *et al.*, 2011; Onder *et al.*, 2012; Zabot *et al.*, 2014).

A glutamina (Gln) é um aminoácido livre mais abundante no plasma e tem uma importante função de preservar a função imune e atenuar os processos inflamatórios intestinais, pois atua sobre o estresse oxidativo (EO) inibindo/reduzindo a geração de ERO. A Gln desempenha um papel crítico na manutenção da função

da barreira intestinal, assim preservando a permeabilidade e a integridade do epitélio intestinal, reduzindo a translocação bacteriana para a circulação sistêmica (Fillmann *et al.*, 2007; Kretzmann *et al.*, 2008; Demirkan *et al.*, 2010; Vasconcelos *et al.*, 2011; Zabot *et al.*, 2014; Spodenkiewicz *et al.*, 2016).

Sendo assim, neste trabalho avaliamos os efeitos da Gln na lesão local intestinal e de órgãos à distância como o fígado, visto que na maioria dos estudos são observados somente os danos locais gerados nos órgãos que foram induzidos ao modelo de I/R.

A estrutura desta tese está dividida em secções: 1– Introdução; 2- referencial teórico, 3- marco conceitual, 4- justificativa, 5- objetivos, 6- referências bibliográficas, 7- artigos, 8- considerações finais, 9- perspectivas futuras, 10- anexos. Seguindo assim, as normas do Programa de Pós Graduação em Medicina: Ciências Médicas da Universidade Federal do Rio Grande do Sul.

2 REFERENCIAL TEÓRICO

2.1 Estratégia para localizar e selecionar informações

A revisão da literatura para o desenvolvimento deste estudo está focada nos aspectos relacionados à isquemia e reperfusão intestinal e seus possíveis tratamentos que envolvem a terapia com antioxidantes. A estratégia de busca envolveu as seguintes bases de dados: PubMed e SciELO, sendo realizada através das palavras-chave e suas combinações (figura 1). Além dessas bases de dados citadas também foram utilizadas dissertações e teses de universidades nacionais e internacionais para contemplar a pesquisa.

PubMed	Palavras chave	SciELO
2874	1. Ischemia and reperfusion intestinal	86
996143	2. Liver	6454
556364	3. Inflammation	3 349
169341	4. Oxidative stress	1684
150727	5. Nitric oxide	992
63236	6. Heat shock protein	112
75	7. Endoplasmatic reticulum stress	1
39440	8. Glutamine	285

PubMed combinações	SciELO combinações
1+2= 460	1+2= 7
1+3= 549	1+3= 8
1+4= 288	1+4= 13
1+5= 310	1+5= 2
1+6= 41	1+6= 0
1+7= 6	1+7= 0
1+8= 59	1+8= 3
1+2+3= 91	1+2+3= 1
1+2+3+4= 10	1+2+3+4= 0
1+2+3+4+5= 1	1+2+3+4+5= 0
1+2+3+4+5+6= 0	1+2+3+4+5+6= 0
1+2+3+4+5+6+7= 0	1+2+3+4+5+6+7= 0
1+2+3+4+5+6+7+8= 0	1+2+3+4+5+6+7+8= 0

Figura 1: Estratégia de busca utilizada para as referências bibliográficas.
Fonte: Autora.

2.2 Isquemia e reperfusão

A isquemia caracteriza-se pela obstrução parcial ou total do fluxo sanguíneo a um órgão específico ou tecido. A diminuição do fluxo sanguíneo compromete o suprimento de oxigênio (O_2) necessário para a função celular normal e leva a uma série de alterações que envolvem acúmulo de metabólitos e de mediadores químicos, vias de sinalização, comprometimento da fosforilação oxidativa, diminuição de O_2 e de nutrientes para o tecido ou órgão lesado. O efeito prejudicial da isquemia está associado à ativação de enzimas citotóxicas, geração de ERO e ERN, produção de mediadores inflamatórios e ativação de alguns fatores de transcrição (Carden e Granger, 2000; Kalogeris *et al.*, 2012; Cámara-Lemarroy, 2014).

No momento da isquemia ocorre uma redução de O_2 e diminuição da nicotinamida adenina dinucleotídeo (NADH) e de adenosina trifosfato (ATP) e acentuado acúmulo de cálcio (Ca^{2+}) e a enzima ATP é degradada em adenosina difosfato (ADP) e adenosina monofosfato (AMP) pela ação de proteases. Durante a isquemia, a enzima xantina desidrogenase (XD) é convertida em xantina oxidase (XO) e acumula-se nos tecidos (Battelli *et al.*, 2014).

A XO depende de O_2 para metabolizar a hipoxantina. E no período da reperfusão, leva ao acúmulo dessa enzima que se degrada em xantina levando à formação de radicais livres (RL) como o radical ânion superóxido ($O_2 \cdot^-$), o qual é dismutado em peróxido de hidrogênio (H_2O_2) que pode reagir com ferro (Fe) e assim formar o potente radical hidroxila (Mc Cord, 1985; Cerqueira *et al.*, 2005; Granger e Kvietys, 2015) (figura 2).

As ERO e os produtos da reação inflamatória são potentes mediadores envolvidos com a destruição tecidual, amplificação do processo inflamatório, quimiotaxia e na ativação de diferentes vias mediadoras da resposta inflamatória, na qual envolvem diversas citocinas inflamatórias como interleucinas (IL) pró-inflamatórias e o fator de necrose tumoral *alfa* (TNF-α) (Granger *et al.*, 1986).

As complicações causadas pela isquemia, nos diferentes tecidos, dependem do seu período de duração e podem resultar em lesões e morte celular, decorrentes do restabelecimento da circulação sanguínea, possivelmente por um fenômeno de

transição de permeabilidade da membrana mitocondrial (Arumugam *et al.*, 2004; Silveira e Yoshida, 2004).
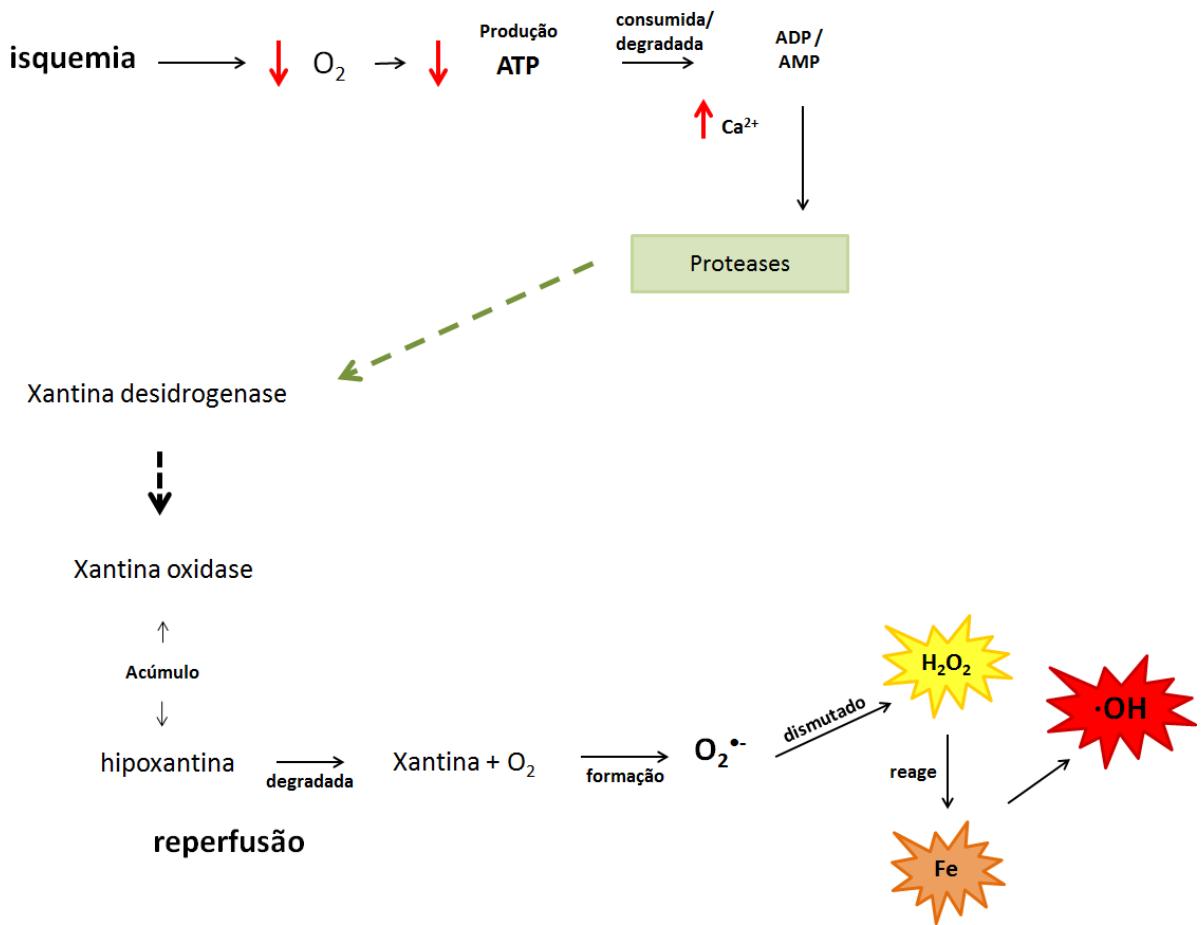
Figura 2: Sequência de eventos químicos que ocorrem na I/R.
Fonte: Adaptada (Granger e Kvietys, 2015).

Paradoxalmente, as lesões causadas pela restauração do fluxo sanguíneo para o órgão isquêmico são na maioria das vezes superiores às lesões causadas pelo evento isquêmico propriamente dito (Mallick *et al.*, 2004; Cámara-Lemarroy, 2014). A reintrodução do fluxo sanguíneo, o qual é um fator necessário para o reequilíbrio da homeostasia no local da isquemia, leva a uma série de alterações funcionais, estruturais, metabólicas e enzimáticas como o aumento da permeabilidade vascular, agregação plaquetária, quimiotaxia de células polimorfonucleares, lesão tecidual, apoptose e necrose celular, danos sistêmicos, além das alterações celulares citadas na figura abaixo (figura 3) (Collard e Gelman, 2001; Eltzschig e Eckle, 2011; Gorsuch *et al.*, 2012; Granger e Kvietys, 2015).

Alteração do potencial de membrana

Desorganização do citoesqueleto de órgãos e tecidos

Aumento do Ca^{2+}/Na intracelular

Alterações celulares I/R

Edema celular

Acidose celular

Diminuição da fosfocreatina

Figura 3: Alterações celulares induzidas pela I/R.
Fonte: Adaptada (Collard e Gelman, 2001).

Os efeitos da I/R estão associados a diferentes órgãos, porém parecem ser agravados quando o órgão em questão é o intestino (tabela 1) (Eltzschig e Eckle, 2011; Abudunaibi *et al*., 2015; Kierulf-Lassen *et al*., 2015; Minutoli *et al*., 2016). Tal comprovação é devido à suscetibilidade desse órgão à lesão de I/R sendo relacionado a lesões em órgãos adjacentes relatado como um evento com alta taxa de morbi/mortalidade (Cerqueira *et al*., 2005).

Tabela 1: Exemplos de lesão por isquemia e reperfusão.

Órgão afetado	Manifestação clínica
Coração	Síndrome coronariana aguda
Rim	Lesão renal aguda
Cérebro	Acidente vascular encefálico
Intestino	Isquemia e reperfusão intestinal, Falência múltipla de órgãos

Fonte: Adaptada (Eltzschig e Eckle, 2011).

2.2.1 Isquemia e reperfusão intestinal

O intestino delgado é o maior órgão endócrino no corpo e é um dos mais importantes órgãos da função imune. Tem como função básica a digestão e a absorção dos componentes dietéticos. O órgão é envolvido por ricos suprimentos vasculares, neurais e linfáticos, todos atravessando o mesentério. A artéria mesentérica superior (AMS) e o tronco celíaco são responsáveis por aproximadamente 95% do fluxo sanguíneo para o fígado, pâncreas e intestino (Lynch e Dauphinee, 2005).

A obstrução completa e definitiva dos vasos pode ocasionar danos às células epiteliais, alteração na integridade, fluidez e permeabilidade da mucosa intestinal, distúrbios hidroeletrolíticos e de equilíbrio ácido-alcalino e com a consequente reintrodução sanguínea pode ocorrer, choque circulatório, formação de ERO e ERN, ativação de leucócitos e liberação de citocinas pró-inflamatórias (Herbert e Steele, 2007; Grootjans *et al.*, 2010; Kalogeris *et al.*, 2012).

A isquemia intestinal pode ocorrer frequentemente em várias situações clínicas e são difíceis de serem diagnosticadas devido às múltiplas etiologias que podem culminar nesse quadro. Desta forma, apresentam uma taxa de mortalidade relativamente alta (50-70%) (figura 4) (Gonzalez *et al.*, 2015; Mastoraki *et al.*, 2016).
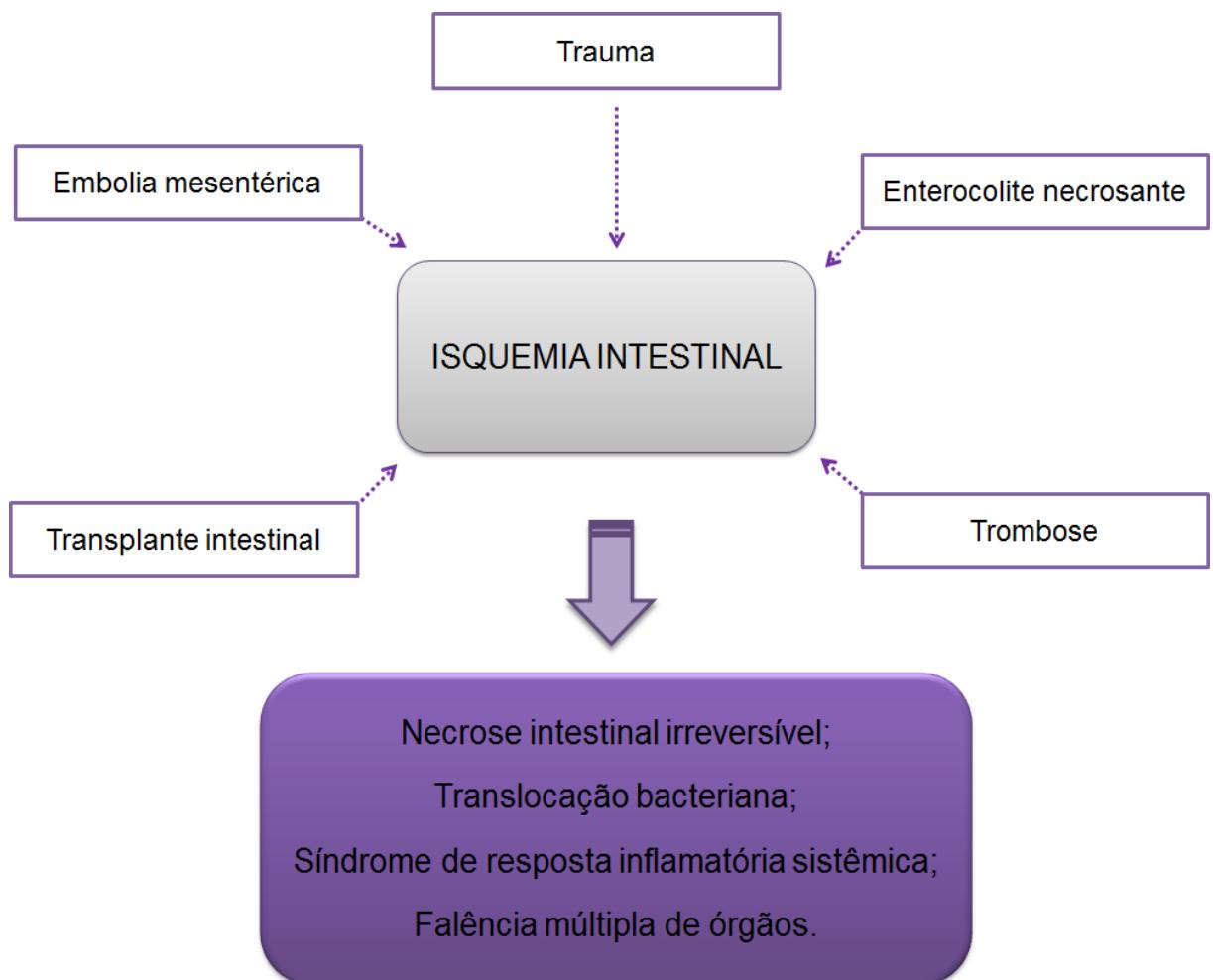
Figura 4: Condições clínicas associadas à lesão isquêmica intestinal.
Fonte: Adaptada (Gonzalez *et al.,* 2015).

O epitélio intestinal é constituído por uma única camada celular presente na mucosa que inclui os enterócitos, células de Paneth, células caliciformes e células enteroendócrinas, amplamente distribuídas no epitélio local e indispensáveis para manter a homeostase normal do intestino (figura 5) (Junqueira e Carneiro, 2013).
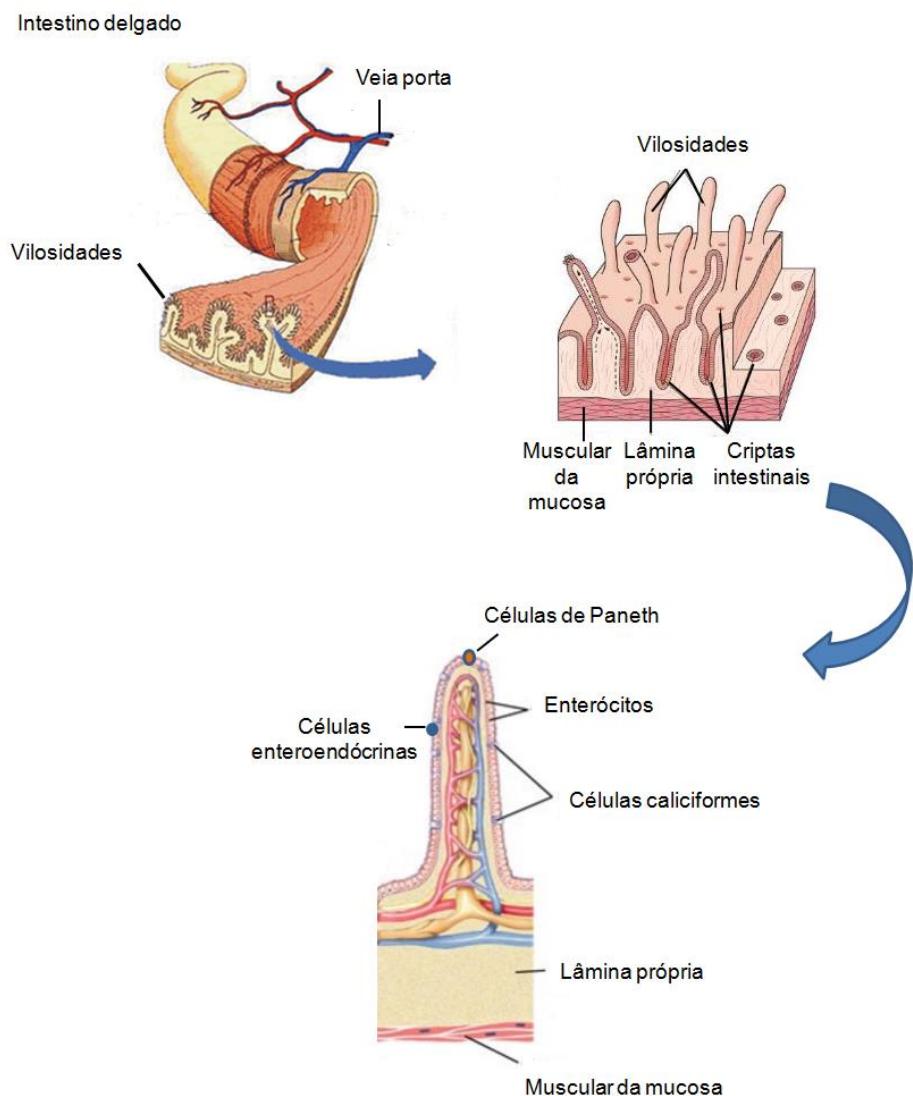
Figura 5: Estrutura do epitélio do intestino delgado.
Fonte: Adaptada (Junqueira e Carneiro, 2013).

Os enterócitos são células de absorção que revestem a camada da mucosa intestinal e essas possuem microvilosidades direcionadas para o lúmem intestinal. As células enteroendócrinas produzem enzimas importantes como colecistocinina e secretina, fundamentais para manter a função fisiológica normal do intestino e regular a digestão (Tóth *et al.*, 2012). As células caliciformes são responsáveis pela

secreção da maioria das glicoproteínas da camada de muco que envolve as células epiteliais do intestino. Esse muco produzido pode bloquear a aderência de endotoxinas que são capazes de alterar a função das células epiteliais, assim levando à alteração da função da barreira intestinal, sendo que a redução desse muco leva ao aumento da permeabilidade e consequente lesão intestinal (Junqueira e Carneiro, 2013; Bhattacharyya *et al.*, 2014).

As células de Paneth, secretoras de substâncias antimicrobianas através da enzima lisozima, estão localizadas na base das criptas de *Lieberkühn* e têm a função de manter a microbiota intestinal e controlar a translocação bacteriana para outros órgãos (Abreu, 2010; Lenaerts *et al.*, 2013; In *et al.*, 2016). Tais células têm uma sensibilidade maior a danos, principalmente os causados pela I/R intestinal, ocorrendo o rompimento dessas células e liberação de conteúdo luminal, incluindo endotoxinas e outros patógenos para a circulação sistêmica (Kaser *et al.*, 2011) (figura 6).
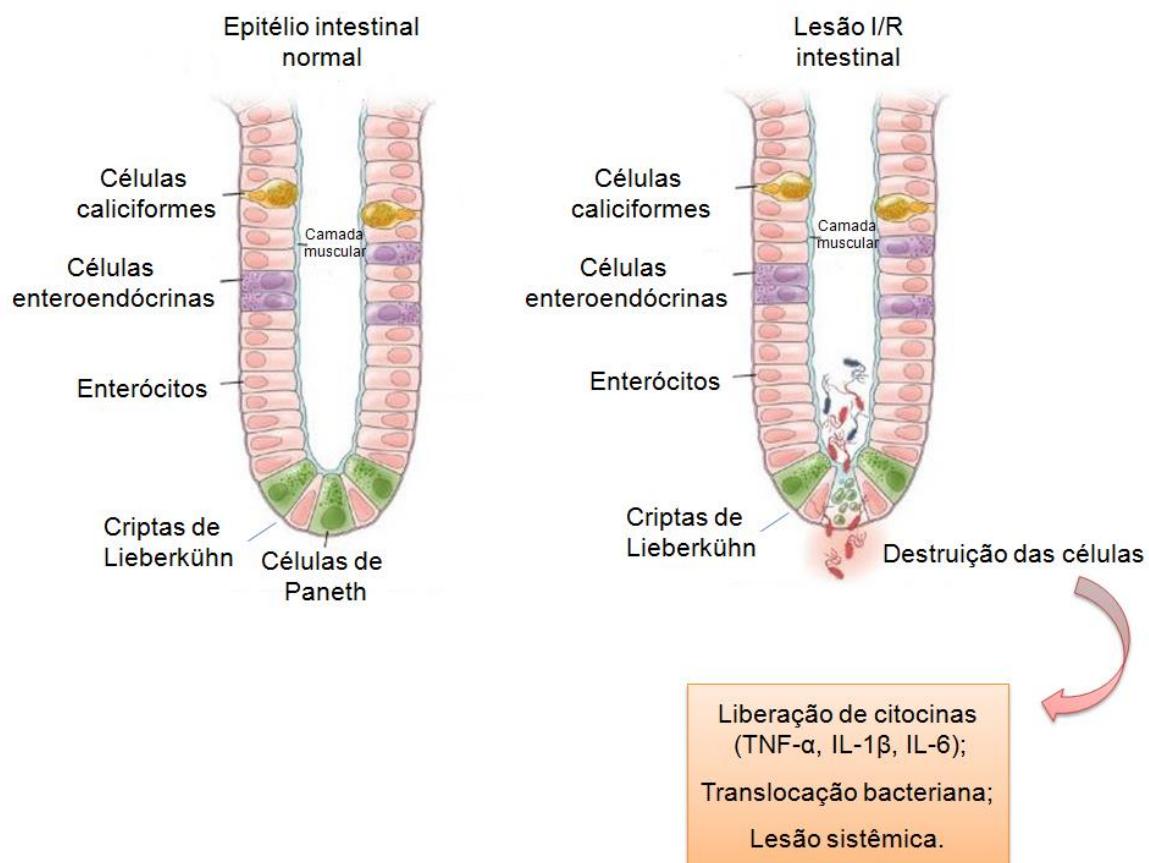
Figura 6: Efeito da lesão de I/R no epitélio intestinal.
Fonte: Adaptada (Kaser *et al.*, 2011).

A lesão na barreira da mucosa intestinal, decorrente da obstrução intestinal, pode provocar a geração de toxinas e ativação de uma intensa resposta inflamatória no intestino. E a reintrodução de sangue no segmento afetado do intestino pode levar à liberação desses mediadores químicos para a circulação portal e sistêmica (Mallick *et al.*, 2004; Cerqueira *et al.*, 2005; Jiang *et al.*, 2011; Chassin *et al.*, 2012).

Os mecanismos moleculares que estão envolvidos nas lesões desencadeadas pela I/R não estão bem esclarecidos, porém sabe-se que a geração exacerbada das ERO representa um importante papel na patogênese da lesão intestinal após um evento de I/R (Bilecová-Rabajdová *et al.*, 2010; Ayada *et al.*, 2015; Yan *et al.*, 2016; Zu *et al.*, 2016).

A I/R intestinal é a base para o aparecimento de lesões em órgãos distantes como na SRIS e na consequente SDMO, levando à falência de múltiplos órgãos, sendo o intestino considerado como o órgão desencadeador desses possíveis danos sistêmicos (Hassoun *et al.*, 2001; Yasuhara et al., 2005; Onder *et al.*, 2012; Zabot *et al.*, 2014; He *et al.*, 2015). Algumas evidências demonstram que a mucosa intestinal produz substâncias altamente lesivas, as quais são liberadas pelo intestino na circulação linfática, como as citocinas pró-inflamatórias e, nesse momento, inicia-se uma reação inflamatória sistêmica que poderá afetar diferentes órgãos, entre eles o fígado (Zhao *et al.*, 2010; Bertoletto *et al.*, 2012; Fan *et al.*, 2014; Ozban *et al.*, 2015).

Estudos apontam que a eficácia dos modelos experimentais pode auxiliar na avaliação da lesão, em aspectos moleculares, além da descoberta de possíveis estratégias terapêuticas (Leung *et al.*, 1992; Gonzalez *et al.*, 2015). A consideração do modelo animal para seleção do estudo é de extrema importância, pois a apresentação clínica é altamente variável nas doenças que envolvem a isquemia. Os modelos experimentais que utilizam roedores são vantajosos pelo seu custo relativamente baixo, a facilidade de manutenção e são bem coerentes aos danos celulares como os associados às ERO e à produção de citocinas pró-inflamatórias (Ozkan *et al.*, 2009; Cámara-Lemarroy *et al.*, 2011; He *et al.*, 2015).

A isquemia por obstrução vascular da AMS é atualmente o método mais comum utilizado para induzir lesão de I/R em modelos de roedores. A duração do período de isquemia mostra-se bem diversificado em diversos estudos, mas dados comprovam que a lesão de I/R intestinal pode ocorrer em 30 minutos de isquemia

com tempo indeterminado de reperfusão. Outra vantagem desse modelo é a opção de estudar os danos sistêmicos, avaliando as lesões em diferentes órgãos distantes ao local da isquemia (Zhao *et al.*, 2010; Fan *et al.*, 2014; Zabot *et al.*, 2014; Jiang *et al.*, 2015; Ozban *et al.*, 2015; Kalimeris *et al.*, 2016).

2.2.2 Isquemia e reperfusão intestinal e danos hepáticos

Nos últimos anos tem sido demonstrado que a lesão de I/R intestinal é o principal mecanismo na etiopatogênia de lesão a órgãos remotos que pode ocasionar na SDMO. O fígado é um órgão normalmente suscetível a essas lesões pela sua vascularização acoplada à circulação intestinal. Uma série de estudos está sendo realizada para esclarecer os processos fisioatológicos envolvidos nos mecanismos das lesões hepáticas induzidas pela I/R intestinal (Balmer *et al.*, 2014; He *et al.*, 2015; Haque e Barritt, 2016).

Os estudos mais atuais sugerem que a perda da integridade da membrana basal leva à perda da função da barreira do intestino, facilitando a produção e liberação de ERO, citocinas e toxinas oriundas do intestino para a circulação portal promovendo a inflamação no fígado (Inan *et al.*, 2013; Liu *et al.*, 2014; Ma *et al.*, 2014). As ERO geradas durante a I/R intestinal desempenham um papel importante nos danos locais e ao fígado podendo iniciar a lipoperoxidação (LPO), oxidação de proteínas, provocar a ruptura de cadeias do DNA e iniciar uma cascata de sinalizadores e mediadores inflamatórios que causam a destruição celular e tecidual aos órgãos afetados (Zhao *et al.*, 2010).

O fígado possui um fluxo sanguíneo intenso fundamental para manter suas funções orgânicas, sendo que aproximadamente 80% é derivado da veia porta e rico em nutrientes absorvidos no intestino. A formação dessa veia deve-se pela confluência da veia esplênica e mesentérica superior formando um leito vascular no interior do fígado, onde o sangue venoso mistura-se ao arterial (Friedman, 2008; Guyton e Hall, 2011). O sangue dos ramos da veia porta e da artéria hepática circula pelo fígado através dos sinusóides de onde converge para a veia central do lóbulo hepático que se localiza em torno da uma veia central e o espaço porta contém um

ramo da artéria hepática, veia porta e o ducto biliar (figura 7) (Friedman, 2008; Mattos e Dantas-Corrêa, 2010; Guyton e Hall, 2011; Schemitt, 2014; Colares, 2016).
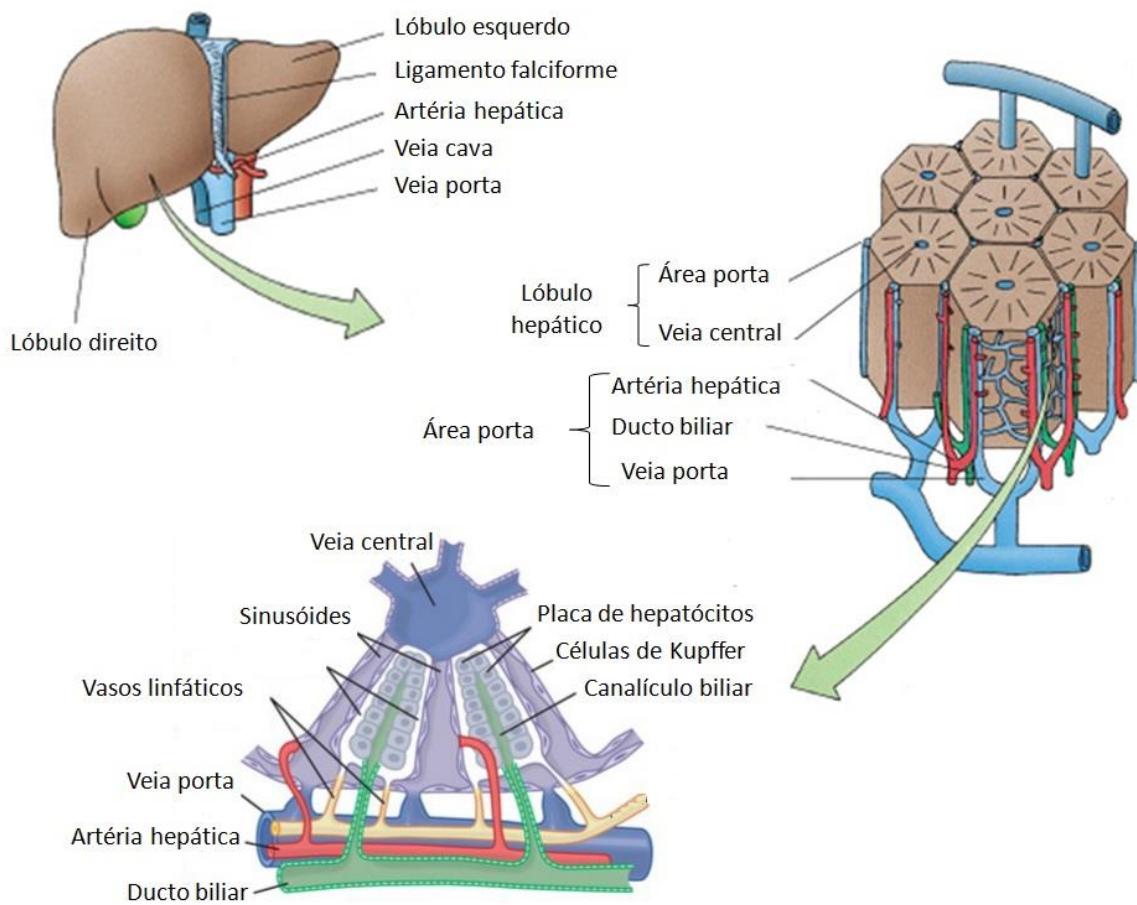
Figura 7: Estrutura hepática.
Fonte: Adaptada (Guyton e Hall, 2011).

O parênquima hepático é dividido em lóbulos hepáticos, constituídos principalmente por quatro tipos de células: células epiteliais (hepatócitos), células endoteliais, células estreladas hepáticas e as células de Kupffer (Friedman, 2008; Bona, 2014). Os hepatócitos, células hepáticas predominantes, organizam-se em cordões rodeados pelos sinusóides revestidos de células endoteliais que estão separadas dos hepatócitos pelo espaço de Disse onde se encontram as células estreladas hepáticas (Bataller e Brenner, 2005; Friedman, 2008).

Essas células estendem-se da periferia do lóbulo até o vaso central nomeado veia centrolobular e vasos subsequentes chegando até a veia porta. As células de

Kupffer, macrófagos hepáticos, encontram-se dentro dos sinusóides e estão presentes em todo o fígado. A principal função dessas células é de impedir que grandes quantidades de toxinas e microrganismos provenientes da microbiota intestinal disseminem-se para a circulação sistêmica (Brenner *et al.*, 2013; Balmer *et al*, 2014; Nastos *et al.*, 2014) (figura 8).
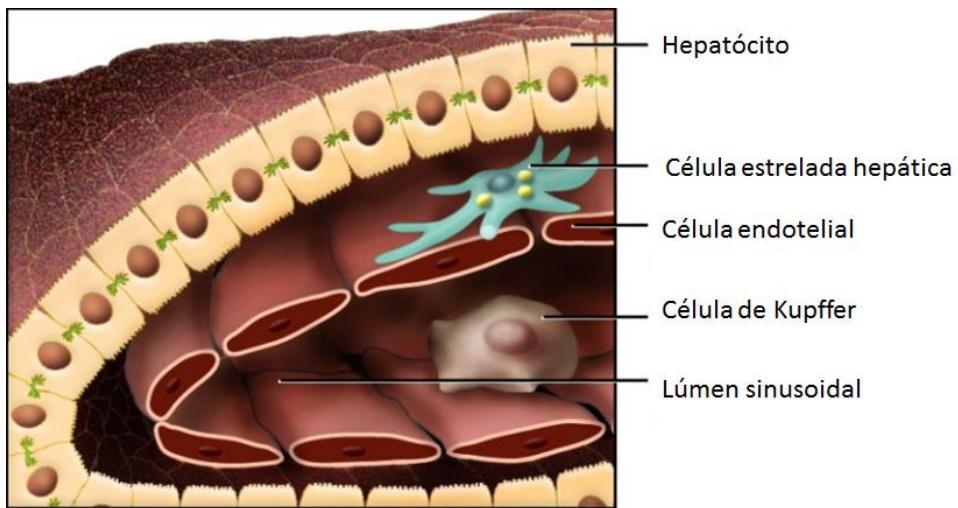
Figura 8: Arquitetura normal do fígado.
Fonte: Adaptada (Bataller e Brenner, 2005).

O fígado é considerado o primeiro filtro de nutrientes, toxinas e metabólitos bacterianos provenientes do intestino delgado. Dessa forma, a integridade do epitélio do intestino delgado faz-se extremamente necessária, pois qualquer desequilíbrio dessa função pode causar danos graves aos tecidos, incluindo o fígado (Sommer e Bäckhed, 2013; Chassaing *et al.*, 2014; Szabo, 2015; Haque e Barritt, 2016).

Os nutrientes são carreados do intestino delgado para a veia portal, diretamente para o fígado que, em seguida, sintetiza as lipoproteínas, metaboliza ou armazena a glicose, processa os lipídeos, armazena vitaminas, e reúne as proteínas para serem utilizadas dentro do fígado ou em outros locais. Uma vez que substâncias químicas e agentes tóxicos e microbianos, incluindo os lipopolissacarídeos (LPS), chegam ao fígado, algumas funções efetoras como a produção de ERO, liberação de neutrófilos e citocinas promovem a amplificação da lesão celular e tecidual, levando a um intenso processo inflamatório no fígado induzido pela I/R intestinal (Adams e Eksteen, 2006; Rock *et al.*, 2010) (figura 9).

Zhao *et al.* (2010) e He *et al.* (2015) demonstraram a presença de substâncias oxidativas (ERO e ERN), citocinas pró-inflamatórias (IL e TNF-α), fatores de transcrição (NF-κB) e lesões teciduais no fígado no modelo de I/R intestinal em ratos (Zhao *et al.* 2010; He *et al.* 2015).

A

Homeostase normal do intestino e fígado

B

Dano hepático induzido pela I/R intestinal
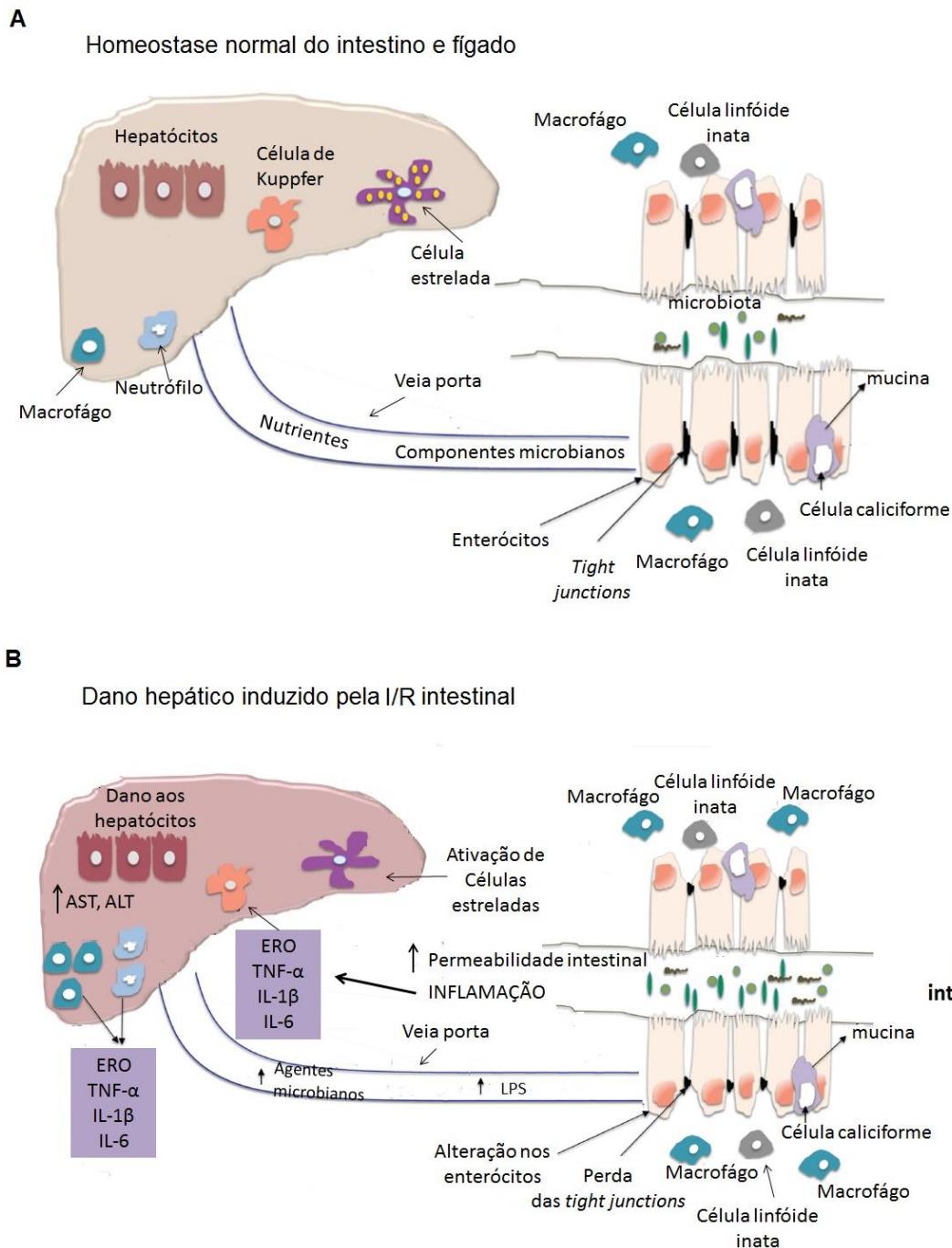
Figura 9: Mecanismos envolvidos nos danos hepáticos decorrentes da lesão de I/R intestinal. A - Homeostase normal do intestino e fígado. B – Danos hepáticos induzidos pela IR intestinal.

Fonte: Adaptada (Szabo, 2015).

2.3 Estresse oxidativo

O metabolismo do O_2 é um dos responsáveis pelo fornecimento de energia celular para as células do organismo, pois mantém as funções bioquímicas intracelulares. Entretanto, alguns efeitos tóxicos como a geração de ERO podem ocorrer durante o processo de redução da molécula de O_2 à água (H_2O) na cadeia respiratória mitocondrial (Boveris e Chance, 1973).

Nesse processo de redução, a maior parte do O_2 (aproximadamente 95%) recebe quatro elétrons de uma só vez. Entretanto, em 5% das vezes, a molécula de O_2 recebe um elétron de cada vez ocorrendo uma redução monovalente, ocasionado a formação de intermediários reativos (Halliwell e Gutteridge, 2007) (Figura 10).
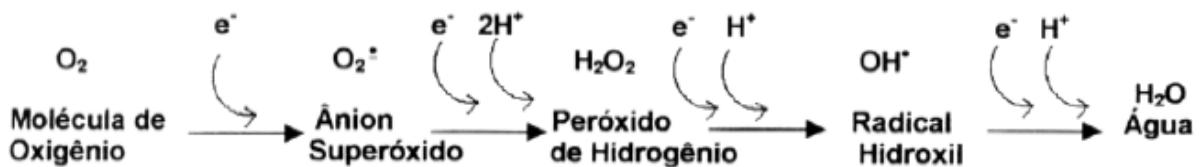
Figura 10: Processo de formação das espécies reativas de oxigênio.
Fonte: Adaptada (Halliwell e Gutteridge, 2007).

Os intermediários da redução monovalente do O_2 são o $O_2^{\bullet-}$, H_2O_2, oxigênio *singlet* e radical $^{\bullet}OH$. Todos são denominados ERO por serem capazes de existir de forma independente. Dois desses intermediários são chamados de radicais livres, são eles o $O_2^{\bullet-}$ e $^{\bullet}OH$ (Dröge, 2002).

Os RL são definidos como qualquer espécie química capaz de existir independente e que contenha um ou mais elétrons desemparelhados, sendo espécies paramagnéticas com alto grau de reatividade química e vida média curta, sendo capazes de atacar qualquer biomolécula. Esses radicais podem ser formados em situações fisiológicas ou patológicas e são danosos às células e ao organismo quando produzidos em grandes quantidades (Del Maestro, 1980; Valko *et al.*, 2007).

O radical $O_2^{\bullet-}$ é constituído pela redução do O_2 molecular por um elétron após aporte energético. Normalmente, é removido pela reação de dismutação que

consiste na reação de dois ânions superóxidos catalisados pela enzima superóxido dismutase (SOD). O H_2O_2 é geralmente produto da dismutação do $O_2^{•-}$ pela enzima SOD. As enzimas oxidativas são capazes de formar e degradar o H_2O_2, sendo normalmente encontradas nos peroxissomas. As mais comuns são a CAT, D-amino oxidase, urato oxidase, B-oxidase de ácidos graxos e GPx. O H_2O_2, apesar de não ser um radical livre, pode reagir com outro $O_2^{•-}$ ou com metais de transição, conforme as reações de Fenton (Reação 1) e Haber-Weiss (Reação 2), formando o radical $OH^{•-}$ (Dröge, 2002).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3} + OH^{•} + OH^{-} \quad \text{(Reação 1)}$$

$$O_2^{•-} + H_2O_2 \longrightarrow O_2 + OH^{•} + OH^{-} \quad \text{(Reação 2)}$$

O radical $OH^{•-}$ é o mais deletério ao organismo, pois devido a sua meia-vida muito curta, dificilmente pode ser sequestrado *in vivo*. Esses radicais frequentemente atacam as moléculas por abstração de hidrogênio e por adição a insaturações. A sua formação ocorre a partir da reação do peróxido de hidrogênio com um ânion superóxido ou com íons de Fe (reação 1). Esse RL possui a capacidade de atravessar as membranas e reagir com biomoléculas, como os lipídios insaturados e DNA (Dröge, 2002; Halliwell, 2012).

Os RL exercem algumas funções importantes com efeitos benéficos ou prejudiciais no organismo dos seres vivos, tais como (Dröge, 2002):

EFEITOS BENÉFICOS:	EFEITOS PREJUDICIAIS:
• Sinalização celular; • Fagocitose; • Regulação do crescimento celular.	• Lipoperoxidação; • Oxidação de proteínas; • Danos ao DNA.

A reação da LPO é um processo natural de renovação das membranas celulares. Essa reação ocorre em cadeia com as etapas de iniciação, propagação e terminação. A iniciação é o primeiro passo dessa reação. O radical livre remove um átomo de hidrogênio de um ácido poli-insaturado. Na fase de propagação ocorrem

duas reações: 1) o carbono radical do lipídio tende a se estabilizar por rearranjo molecular, produzindo dienos conjugados que rapidamente reagem com o O_2 formando um radical peroxil; 2) o radical peroxil capta um próton de outra molécula de lipídio, formando um hidroperóxido. Na etapa final, dois radicais peroxil reagem entre si, formando um tetróxido instável que se decompõe dando origem ao $^1O_2$ e a carbonilas excitadas (figura 11) (Buege e Aust, 1978; Halliwell e Gutteridge, 2007).
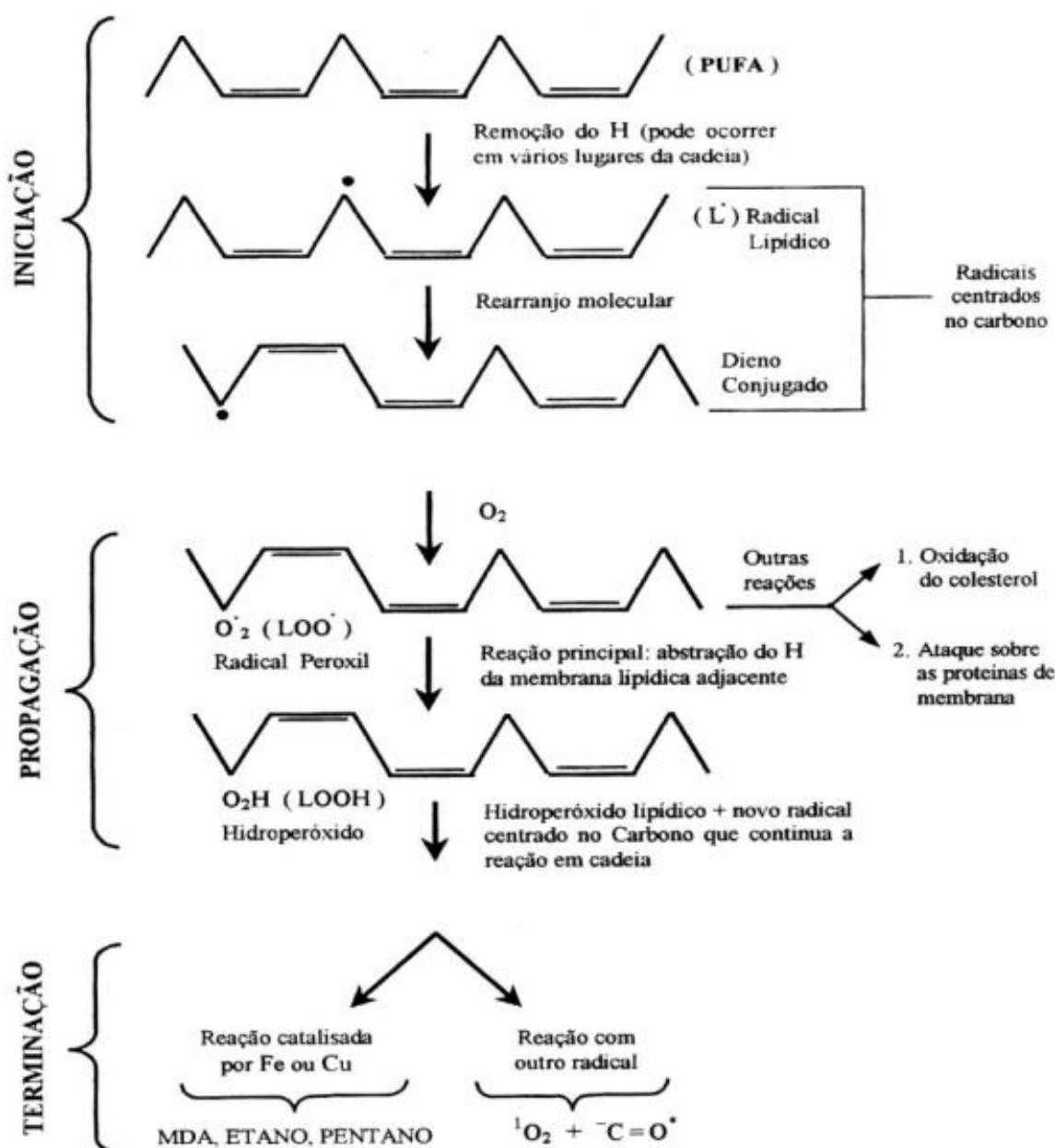
Figura 11: Representação esquemática da reação da lipoperoxidação.
Fonte: Adaptada (Halliwell e Gutteridge, 2007).

A produção excessiva dos RL, quando ultrapassam a capacidade antioxidante ou quando ocorre uma diminuição das defesas antioxidantes no organismo, favorece o que denominamos de EO (Dröge, 2002; Halliwell, 2012).

Estudos clínicos e experimentais indicam que a elevada produção de ERO, ativação de neutrófilos e liberação de citocinas pró-inflamatórias possuem papel central na lesão local e de órgão remotos decorrente da I/R intestinal (He *et al.*, 2015). Os danos causados pela I/R intestinal na mucosa do intestino levam à perda da barreira intestinal, assim ocorrendo a translocação de bactérias, a geração e liberação de RL e citocinas para a circulação sanguínea desencadeando uma reação inflamatória sistêmica, sendo essa a principal causa de lesões em órgãos remotos a I/R intestinal (Jiang *et al.*, 2015).

Estudos sugerem que o EO está envolvido na lesão de I/R intestinal, pois ocorre um acúmulo de ERO que pode levar à oxidação de proteínas, LPO, danos às bases de DNA e desequilíbrio no sistema antioxidante (Onder *et al.*, 2012; Sun *et al.*, 2012; Fan *et al.*, 2014; Ayada *et al.*, 2015). Cámara-Lemarroy *et al.* (2011) avaliaram os possíveis efeitos antioxidantes e anti-inflamatórios do triflusal, S-adenosilmetionina, dextrometorfano, sugerindo que a administração dessas substâncias reduziu os níveis de malondialdeído (MDA) nos animais com I/R intestinal (Cámara-Lemarroy *et al.*, 2011). Shafik (2013) encontrou um aumento nos níveis séricos de MDA de animais com I/R. Após a administração de febuxostat, estes níveis foram reduzidos, demonstrando uma possível ação antioxidante desta substância (Shafik, 2013).

2.4 Estresse Nitrosativo

O NO é considerado um radical livre inorgânico que participa de muitos processos fisiológicos e patológicos, sendo um sinalizador ou fator de relaxamento derivado do endotélio. Também age como um regulador na adesão plaquetária, agregação de neutrófilos, citotoxicidade de macrófagos entre outras funções (Fang, 1997; Liaudet *et al.*, 2000). A síntese do NO ocorre pela ação da enzima óxido nítrico sintase (NOS), através da conversão de L-arginina e oxigênio em L-citrulina e NO (figura 12).

NOS

L-arginina $\longrightarrow$ L-citrulina
+ +
O_2 NO
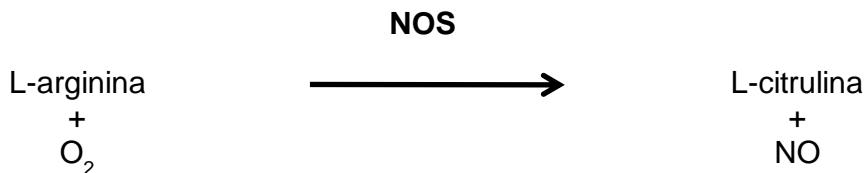
Figura 12: Síntese do óxido nítrico.
Fonte: Adaptada (Liaudet *et al.*, 2000).

Existem três formas de NOS identificadas: óxido nítrico sintase endotelial (eNOS), encontrada no endotélio vascular; e a óxido nítrico sintase neuronal (nNOS), que regula a transmissão neuronal, ambas constitutivas. A óxido nítrico sintase induzível (iNOS), identificada primeiramente nos macrófagos, está relacionada aos danos teciduais e presente na inflamação e apoptose celular (Knowles e Moncada, 1994; Davis *et al.,* 2001).

O NO pode ser dividido de acordo com o mecanismo de ação da molécula com sua célula-alvo. Esse mecanismo pode ocorrer de duas formas: quando interage diretamente com a molécula do sistema-alvo e quando existem intermediários de ERO como o radical $O_2^{\bullet-}$ que podem reagir com o NO, podendo levar à formação de ERN, como o peroxinitrito ($ONOO^-$) (Kolios *et al.*, 2004). A produção exacerbada de espécies reativas de nitrogênio (ERN) é conhecida como estresse nitrosativo. Isso ocorre quando a geração de ERN em algum sistema excede a habilidade do sistema em neutralizá-las e eliminá-las (Villanueva e Giulivi, 2010).

O NO, sob condições fisiológicas, desempenha um papel essencial na homeostase vascular, bem como no sistema imune, e atua como uma molécula moduladora em diferentes processos fisológicos como na regulação da integridade e motilidade intestinal (Wolin, 2000; Krieglstein *et al.*, 2001; Soufli *et al.*, 2016). Estímulos como pela I/R levam ao aumento dos níveis de NO e à produção de $ONOO^-$, causando acentuada peroxidação lipídica, protéica e modificações no DNA, resultando em danos celulares (Liu *et al.*, 2007; Roberts *et al.*, 2013).

Na I/R intestinal a produção aumentada de NO leva à disfunção celular da mucosa, acarretando diversos eventos prejudiciais ao órgão isquêmico, como perda da integridade da barreira da mucosa e uma consequente liberação de mediadores

inflamatórios para outros sistemas, podendo levar à inflamação sistêmica característica da SDMO (Carden e Granger, 2000; Krieglstein *et al.*, 2001; Granger e Kvietys, 2015).

O aumento dos níveis de NO é decorrente da expressão da forma induzível iNOS que não está presente em condições normais, mas pode ser induzida em resposta a citocinas e fatores de transcrição nuclear, levando ao aumento dos níveis de NO, o que contribui para a fisiopatologia da lesão por I/R intestinal e consequentes danos sistêmicos (Takizawa *et al.*, 2011; Granger e Kvietys, 2015).

Sun *et al.* (2015) utilizaram a dexmedetomidina como tratamento na lesão intestinal induzida por I/R e demonstraram que os níveis de NO foram significativamente reduzidos em comparação com o grupo I/R e sugeriram que o NO é um mediador crítico da resposta inflamatória durante o desenvolvimento da lesão intestinal (Sun *et al.*, 2015). Estudos sugerem que a iNOS desempenha um papel importante no agravamento da lesão de I/R e que a sua inibição implica a redução da produção de NO (Liu *et al.*, 2007; Kudoh *et al.*, 2014; Akcılar *et al.*, 2015; Sun *et al.*, 2015). Takizawa *et al.* (2011) observaram um aumento nos níveis de NO e uma expressão aumentada do gene da iNOS em modelo animal de lesão intestinal I/R (Takizawa *et al.*, 2011).

2.5 Antioxidantes

O organismo, para compensar os danos causados por processos oxidativos, possui um sistema antioxidante, constituído por componentes enzimáticos e não enzimáticos que atuam conjuntamente na proteção celular impedindo ou retardando o dano oxidativo. A função desses compostos é manter os níveis intracelulares de ERO em baixas concentrações e para isso atuam prevenindo a formação dessas espécies ou combatendo-as uma vez que tenham sido formadas (Sies, 1993; Cadenas, 1997).

A geração de ERO e ERN pode ser remediada pela expressão e atividade de diversas enzimas antioxidantes, que são reguladas por uma série de fatores de transcrição, incluindo o fator de transcrição nuclear eritróide 2 (Nrf2) o qual é uma proteína reguladora de respostas celulares a danos oxidativos que, em condições

normais, está mantido no citoplasma associado à proteína Keap1 (*Kelch-like ECH associated protein 1*) (Kaspar *et al.*, 2009; Zhao *et al.*, 2010; Fuse e Kobayashi, 2017).

O Nrf2 desempenha um papel de proteção contra os danos oxidativos induzidos por lesões agudas, estresse nitrosativo, estresse do RE. Após estímulos, como pelo EO, ocorre à perda dessa ligação Keap1/Nrf2 e o fator de transcrição Nrf2 é translocado para o núcleo e se liga a uma sequência promotora chamada de elemento de resposta antioxidante (ARE) (Jaiswal, 2004; Osburn *et al.*, 2006; Thimmulappa *et al.*, 2006; Sun *et al.*, 2013; Espinosa-Diez *et al.*, 2015).

Após esse processo ocorre a regulação positiva de genes citoprotetores e defesas antioxidantes, como a NADPH quinona oxidorredutase 1 (NQO1) e a enzima SOD (Zhao *et al.*, 2010; Sykiotis *et al.*, 2011; Yao *et al.*, 2014). Essas proteínas atuam direta ou indiretamente na eliminação dos RL, regulação da síntese de glutationa (GSH) e na proteção das células contra a apoptose (figura 13).
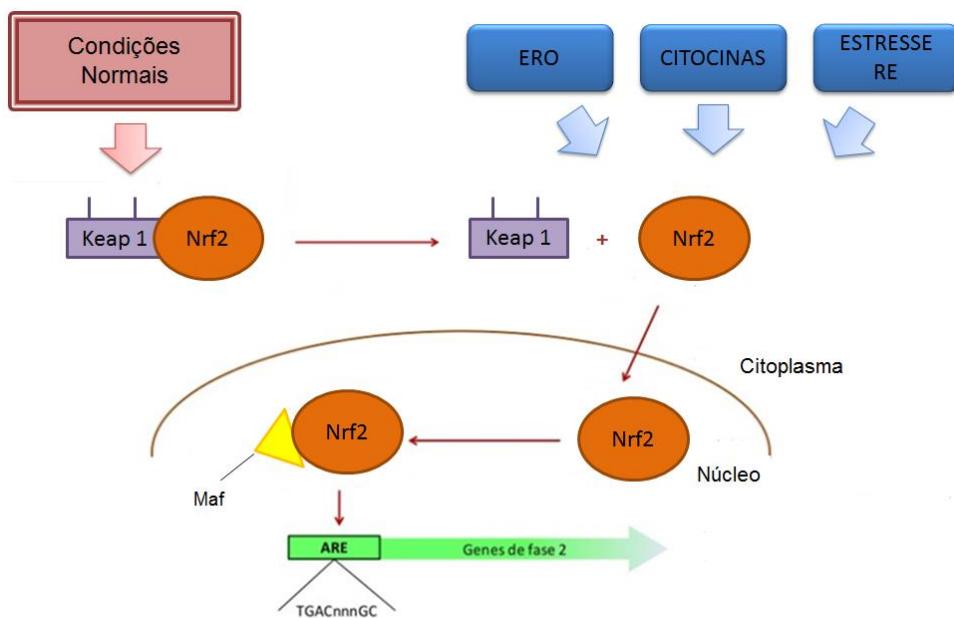
Figura 13: Via do Nrf2/Keap1.
Fonte: Adaptado de (Sykiotis *et al.*, 2011).

Os efeitos protetores de Nrf2 foram avaliados no intestino, demonstrando uma proteção na integridade intestinal, através da regulação de citocinas pró-inflamatórias e enzimas antioxidantes (Sun *et al.*, 2012). Zhao *et al.* (2010) avaliaram o efeito do sulforafano, produto natural que está presente em vegetais, e

demonstraram que o pré-tratamento com sulforafano levou à ativação da via do Nrf2, o que resultou no aumento da capacidade antioxidante, protegendo o fígado contra as lesões da I/R intestinal (Zhao *et al.*, 2010).

O sistema enzimático é considerado a linha de defesa primária, uma vez que evita o acúmulo do ânion radical superóxido e do peróxido de hidrogênio. Existem, também, as defesas secundárias que impedem a propagação da LPO e as terciárias, enzimas responsáveis pelo reparo de danos já instalados (Halliwell, 2012). O sistema enzimático encarregado de detoxificação das ERO é formado por muitas enzimas e dentre elas estão: SOD, CAT e GPx (Harris, 1992; Halliwell e Gutteridge, 2007).

A SOD tem por principal função atuar na dismutação do $O_2^{\bullet-}$ em H_2O_2 e oxigênio, sendo o primeiro menos reativo e pode ser degradado por outras enzimas (reação 1) (Sies, 1993).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \quad \xrightarrow{\text{SOD}} \quad H_2O_2 + O_2 \qquad \text{Reação 1}$$

O H_2O_2, apesar de não ser considerado um RL, reage facilmente originando o $^{\bullet}OH$. A remoção dos peróxidos ocorre por meio das enzimas CAT e da GPx. A CAT mostra mais afinidade ao peróxido de hidrogênio , de metila e etila, enquanto a GPx catalisa a redução do peróxido de hidrogênio e hidroperóxidos orgânicos (reação 2) (Sies, 1993).

$$2H_2O_2 \quad \xrightarrow{\text{CAT}} \quad O_2 + 2H_2O \qquad \text{Reação 2}$$
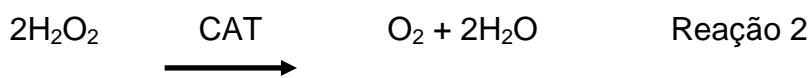
Entre as peroxidases que geralmente usam o grupo heme sobressai-se a atividade da GPx que está localizada no citosol e na matriz mitocondrial. Essa enzima catalisa a redução do peróxido de hidrogênio através da GSH que será regenerada pela ação da glutationa redutase com consumo de fosfato nicotinamida adenina dinucleotídeo (NADPH) ocorrendo à razão celular entre a GSH e a glutationa oxidada (GSSG) (reação 3).

$$R\text{-}OOH + 2\,GSH \quad \xrightarrow{\text{GPx}} \quad R - OH + GSSG + H2O$$

Reação 3

$$GSSG + 2NADPH \quad \xrightarrow{\text{GR}} \quad 2GSH + 2NADPH+$$
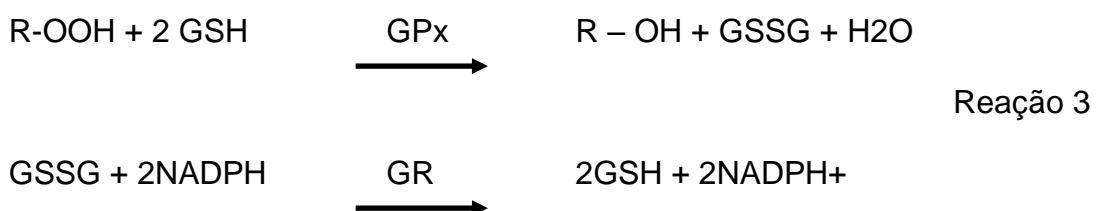
O sistema de defesa não-enzimático é formado por antioxidantes hidrossolúveis, como o α-tocoferol, também conhecido como vitamina E, promovendo a inibição da peroxidação de lipídios. O ácido ascórbico, conhecido também por vitamina C, atua como *scavenger* do radical $O_2^{\cdot-}$ e radical $\cdot OH$ (Harris, 1992; Halliwell, 2013; Espinosa-Diez *et al.*, 2015).

Zhao *et al.* (2010) observaram uma redução na atividade de SOD e GPx e nos níveis de GSH no fígado de animais submetidos a um modelo I/R intestinal. A administração do composto sulforafano, presente em várias plantas, foi capaz de aumentar a atividade dessas enzimas no fígado, sugerindo um papel protetor contra o dano oxidativo (Zhao *et al.*, 2010). Onder *et al.* (2012) avaliaram o efeito da curcumina nas lesões ao intestino, rim, fígado e pulmão no modelo de I/R intestinal e observaram que o tratamento reduziu os danos teciduais e aumentou a capacidade antioxidante nos tecidos estudados (Onder *et al.*, 2012).

2.6 Mediadores inflamatórios envolvidos na isquemia e reperfusão intestinal

2.6.1 Interleucinas e fator de necrose tumoral

A reposta inflamatória na I/R intestinal é desencadeada por diversos mecanismos como a geração de mediadores inflamatórios que podem ser liberados pelo sistema linfático mesentérico para outros órgãos desencadeando uma inflamação sistêmica (Carden e Granger, 2000; Cerqueira *et al.*, 2005; Sakrak *et al.*, 2008; Ma *et al.*, 2014).

O TNF-α está entre as principais citocinas envolvidas nos processos inflamatórios e possui alguns efeitos biológicos como a ativação de macrófagos e neutrófilos e aumento das moléculas de adesão envolvidas com o rolamento dos leucócitos, diferenciação celular e apoptose (Aggarwal, 2003). O TNF-α é induzido por uma série de estímulos que incluem microrganismos, mediadores lipídicos, células tumorais e citocinas. O seu papel pró-inflamatório está associado à indução/produção de IL e expressão de moléculas de adesão, assim como na

ativação de fatores apoptóticos e regulação do sistema imune durante a inflamação aguda e crônica (Hu *et al.*, 2007).

A interleucina-1 (IL-1) é encontrada nas formas *alfa* (IL-1α) e *beta* (IL-1β). Sendo uma das primeiras citocinas a ser descoberta, desempenha uma função central na regulação das respostas imunes e processos inflamatórios. A IL-1β promove a migração de leucócitos para locais de lesão ou infecção e sua produção normalmente é realizada por monócitos e macrófagos. A sua indução e consequente produção pode levar ao aumento da expressão de moléculas de adesão endotelial e estimulação da produção de interleucina-6 (IL-6), juntamente com o TNF-α (Ren e Torres, 2009; Rock *et al.*, 2010).

A IL-6 é uma citocina que desempenha um papel importante na homeostase do sistema imunológico e também no equilíbrio de vias pró e anti-inflamatórias em respostas ao estresse. A IL-6 pode ser produzida por diferentes células como monócitos, macrófagos e endoteliais e sua síntese e secreção pode ser induzida durante diferentes condições inflamatórias pela ativação dos receptores *toll-like*, LPS e pelo TNF-α. Essa citocina também tem sido considerada como marcador de quadros graves de inflamação em situações de trauma gastrointestinal (Ershler e Keller, 2000; Cerqueira *et al.*, 2005; Grootjans *et al.*, 2015).

A I/R Intestinal leva à geração desses mediadores inflamatórios e oxidativos como as ERO, IL-1β, IL-6 e TNF-α que levam à alteração da função protetora da barreira intestinal e, consequentemente, são liberados para a circulação extraintestinal e contribuem para lesão hepática (Grootjans *et al.*, 2010; Kalimeris *et al.*, 2016). Fan *et al.* (2014) observaram que a curcumina reduziu os níveis das citocinas TNF-α e IL-6. Tais resultados foram associados à inibição do NF-κB, o que levou à redução da inflamação local e sistêmica, contribuindo para a melhora da lesão hepática induzida pela I/R intestinal (Fan *et al.*, 2014).

Sakrak *et al.* (2008) sugeriram que o composto natural ergotioneína, sintetizado pelo fungos e pelas micobactérias e que apresenta um papel antioxidante foi capaz de reduzir os níveis séricos do TNF-α e da IL-1β que ocorreram após prolongados períodos de até 4 horas de reperfusão no modelo de I/R intestinal (Sakrak *et al.*, 2008). Zu *et al.* (2016) demonstraram no seu estudo com animais submetidos a I/R intestinal e pré-tratados com o princípio ativo do *Ginseng,* a redução significativa dos níveis de IL-1β, IL-6 e TNF-α, relacionando esses achados

com a atividade anti-inflamatória e antioxidante desse princípio ativo estudado (Zu *et al.*, 2016).

2.6.2 Fator de transcrição nuclear kappa B

O NF-κB desempenha um importante papel em situações normais como na coordenação de respostas imunes adaptáveis, regulando a expressão de muitos mediadores celulares. O NF-κB está expresso na maioria dos tipos celulares sendo constituído por um dímero composto dos membros da família da Rel. A família do NF-κB/Rel compreende cinco subunidades, chamadas p50, p52, p65 (RelA), c-Rel, e RelB. Geralmente, o NF-κB consiste em dois polipeptídeos um de 50 kDa (p50) e um de 65 kDa (p65) (Zingarelli *et al.*, 2003; Espinosa-Diez *et al.*, 2015).

Na homeostase, o NF-κB mantém-se no citoplasma em sua forma inativa associado com as proteínas inibidoras do sítio κB chamadas de inibidores κB (IκB). O NF-κB pode ser ativado por uma variedade de sinais relevantes à etiologia e à fisiopatologia inflamatória. Para ocorrer à ativação do NF-κB são necessários estímulos intracelulares e ou extracelulares, onde os ativadores podem ser: produtos bacterianos (endotoxinas, peptideoglicanos), vírus e componentes virais, protozoários, citocinas e RL (Yamaoka *et al.*, 1998; Zingarelli *et al.*, 2003).

A ativação do NF-κB requer a fosforilação de seus inibidores fisiológicos (particularmente o IκBα) em resíduos específicos de Serina (Ser-32 e Ser-36). Esta fosforilação é mediada por um complexo protéico. O complexo kinase *kappa* B (IKKs) é composto de três subunidades, duas unidades catalíticas IKK-α, IKK-β, e uma unidade reguladora IKKγ. Após a fosforilação ocorre a subsequente degradação das IκBs através das ubiquitinas, formando um proteossoma 26S. A degradação proteolítica dos IκBs permite a translocação do NF-κB ao núcleo, onde é regulada a expressão de centenas de genes que são importantes à resposta imune inflamatória (figura 14) (Cadenas e Davies, 2000; Siomek, 2012).
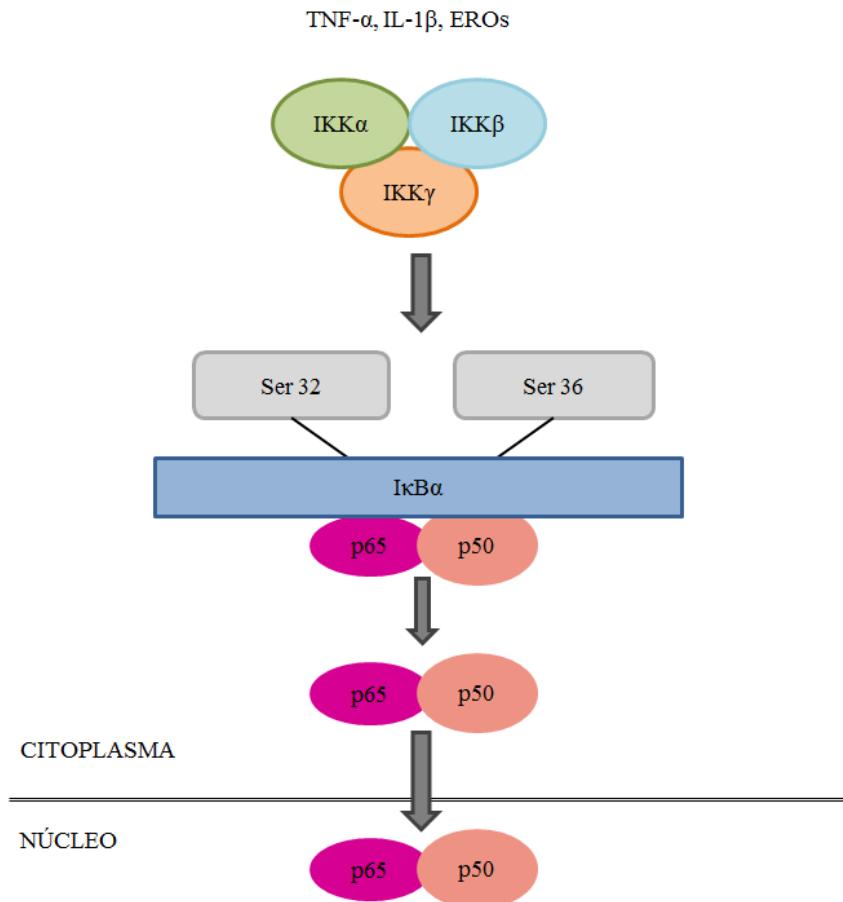
Figura 14: Via de ativação do NF-κB por citocinas e ERO.
Fonte: Adaptada (Siomek, 2012).

O mecanismo pelo qual as ERO participam da resposta inflamatória é iniciado pelo aumento na produção de diversos marcadores inflamatórios oriundos das células de defesa. A síntese de inúmeras citocinas pró-inflamatórias depende de ativação do NF-κB, a qual depende do potencial redox celular e consequentemente pode ser regulada pela relação de GSH (Medling *et al.*, 2010; Bertoletto *et al.*, 2012).

Estudos têm demonstrado que as ERO podem estimular a ativação dos fatores de transcrição celular, como o NF-κB. E com o aumento da produção de citocinas inflamatórias TNF-α e de diversas interleucinas (IL-1β, IL-6, IL-8) ocorre a resposta inflamatória sistêmica. Assim, o NF-κB funciona como um marcador para o tratamento anti-inflamatório (Cadenas e Davies, 2000; Cerqueira *et al.*, 2005; Medling *et al.*, 2010; Cámara-Lemarroy *et al.,* 2011; Zeng *et al.,* 2012).

Jing *et al.* (2012) administraram doses de MG132, um inibidor de proteases com ação antioxidante, nos animais com I/R intestinal e observaram uma redução do NF-κB sugerindo que o MG132 tem um efeito benéfico na proteção contra a lesão hepática induzida por I/R intestinal devido à modulação da via do NF-κB (Jing *et al.*, 2012). Ma *et al.* (2014) sugeriram que o ácido protocatecuico, composto polifenólico derivado do chá verde, frutas e ervas medicinais, inibiu a expressão do NF-κB (p65) no intestino e fígado de animais submetidos à isquemia intestinal de 45 minutos seguida de uma reperfusão de 90 minutos (Ma *et al.*, 2014).

Yao *et al.* (2009) demonstraram que o pré-tratamento com carnosol, princípio ativo da planta *Rosmarinus officinalis L.* que possui ação antioxidante e anti-inflamatória, atenuou a lesão hepática induzida por I/R intestinal, incluindo as lesões teciduais e danos oxidativos, níveis séricos das enzimas de integridade hepática ALT e AST, bem como atuou na regulação negativa da expressão do NF-κB e consequentemente na diminuição da IL-6 (Yao *et al.*, 2009).

2.7 Proteínas de choque térmico

A família das proteínas de choque térmico (HSP) é considerada um grupo de moléculas altamente conservadas, presentes em diferentes espécies e tem como principal função promover o enovelamento correto de outras proteínas. As HSP são classificadas de acordo com seu peso molecular e são divididas em ATP-dependentes como as HSP100, HSP90, HSP70, HSP60 e as ATP-independentes que incluem as HSP27 e HSP10. Tais proteínas, em condições fisiológicas, estão localizadas em locais distintos dentro da célula, como citoplasma, mitocôndria, RE e núcleo (Bozaykut *et al.*, 2014; Jee, 2016).

A proteína de choque térmico 70 (HSP70) é considerada a mais conservada dentro dessa família, sendo encontrada na forma constitutiva e também na forma induzível. A HSP70 vem sendo estudada em diversas enfermidades pelo seu papel na proteção celular contra estímulos nocivos como infecções virais ou bacterianas, EO e do RE e isquemia (Shiota *et al.*, 2010; Liu *et al.*, 2012).

Em condições normais, as HSP estão vinculadas ao fator de choque térmico (HSF), que se encontra inativo no citoplasma. Após algum estímulo, como pelo EO, ocorre a dissociação da ligação do HSF e HSP, resultando na ativação do fator HSF. A sua forma ativa é fosforilada e ocorre a formação de trímeros de HSF-1 que migram para o núcleo e se ligam à região promotora de elemento de choque térmico (HSE), induzindo a transcrição dos genes das HSPs que mantêm a homeostase do organismo (Fleming *et al.*, 2002; Jee, 2016) (figura 15).
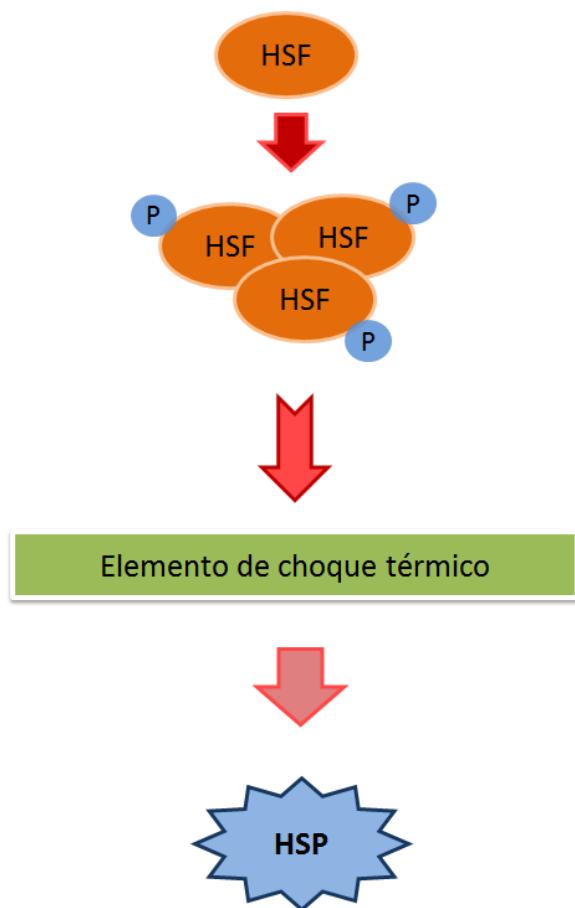
Figura 15: Produção das proteínas de choque térmico.
Fonte: Adaptada (Jee, 2016).

O mecanismo do efeito protetor das HSP, principalmente da HSP70, contra a lesão de I/R parece estar envolvido com a inibição da infiltração de neutrófilos e leucócitos, aumento da capacidade antioxidante e redução das citocinas pró-inflamatórias. Estudos indicam que essa regulação positiva da HSP70 resulta na melhora dos danos teciduais e celulares decorrentes da I/R em diferentes órgãos

(Fleming *et al.*, 2002; Sakrak et al., 2008; Abudunaibi *et al.*, 2015; Kierulf-Lassen *et al.*, 2015).

2.8 Estresse do Retículo Endoplasmático

O RE, presente em células eucarióticas, é uma organela que desempenha diversas funções como no transporte e armazenamento de Ca^{2+}, na síntese de lipídios e principalmente no enovelamento e secreção de proteínas recém-sintetizadas (Braakman e Hebert, 2013; Elshaer e Begun, 2017). Em condições normais, após o enovelamento correto, as proteínas são transportadas para os locais nos quais desempenharam suas funções celulares corretamente. Entretanto, alterações nesse processo podem levar ao acúmulo de proteínas mal enoveladas, consequentemente comprometendo a homeostasia do RE e caracterizando o estresse do RE. Alguns fatores podem estar envolvidos nesse processo como as ERO, privação de nutrientes, mutações gênicas, infecções, hipóxia e isquemia (Doroudgar *et al.*, 2009; Braakman e Hebert, 2013; Khan *et al.*, 2015).

Em resposta ao estresse do RE, ocorre a ativação de uma via de sinalização conhecida como UPR (do inglês *Unfolded protein response*) que desencadeia a indução de chaperonas para aumentar o dobramento das proteínas, sendo a proteína regulada por glicose 78 kDa (GRP78) a principal chaperona residente no RE (Braakman e Hebert, 2013). A via da UPR possui algumas moléculas sinalizadoras como a *inositol-requiring enzyme-1 (*IRE-1), *protein kinase RNA-like ER-kinase* (PERK) e a *activating transcription fator-6* (ATF-6), que são mantidas inativadas no citoplasma pela ligação com a proteína GRP78. O acúmulo de proteínas mal formadas leva a perda da associação entre a GRP78 e a ATF-6, assim permitindo a translocação da ATF-6 para o núcleo onde se liga a região promotora conhecida como elemento de resposta ao estresse de RE (ERSE) ocasionando a expressão de genes como das chaperonas (figura 16) (Xu *et al.*, 2005; Doroudgar *et al.*, 2009).
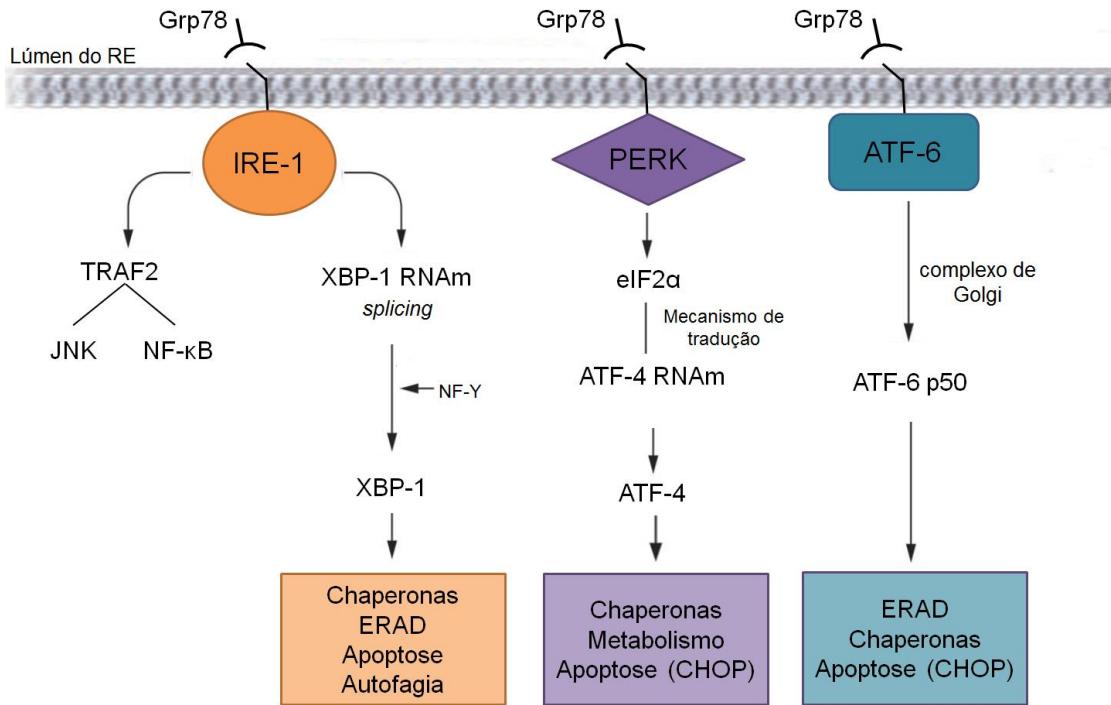
Figura 16: Mecanismo de ação das moléculas sinalizadoras da via UPR.
Fonte: Adaptada (Xu *et al.*, 2005).

A redução de fornecimento sanguíneo e, principalmente, o restabelecimento do fluxo sanguíneo podem estar associados ao estresse do RE pelo requerimento de O_2 no momento da formação e síntese de proteínas no RE. Sendo assim, a ativação do estresse do RE pode ser ativado no momento da isquemia e seus efeitos prejudiciais serem agravados pela reperfusão ocorrendo a liberação de ERO e ERN e citocinas que podem resultar em alterações no enovelamento das proteínas em diferentes órgãos (Bilecová-Rabajdová *et al.*, 2010; Elshaer e Begun,2017).

Na I/R intestinal, as células caliciformes são mais suscetíveis ao estresse do RE, sendo que a inibição do estresse de RE faz-se necessária para manter o equilíbrio na homeostase e da função intestinal, impedindo a ativação de citocinas que podem levar a uma inflamação local e sistêmica (Kaser *et al.*, 2011; Elshaer e Begun, 2017).

Sun *et al.* (2014) sugeriram que o tratamento com N-acetilcisteína proporcionou proteção contra a lesão de I/R hepática, o que foi observado pela redução do estresse do RE no fígado dos animais (Sun *et al.*, 2014). Atualmente, a regulação desse estresse está sendo associada com terapias antioxidantes que

demonstram um papel protetor contra os marcadores de estresse do RE (GRP78 e ATF-6) em diferentes modelos experimentais de I/R (Sun *et al.*, 2014; Hadj Ayed Tka *et al.*, 2015; Jian *et al.*, 2016).

2.9 Glutamina

A glutamina (Gln), considerada o aminoácido mais abundante no organismo, possui propriedades biologicamente importantes e pode ser encontrada no plasma e em diferentes tecidos. A Gln desempenha um papel importante em pacientes graves, como aqueles internados nas Unidades de Terapia Intensiva (UTI), pois ocorre um consumo maior dessa substância nesses casos e a Gln mostra-se um aminoácido essencial nessas situações, reduzindo a taxa de mortalidade desses pacientes críticos (figura 17) (Curi *et al.*, 2005; Wernerman, 2008).
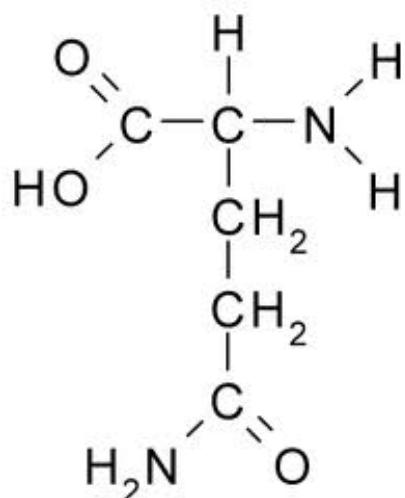
Figura 17: Estrutura química da glutamina.
Fonte: Adaptada (Schemitt, 2014).

A Gln possui algumas funções de extrema importância ao organismo como no equilíbrio ácido-básico, gliconeogênese, biossíntese de nucleotídeos, modulação da resposta inflamatória e pode aumentar a taxa de captação de oxigênio celular, função respiratória mitocondrial de enterócitos e melhora o suprimento de sangue intestinal entre outras funções (figura 18) (Borges *et al.*, 2008; Spodenkiewicz *et al.*, 2016). Além disso, a Gln é precursora da GSH considerada um importante

antioxidante que protege as células epiteliais do intestino contra os danos oxidativos e inibe o processo inflamatório da mucosa intestinal (Wernerman, 2008; Demirkan *et al.*, 2010).

A Gln apresenta um papel importante na defesa imunológica intestinal induzindo a produção de IgA sendo uma fonte essencial para o trato gastrointestinal. E evidências indicam que Gln desempenha um papel crítico na manutenção da função da barreira intestinal, assim preservando a permeabilidade e integridade do epitélio intestinal e reduzindo a translocação bacteriana para a circulação sistêmica (Demirkan *et al.*, 2010; Zabot *et al.*, 2014).
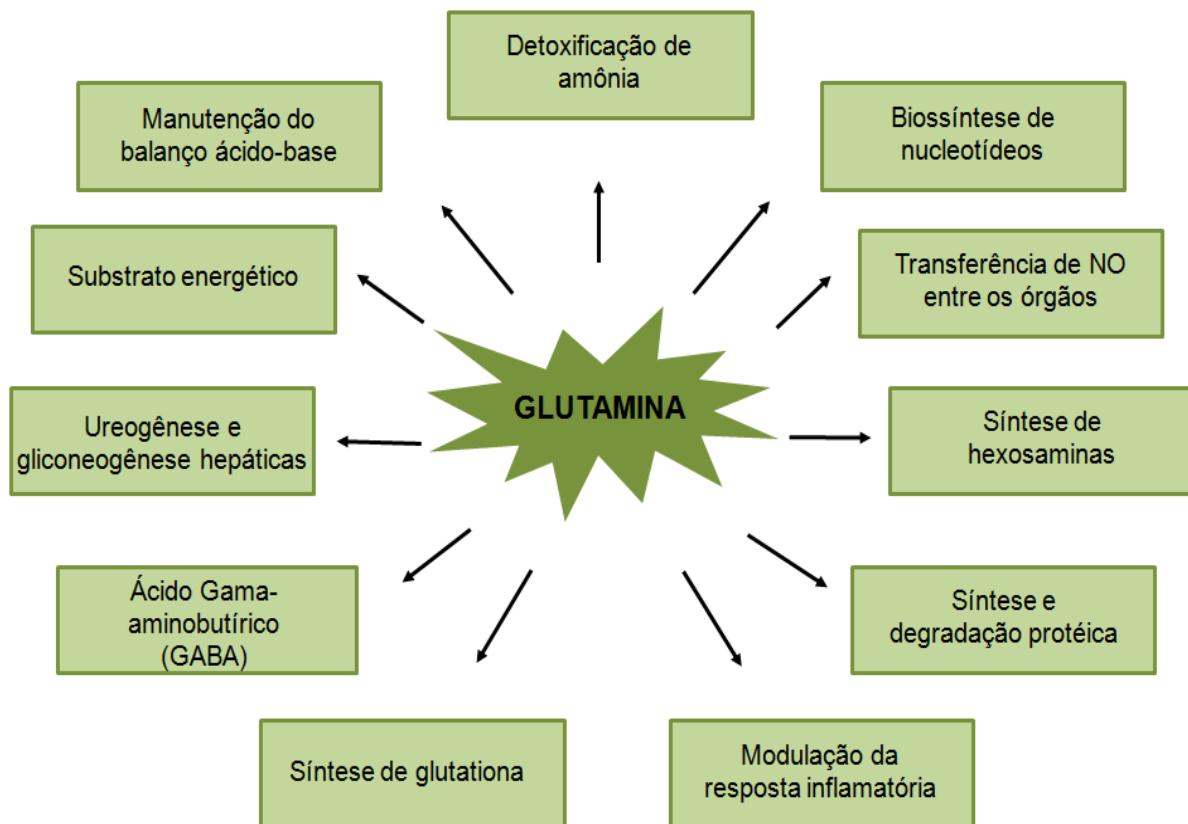
Figura 18: Funções da glutamina no organismo.
Fonte: Adaptada (Borges *et al.*, 2008).

Estudos realizados no nosso grupo de pesquisa demonstram que a administração da Gln melhorou a resposta inflamatória reduzindo a geração de RL e citocinas envolvidos em modelo experimental de colite induzida por ácido acético e ácido 2,4,6-trinitrobenzenosulfônico (Fillmann *et al.*, 2007; Kretzmann *et al.*, 2008). Outros estudos do grupo com modelo experimental de ligadura parcial de veia porta

e de insuficiência hepática aguda grave sugeriram que tratamento com a Gln protege a mucosa gástrica e tecido hepático reduzindo o dano oxidativo e inflamatório por sua possível ação antioxidante (Marques *et al.*, 2011; Schemitt *et al.*, 2016).

A Gln está sendo investigada na proteção contra a lesão de I/R no intestino, fígado e rins sugerindo que a sua administração exerce uma proteção a esses órgãos contra as lesões teciduais e danos gerados pelas ERO e ERN (Gouvêa Junior *et al.*, 2011; Sözen *et al.*, 2011; Zabot *et al.*, 2014). Wu *et al.* (2004) demonstraram que o pré-tratamento com Gln em ratos com I/R intestinal diminuiu os níveis de lactato desidrogenase e as endotoxinas no plasma, aumentou a permeabilidade intestinal e reduziu a incidência de translocação bacteriana (Wu *et al.*, 2004).

Zhang *et al.* (2011) sugeriram que o pré-tratamento com Gln foi capaz de aumentar a capacidade antioxidante em um modelo de I/R hepática. Os animais tratados com diferentes doses de Gln mostraram um aumento na atividade de SOD, sugerindo um papel protetor dessa substância no fígado contra o dano oxidativo causado pela I/R (Zhang *et al.*, 2011).

Tendo em vista essas considerações, analisamos o efeito protetor da glutamina no intestino e fígado dos animais submetidos ao modelo experimental de I/R intestinal, avaliando esses processos envolvidos como o estresse oxidativo, processo inflamatório e estresse do reticulo endoplasmático.

3 MARCO CONCEITUAL

A isquemia intestinal pode ocorrer frequentemente em várias situações clínicas e são difíceis de serem diagnosticadas devido às múltiplas etiologias que podem culminar nesse quadro. Desta forma, apresentam uma taxa de mortalidade relativamente alta.

O intestino tem uma sensibilidade maior a danos, principalmente os causados pela I/R intestinal, o que leva ao rompimento das células intestinais e liberação de conteúdo luminal, incluindo endotoxinas e outros patógenos para a circulação sistêmica. A I/R intestinal tem grande associação nas lesões em órgãos distantes como o fígado e esses danos podem ser desencadeados pela perda da integridade da membrana basal intestinal, facilitando a produção e liberação de ERO, citocinas e toxinas oriundas do intestino para a circulação portal promovendo processo inflamatório no fígado

A Gln está sendo investigada na proteção contra a lesão de I/R, sugerindo que a sua administração exerce uma proteção a esses órgãos contra as lesões teciduais e danos gerados pelas ERO, citocinas e NO, reduzindo possíveis danos sistêmicos (figura 19).
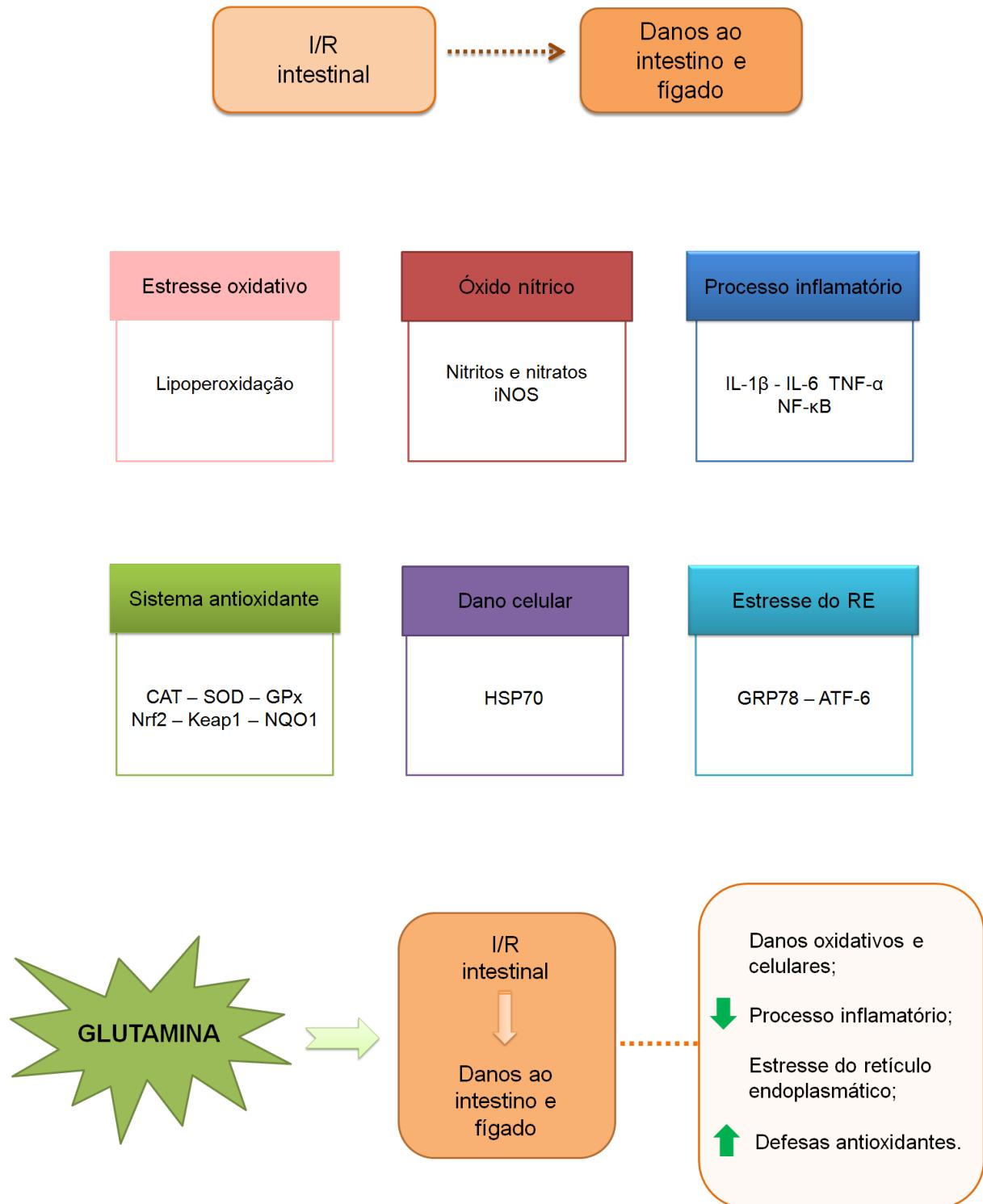
Figura 19: Marco conceitual da I/R intestinal e danos ao fígado.

Fonte: Autora.

4 JUSTIFICATIVA

As situações de isquemia mesentérica são muito comuns na prática clínica diária, sobretudo em pacientes de Unidade de Terapia Intensiva (UTI), pacientes idosos e pós-operatório de cirurgias de médio e grande porte. A hipotensão arterial, causada principalmente por hipovolemia, sepses ou distúrbios cardiológicos, leva a uma diminuição imediata do fluxo mesentérico. O cuidado com o fluxo sanguíneo no intestino delgado e grosso é muitas vezes relegado a um segundo plano, priorizando a manutenção de órgãos agudamente mais vitais como cérebro, coração e rins. Entretanto, as repercussões desta isquemia mesentérica podem trazer graves consequências ao indivíduo, podendo, inclusive levá-lo a óbito.

A lesão por I/R intestinal, além de causar lesões locais, pode desencadear lesões em órgãos distantes e iniciar uma reação inflamatória sistêmica associada à lesão endotelial intestinal, translocação bacteriana e liberação de substâncias inflamatórias que, através da veia porta, afetará o fígado. Tal lesão está associada à alta morbidade e mortalidade dos pacientes e por este motivo ocorre à necessidade do estudo de substâncias que possam impedir ou diminuir a injúria local e danos sistêmicos.

O excesso na produção de ERO e citocinas que ocorre na lesão de I/R intestinal ocasiona alteração na integridade do epitélio do intestino levando à liberação dessas substâncias deletérias diretamente para o fígado, via veia porta, ocorrendo um intenso processo inflamatório no fígado. Desta forma, experimentos utilizando agentes antioxidantes mostram-se uma opção útil para novas terapias.

A Gln está sendo investigada na proteção contra a lesão de I/R no músculo cardíaco, músculo esquelético, intestino e rins. Alguns estudos com modelos experimentais de I/R demonstram que a administração de Gln pode prevenir as lesões locais pela redução nos danos teciduais e oxidativos. O estudo do papel da Gln no modelo de I/R intestinal mostra-se viável por ser uma possível estratégia para novas terapias na tentativa de melhorar o prognóstico e o índice global de complicações sistêmicas nos pacientes com I/R intestinal.

Por este motivo, avaliamos o efeito da Gln no intestino e fígado dos animais submetidos ao modelo experimental de I/R intestinal na tentativa de elucidar o eixo

intestino/fígado e quais os mecanismos oxidativos e moleculares estariam envolvidos nessas rotas celulares.

5 OBJETIVOS

5.1 Objetivo geral

Avaliar os efeitos da Gln no intestino e fígado de animais submetidos à isquemia e reperfusão intestinal.

5.2 Objetivos Específicos

5.2.1 Objetivos específicos do artigo I

Respondidos no artigo intitulado, "*Effect of glutamine on liver injuries induced by intestinal ischemia-reperfusion in rats*".
Aceito para publicação na Revista *Nutrición Hospitalaria*.

- Avaliar as enzimas de integridade hepática integridade hepática através da análise sérica das enzimas AST, ALT e FA;
- Avaliar o dano ao DNA pelo ensaio cometa;
- Analisar os níveis de LPO no fígado através da técnica de TBARS;
- Determinar a atividade das enzimas antioxidantes CAT, SOD e GPx no fígado;
- Analisar os níveis de glutationa no fígado;
- Analisar e quantificar a expressão da IL-6 e do NF-κB no fígado pela técnica de imunohistoquímica;
- Quantificar a expressão do NF-κB pela técnica de *Western blot*.

5.2.2 Objetivos específicos do artigo II

Respondidos no artigo intitulado, "*Protective effect of glutamine on the main and adjacent organs damaged by ischemia/reperfusion in rats*".
Publicado na Revista *Protoplasma (maio de 2017)*.

- Avaliar a integridade hepática através da análise sérica das enzimas AST e ALT;
- Analisar os níveis de LPO no intestino e fígado através da técnica de TBARS;

- Analisar as alterações histológicas no intestino e fígado;

- Avaliar os níveis dos metabólitos do óxido nítrico no intestino e fígado pela técnica de nitritos e nitratos;

- Analisar e quantificar a expressão da IL-1β e do TNF-α no intestino e fígado pela técnica de imunohistoquímica

- Quantificar a expressão das proteínas iNOS, Nrf2, Keap1, NQO1,SOD, HSP70, GRP78 e ATF-6 no intestino e fígado pela técnica de *Western blot*.

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7 ARTIGOS

7.1 Artigo I:

"*Effect of glutamine on liver injuries induced by intestinal ischemia-reperfusion in rats*".

Publicado na Revista *Nutrición Hospitalaria* (2017).

7.2 Artigo II:

"*Protective effect of glutamine on the main and adjacent organs damaged by ischemia/reperfusion in rats*".

Publicado na Revista *Protoplasma* (2017).

Protective effect of glutamine on the main and adjacent organs damaged by ischemia/reperfusion in rats

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Abstract

Background and Aim: Intestinal ischemia and reperfusion (I/R) causes cellular and tissue damage to the intestine and remote organs such as the liver. Increased production of ROS and nitric oxide, desregulation of cytoprotective enzymes, may be involved in intestinal I/R. Aim was to evaluate the protective effects of glutamine on the intestine and liver of rats with intestinal I/R injury.

Methods: Twenty male Wistar rats (300 g) were divided into four groups: sham-operated (SO), glutamine + SO (G+SO), ischemia and reperfusion (I/R), and glutamine + I/R (G+I/R). Occlusion of the SMA for 30 minutes followed by 15 minute reperfusion. Glutamine (25 mg/kg/day) was administered once daily 24 and 48 hours before I/R induction. Blood and tissue of were collected for aspartate aminotransferase (AST), alanine aminotransferase (ALT) levels, histopathological analysis, immunohistochemistry of IL-1β and TNF-α, TBARS and nitric oxide, Nrf2/keap1, superoxide dismutase (SOD), NADPH quinone oxidoreductase1 (NQO1), inducible nitric oxide synthase (iNOS), heat shock protein (HSP70), glucose-regulated protein 78 (GRP78) and activating transcription factor 6 (ATF-6) by western blot. Statistic analysis by ANOVA – Student -Newman-Keuls test (mean ± SE) significantly was $p < 0.05$.

Results: Tissue damage, AST, ALT, IL-1β, TNF-α, TBARS, NO, Keap1, iNOS, GRP78 and ATF-6 expression were significantly lower in the G+I/R group as compared to the I/R group. Expression of Nrf2, SOD, NQO1, HSP70, was significantly higher in the G+I/R group as compared to I/R group.

Conclusion: Pretreatment with glutamine provided protection against oxidative damage in the intestine and liver in an experimental model of intestinal I/R.

Key Words: cytoprotective enzymes; endoplasmic reticulum stress; inflammation; nitric oxide; oxidative stress

Abbreviations: ANOVA - one-way analysis of variance; ATF-6 - activating transcription factor 6; ERS - endoplasmic reticulum stress; Gln - glutamine; GRP78 - glucose-regulated protein 78; HSP 70 - heat shock protein 70; iNOS - inducible nitric

oxide synthase; I/R – ischemia/reperfusion; LPO - lipid peroxidation; NQO1 - NADPH quinone oxidoreductase 1; Nrf2 - nuclear factor erythroid 2; RNS - reactive nitrogen species; ROS - reactive oxygen species; SOD - superoxide dismutase.

Introduction

Intestinal ischemia and reperfusion injury (I/R) is a life-threatening event that frequently results from pathological factors such as hemorrhagic shock, strangulated hernias, and vascular surgery. In critically ill patients, the mortality risk associated with I/R is between 60% and 80% (Bolekova et al. 2011; Ozban et al. 2015). Intestinal I/R injury results from the restoration of blood flow (reperfusion) following a period of reduced blood flow (ischemia) in the intestine. Reperfusion may compromise the integrity of the intestinal epithelial barrier and is associated with the generation of reactive oxygen (ROS) and nitrogen species (RNS) and cytokines causing damage to the ischemic site as well as to remote non-ischemic organs, such as the liver (Takizawa et al. 2011; He et al. 2015).

The intestine and the liver are closely associated, with continuous two-way communication through bile, hormones, inflammatory mediators, and products of digestion. The intestinal microbiota also plays an important role in liver function, because intestinal substances of bacterial origin may dysregulate normal liver metabolism (Liu et al. 2014; Vaikunthanathan et al. 2016). Even though the mechanisms underlying intestinal I/R-induced liver damage have not been elucidated, factors such as ROS, cytokines (interleukins IL-1β and IL-6) and tumor necrosis factor (TNF-α), endoplasmic reticulum stress and activation of nuclear factor have been positively linked to this process (Zhao et al. 2010; Fan et al. 2014; Liu et al. 2014). Fan et al. (2014) have observed that animals with induced intestinal I/R had tissue changes such as infiltration of neutrophils and liver edema, along with elevated levels of serum enzymes and changes in markers of oxidative damage and inflammation in the liver (Fan et al. 2014).

Studies suggest that oxidative stress is involved in local and systemic lesions in intestinal I/R, because the accumulation of ROS can lead to protein oxidation, lipid peroxidation, and DNA damage (He et al. 2015). Nuclear factor (erythroid-2)-related factor 2 (Nrf2) is a regulator of cellular responses to oxidative damage. Under normal conditions, Nrf2 is maintained in the cytoplasm by binding to the kelch-like ECH-associated protein 1 (Keap1). Stimuli such as oxidative stress cause Nrf2 to dissociate from Keap1, with translocation of Nrf2 into the cell nucleus, where it binds to promoter sequence denominated to as antioxidant response element (ARE). After

this process, cytoprotective genes and antioxidant defenses, such as NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) and superoxide dismutase (SOD), are upregulated (Zhao et al. 2010; Fan et al. 2014; Yao et al. 2014; Wang et al. 2014) .

The excess production of nitric oxide from L-arginine by the action of the enzyme nitric oxide synthase (NOS) could also be associated with intestinal I/R injury. Three isoforms of NOS (neuronal, nNOS; endothelial, eNOS; and inducible, iNOS) are expressed in response to inflammatory mediators. The increased expression of iNOS contributes to inflammation of intestinal tissue and distal organs (Bolekova et al. 2011; He et al. 2015). Using an experimental model of intestinal I/R injury, Liu et al. (2007) have demonstrated that the systemic inflammatory response and damage observed in lung tissue were related to the positive expression of iNOS and the increased levels of NO (Liu et al. 2007).

Heat shock protein 70 (HSP 70) is an endogenous protein that plays a protective role in cellular function, assisting in protein synthesis. Studies have shown that induction of HSP70 occurs in response to various stimuli, such as exposure to toxins, glucose deprivation, and ROS formation (Fleming et al 2002; Shiota et al. 2010). Experimental studies demonstrate that increased expression of HSP70 may be beneficial against I/R damage in different organs, suggesting a protective effect of this protein (Abudunaibi et al. 2015; Kierulf-Lassen et al. 2015).

The endoplasmic reticulum (ER) is a multifunctional organelle, that ensures the correct structure of proteins and plays a key role in the lipids' synthesis, sterols and the maintenance of intracellular calcium (Doroudgar et al. 2009). ER is sensitive to imbalance in cellular homeostasis triggered by different types of endogenous or exogenous stress, such as ischemia, chemical damage, increased protein synthesis, nutrient insufficiency and infections. Such changes may lead to endoplasmic reticulum stress (ERS), causing the activation of the intracellular signaling pathway known as the unfolded protein response (UPR) and leading to the expression of certain markers such as glucose-regulated protein 78 (GRP78) and activating transcription factor 6 (ATF-6) related to ERS (Elshaer and Begun 2016). ERS may play an important role in the progression of intestinal I/R and studies indicate that the inhibition/regulation of ERS provides protection against I/R at the initial site and in different organs that may be injured (Kaser et al. 2011; Khan et al. 2015).

Antioxidant therapy has been studied in experimental models as an alternative to prevent the organ injury associated with I/R. The results of various studies have shown a decrease in tissue and oxidative damage in intestinal I/R as well as a reduction in systemic inflammatory response (Fleming et al 2002; Liu et al. 2007; Zhao et al. 2010; Takizawa et al. 2011; Fan et al. 2014).

Glutamine is the most abundant amino acid in plasma and skeletal muscle. It contributes to the synthesis of amino acids, proteins, and nucleotides, and also provides nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH) for the maintenance of redox homeostasis (Wu et al. 2004; Vasconcelos et al. 2011; Chen and Cui 2015). Glutamine supplementation in critically ill patients can improve immune function, thus preventing potential infection and sepsis (Wernerman 2008). A study by Wu et al. (2004) showed that pretreatment with glutamine in rats with intestinal I/R decreased lactate dehydrogenase levels and endotoxins in plasma, increased intestinal permeability, and reduced the incidence of bacterial translocation (Wu et al. 2004). Xu et al. (2014) found that treatment with glutamine in rats exposed to hypobaric hypoxia was effective in reducing intestinal damage by decreasing the levels of inflammatory cytokines TNF-α and IL-6 and of malondialdehyde. This suggests an antioxidant effect and anti-inflammatory effect of glutamine (Xu et al. 2014).

Therefore, the aim of the present study was to evaluate the effects of glutamine on oxidative damage in a rat model of induced intestinal I/R. For that, we analyzed the tissue damage, activation of Keap1/Nrf2, the expression of SOD, NQO1, HSP70, iNOS, GRP78, ATF-6 and the levels of lipid peroxidation and nitric oxide metabolites in the intestine and liver. We hypothesized that pretreatment with glutamine would restore protection against oxidative damage following I/R.

Materials & Methods

Animals

The care of laboratory animals followed local and international guidelines with animals were in accordance with the recommendations by the Research and Ethics Committee on Health Research Group and Graduate Studies (GPPG) Porto Alegre

Clinical Hospital (HCPA) and the recommendations of the European Directive 2010/63/EU (Goldim and Raymundo 1997; European Commission 2010).

Twenty male Wistar rats (mean weight: 300 grams) were divided into four groups: sham-operated (SO) (n = 5), glutamine + sham-operated (G + SO) (n = 5), ischemia and reperfusion (I/R) (n = 5), and glutamine + ischemia and reperfusion (G + I/R) (n = 5). The animals were housed in the Animal Experimentation Unit (UEA) at Porto Alegre University Hospital, in plastic boxes measuring 47x34x18 cm, lined with wood shavings, at 20-25 °C, in a 12 hour light/dark cycle (light from 7 a.m. to 7 p.m.). Water and feed were given ad libitum.

Glutamine administration

Glutamine was administered intraperitoneally at a dose of 25 mg/kg diluted in 1 mL of saline. This dose was given once daily for 48 hours before ischemia induction (Zabot et al. 2014).

Experimental model

The animals were anesthetized by intraperitoneal injection of xylazine (8 mg/kg body weight) and ketamine hydrochloride (92 mg/kg body weight). Laparotomy and evisceration were performed to identify the superior mesenteric artery in all groups. In the SO and G + SO groups, the artery was not obstructed. In the I/R and G + I/R groups, the artery was occluded for 30 minutes using a microvascular dissecting forceps. After the intestinal ischemia period, the vascular clamp was removed for 15 minutes of reperfusion (Cho et al. 2013). After the reperfusion period, a portion of the intestine and liver and blood samples were removed for further analysis. Finally, the animals were euthanized by exsanguination under deep anesthesia (THE NATIONAL ACADEMIES PRESS 2011).

Liver function tests based on aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

Levels of serum AST, ALT were determined by the Biochemistry Center of the Hospital de Clínicas de Porto Alegre. To determine AST and ALT levels in the plasma, we used the commercially available enzymatic method (Boehringer Mannheim, Germany) and the results were obtained by kinetic measurement at 567 nm.

Histopathological analysis

The sections of intestine and liver were removed and fixed in 10% buffered formalin for 24 h. After paraffin blocks were cut with a rotary microtome to create 3-mm sections. The tissues were stained with hematoxylin and eosin and the slides were analyzed by a pathologist who had no prior knowledge of the groups. The slides were photographed using a NIKON Labophot binocular microscope at a magnification of 200× and the damage was evaluated in accordance with the criteria below.

Intestinal injury was classified using scale defined by Chiu et al. (1970) as follows:

0 = no diagnostic change; grade 1 = epithelial layer lifting from the lamina propria, usually at the apex of the villus; grade 2 = moderate epithelial cell layer lifting from the lamina propria; grade 3 = loss of a few villi with massive epithelial lifting from the lamina propria with a few denuded villi; and grade 4 = disintegration of the lamina propria with ulceration and hemorrhage.

Liver injury was evaluated by Onder et al. (2012) as follows:

0 = normal; 1 = mild damage; 2 = moderate damage and infiltrate, and 3 = intense damage, hemorrhage, necrosis with loss of hepatic architecture.

Each slide of intestine and liver was evaluated by an experienced pathologist who was blinded to the experiment and the data. The slides were randomly numbered and then given to the pathologist for scoring.

Immunohistochemistry and quantification of the expression of IL-1β and TNF-α

The expression of IL-1β and TNF-α in intestine and liver tissue was determined by immunohistochemical analysis. Antigen retrieval was performed using buffer at 60 °C, and endogenous peroxidase activity was blocked by incubation in absolute methanol. The slides were incubated with rabbit polyclonal antibody (IL-1β - Santa Cruz Biotechnology, USA) at 1:200 and (TNF-α - Santa Cruz Biotechnology, USA) at 1:200 overnight at 4 °C. The slides were washed with buffer and incubated with the secondary antibody (anti-mouse IgG-HRP, anti-goat IgG-HRP, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:300 for 30 minutes at room temperature. The slides were examined by a pathologist, who was unaware of the groups, using a microscope equipped with a digital analysis system including a Zeiss Axioskop 40 microscope (Oberkochen, Germany) connected by a Roper Scientific camera (Media Cybernetics, Rockville, USA) to a computer with an image capture software. The Image-Pro Plus version 4.5 software (Media Cybernetics, Rockville, USA) was used to analyze digital images. The expression was determined by multiplying the mean density of the image by the percentage of positively stained areas (brown-stained areas).

Thiobarbituric acid reactive substances (TBARS)

The amount of aldehydes generated by lipid peroxidation (LPO) was determined by a method that measures the amount of thiobarbituric acid reactivity. Thiobarbituric acid was added to the samples at 0.37%, whereas trichloroacetic acid was added at 15%. The samples were incubated at 100 °C for 15 minutes and centrifuged at 3,000 rpm (1,612 x g) for 10 minutes at 4 °C. Absorbance was determined by spectrophotometry at 535 nm (Buege and Aust 1978).

Metabolites of nitric oxide (nitrites and nitrates)

Nitric oxide production was measured indirectly using the quantitative Griess colorimetric assay. This assay is based on enzymatic reduction of nitrates (NO_3-) to

nitrites (NO_2-) in the presence of nitrate reductase and NADPH, with subsequent colorimetric determination of NO_2- by the Griess reagent (a mixture of sulfanilamide and naphthylethylenediamine specific for NO_2-). Because excess NADPH inhibits the Griess reaction, it is necessary to oxidize all NADPH that was not used in the reduction of NO_3-. This is achieved by adding nitrate reductase. The reading was performed in a microplate reader at 540 nm. The results are expressed as mmol of NO_2-/NO_3- (Granger et al. 1999).

Expression of Nrf2/Keap1, NQO1, SOD and HSP70, GRP78 and ATF-6 in intestine and liver

Western blot analysis was performed on nuclear and cytosolic extracts prepared from intestine and liver homogenates as previously described (Laemmli et al. 1970; Towbin et al. 1992). Briefly, the supernatant was collected and stored at -80 °C in 200 µL aliquots until further analysis. Lysate proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) for 1 hour at room temperature and probed overnight at 4 ºC with polyclonal iNOS (120 kDa), GRP78 (78 kDa), Keap1 (69 kDa), Nrf2 (57 kDa), ATF-6 (50 kDa), SOD (32 kDa), and NQO1 (31 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200-1,000 dilution with TTBS in 5% nonfat dry milk. After that, the membranes were incubated with anti-β-actin antibody (A5060/42kDa) (Sigma Aldrich, St Louis, MO, USA) at 1:2,000 dilution with TTBS in 5% nonfat dry milk. After washing with TTBS, the membranes were incubated for 1 hour at room temperature with secondary anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:4,000). Protein detection was performed by chemiluminescence using a commercial ECL kit (Amersham Pharmacia Biotech, Little Chalfont, UK). The density of the specific bands was quantified using Scion Image software (Scion Corp., Frederick, MD, USA).

Statistical analysis

Data are expressed as means ± standard error. Statistical significance was calculated using Graphpad Instat, version 3.0 for Windows. We used one-way analysis of variance (ANOVA) and Student-Newman-Keuls for multiple analysis. Results were considered statistically significant when $p<0.05$.

Results

Liver function tests based on aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

The serum levels of AST and ALT were significantly increased in the I/R group as compared with the SO and G+SO groups, whereas there was a reduction in these levels in the I/R+G group in comparison with the I/R group (Figure 1, AST *, #p<0.001; ALT *, p<0.001).

Histopathological analysis

In the intestine tissue, we observed that the damage in I/R group was significantly higher than in the control (SO and G+SO) and pre-treated groups (G+I/R). The microscopic analysis of the I/R group showed edema, hemorrhage, alterations such as loss and shortening of the villi and some glandular structures. In the glutamine pre-treated group (G+I/R) a preservation of the epithelial layer occurred, without loss of villi when compared to I/R group. The SO and G+SO groups did not show any tissue changes (figure 2, *,# p<0.001).

In the hepatic tissue, significant tissue changes were observed in I/R group, loss of hepatic architecture, hemorrhage, necrosis and inflammatory infiltrate. In the G+I/R group it was possible to observe an improvement in those lesions as well as a reduction of the necrosis and preservation of the liver parenchyma. The SO and

G+SO groups showed no significant tissue changes. The score for the damage in I/R group, in both organs, was significantly higher than in the control (SO and G+SO) and pre-treated (G+I/R) groups (Figure 3, *,# $p<0.01$).

Immunohistochemistry and quantification of the expression of IL-1β and TNF-α

The I/R group showed strong positive staining for IL-1β in the intestine (figure 3a) and liver (figure 3b) visible in brown staining. The SO and G+SO groups showed no positive staining. Pretreatment with Gln reduced the staining for IL-1β in the G+I/R group. In the same way, the quantification of the expression of IL-1β showed an increase in the expression of inflammatory mediators in the I/R group as compared with the SO and G+SO groups and a significant reduction in the G+I/R as compared with the I/R group (Figure 4a, Figure 4b, *$p<0.001$, #$p<0.001$). All images were magnified 200x.

In the TNF-α results we observed strong positive staining for TNF-α in the intestine (figure 4a) and liver (figure 4b) of I/R group, visible in brown staining. The pretreatment with Gln group showed reduced the staining for TNF-α. The SO and G+SO groups showed no positive staining. In quantification of the expression of TNF-α showed an increase in the expression of I/R group as compared with the SO and G+SO groups and a significant reduction in the G+I/R as compared with the I/R group (Figure 5a, Figure 5b, *$p<0.001$, #$p<0.001$). All images were magnified 200x.

Lipid peroxidation (TBARS)

LPO levels in intestine and liver were significantly higher in the I/R group as compared to the SO and G+SO groups, and significant lower in the G+I/R group as compared to the I/R group (Table 1) (*, # $p<0.001$).

Metabolites of nitric oxide (nitrites and nitrates) and expression of iNOS

The levels of nitric oxide metabolites (nitrites and nitrates) were significantly higher in I/R as compared to the SO and G+SO groups, and significantly lower in the G+I/R group as compared to the I/R group. Expression of iNOS was significantly higher in I/R group as compared to the SO and G+SO groups, and significantly lower in the G+I/R group as compared to the I/R group (Figure 6a, Figure 6b, NO *,# p <0.05 iNOS *, # p <0.001).

Expression of NRF2 / Keap1, NQO1, SOD, HSP70, GRP78 and ATF-6 in intestine and liver

Nuclear expression of Nrf2 was significantly lower in I/R as compared to the SO and G+SO groups, and significant higher in the G+I/R group as compared to the I/R group. Cytoplasmic expression of Keap1 was significantly higher in I/R as compared to the SO and G+SO groups, and significantly lower in the G+I/R group as compared to the I/R group (Figure 7a, Nrf2 *, # p <0.01; Keap1 *, # p <0.05). Expression of NQO1 and SOD was significantly lower in I/R vs. SO and G+SO, and significantly higher in the G+I/R group than as compared to the I/R group (Figure 7b, SOD and NQO1 *, #p <0.01). HSP70 expression was significantly lower in I/R vs. SO and G+SO, and significantly higher in the G+I/R group than in the I/R group (Figure 8, HSP70 *, # p < 0.01). Stress of endoplasmic reticulum, evaluated by markers GRP78 and ATF-6, expression was significantly increased in I/R as compared to the SO and G+SO, and significantly decreased in the G+I/R group than in the I/R group (Figure 9a, Figure 9b, GRP78 and ATF-6 *, # p <0.01).

Discussion

Intestinal I/R injury is associated with high morbidity and mortality in critically ill patients. Restoration of blood flow is required to recover organ function, but

reperfusion causes cell damage as well as systemic damage (Bolekova et al. 2011; Takizawa et al. 2011; Liu et al. 2011; Inan et al. 2013; He et al. 2015).

The liver-intestine axis plays a key role in the deleterious effects induced by intestinal I/R, since intestinal circulation is directly linked to the vasculature of the liver. Intestinal I/R leads to increased release of harmful substances such as ROS, nitric oxide, and inflammatory mediators to the liver through the portal vein, causing severe liver injury (Liu et al. 2011; Onder et al. 2012; Inan et al. 2013; Chassaing et al. 2014). Studies suggest that the increased generation of free radicals is a major cause of local and systemic injury following I/R (Wernerman 2008; Onder et al. 2012; Sun et al. 2014; Xu et al. 2014; He et al. 2015).

Several mechanisms have been implicated in the development of intestinal I/R injury, such as the increase in ROS, which may lead to impaired gut barrier function, hampering motility. These changes in the intestinal mucosal barrier may lead to bacterial translocation, release of inflammatory mediators, and reduction of the antioxidant system, thus causing systemic inflammation (He et al. 2015; Ozban et al. 2015; Tas et al. 2015).

We evaluated tissue damage in the intestine and liver and found increased lesions in the I/R group, as well as in the assessment of liver enzyme function AST and ALT. In the pretreated group with glutamine an improvement in the tissue lesions was observed in both organs and liver enzymes evaluated. Our results corroborate with the studies of Zhao et al. (2010), Inan et al. (2013) and Liu et al. (2014) who observed morphological and tissue changes, such as hemorrhage, inflammatory infiltrate and edema in the intestine and liver, as well as a marked increase in serum levels of AST and ALT in the animals submitted from intestinal I/R induced liver injury and demonstrated a significance decreased when treated with sulforaphane, sindenafil and mesenchymal stem cells (Zhao et al. 2010; Inan et al. 2013; Liu et al. 2014).

In our study, we observed an increase in lipid peroxidation levels in the intestine and liver of animals undergoing occlusion of the superior mesenteric artery. In the glutamine-treated group, these increased levels were reduced; this might be explained by an antioxidant effect of glutamine. Similar results have been observed by Ozban et al. (2015) using pretreatment with melatonin. Those authors report that the beneficial effect of melatonin may be explained by the hormone's ability to

scavenge free radicals, thereby reducing LPO levels in the treated group (Ozban et al. 2015). Other substances, such as aminoguanidine, curcumin, thymoquinone and resveratrol, all of which have antioxidant and anti-inflammatory effects, have also been successfully employed to reduce lipid peroxidation levels in intestinal I/R (Takizawa et al. 2011; Onder et al. 2012; da Silva de Souza et al. 2015; Tas et al. 2015).

Nitric oxide has also been associated with I/R injury and iNOS is expressed in inflammatory conditions in response to pro-inflammatory cytokines. The increase in ROS as well as the increase in NO and iNOS expression and cytokines contribute to the progression of systemic and local I/R injury (Liu et al. 2011). Studies have suggested that the release of these cytokines into the systemic circulation is associated with remnant organ damage after intestinal I/R (Fan et al. 2004; Xu et al. 2004). We observed that the I/R group had higher levels of NO, iNOS expression and cytokines (IL-1β and TNF-α) when compared to the other groups. However, in the I/R group treated with glutamine, we observed a reduction in these parameters, demonstrating the antioxidant potential of pretreatment with glutamine. The increased levels of nitric oxide may have resulted from increased production of iNOS (Kudoh et al. 2014; Sun et al. 2015). In that sense, our results corroborate the findings of Akcılar et al. (2015), who demonstrated that iNOS plays an important role in the aggravation of I/R injury, and that iNOS inhibition entails reduced NO production (Akcılar et al. 2015). Takizawa et al. (2011) observed an increase in NO and iNOS levels in an animal model of intestinal I/R injury. After pretreatment with aminoguanidine and inhibition of iNOS, those authors observed a reduction in NO levels and iNOS gene expression (Takizawa et al. 2011). Ma et al. (2014) observed in their study that of interleukins and TNF-α levels were significantly increased in the intestinal I/R group and pretreatment with protocatechuic acid (PCA) reduced these levels as well as liver enzymes and this improvement was associated to the anti-inflammatory and antioxidant effect of PCA (Ma et al.2014).

Intestinal I/R trigger the initiation of various pathways, such as the Nrf2/Keap1 pathway, which has a protective effect against oxidative damage. Under stress conditions, Nrf2 is separated from Keap1; it is then translocated to the nucleus and activates the expression of genes encoding several antioxidant enzymes, such as superoxide dismutase and NQO1 (Zhao et al. 2010; Sun et al. 2013; Yao et al. 2014;

Wang et al. 2014). In this study, we evaluated the nuclear expression of Nrf2, which was significantly reduced in I/R, as well as the cytoplasmic expression of Keap1, which was increased in the I/R group in both liver and intestine. Similarly, we observed that glutamine pretreatment significantly increased the expression of Nrf2 and of cytoprotective enzymes SOD and NQO1, suggesting that glutamine restored the antioxidant system, reducing oxidative stress in intestine and liver through modulation of the Nrf2/Keap1 pathway. Other studies have also observed an increase in Nrf2 expression when using substances with antioxidant effects, such as sulforaphane, ginsenoside, mangiferin and α-lipoic acid in different models of ischemia and reperfusion (Zhao et al. 2010; Sun et al. 2013, da Silva de Souza et al. 2015; Sun et al. 2015). Zhao et al. (2010) analyzed the expression of Nrf2 in liver tissue in a model of intestinal I/R. They observed increased expression in the group treated with sulforaphane, a natural compound derived from vegetables, suggesting an antioxidant and protective effect through the Nrf2-ARE pathway (Zhao et al. 2010).

Kudoh et al. (2014) used a derivative of PGJ_2 omega 6 as treatment in a rat model of hepatic I/R injury and observed increased NQO1 expression in the treated group. That study also used immunofluorescence to evaluate Nfr2, and showed nuclear translocation of Nrf2 in the treated group, reflected by the strong staining in the nucleus of hepatocytes (Kudoh et al. 2014). Taken together, the results of the present and earlier studies confirm that the pre-administration of antioxidants in models of I/R injury activates Nrf2, which reduces local and systemic damage by regulating SOD and NQO1 (Akcılar et al. 2015).

Heat shock proteins are also important for cell protection. In particular, HSP70 is constitutively expressed and can be induced in response to various stresses, including trauma, inflammatory diseases, oxidative stress, and I/R injury (Shiota et al. 2010; Kiang et al. 2011; Abudunaibi et al. 2015). In the present study, we observed decreased expression of HSP70 in animals with I/R; however, glutamine treatment led to significant increase in HSP70 expression in intestine and liver. This suggests that glutamine, possibly as a result of its important antioxidant effect, effectively regulated HSP70 in our intestinal I/R model. Our results are similar to those of Abudunaibi et al. (2015), who reported a decrease in HSP70 expression in a model

of myocardial I/R injury, followed by increased HSP70 expression when the animals were treated with different doses of Munziq (Abudunaibi et al. 2015).

Inhibition of ROS and ERS generation is necessary to maintain balance in homeostasis and intestinal function, thus preventing the activation of cytokines that may lead to local and systemic inflammation (Elshaer and Begun 2016). In our study we observed the presence of ERS in the animals of the I/R group, demonstrated by increased expression of GRP78 and ATF-6. Positive transcription of these ERS markers is triggered by several stimuli that evade the imbalance of ER function, for example, intestinal I/R. We have demonstrated that glutamine promoted a significant decrease in the expression of GRP78 and ATF6, observed in animals that received pretreatment with glutamine. Sun et al. (2014) suggested that treatment with N-acetylcysteine provided protection against hepatic I/R injury, observed by the reduction of ER stress in the liver of the animals. Currently, ERS regulation is being associated with antioxidant therapies that demonstrate a protective role against ERS in different experimental models of I/R (Espinosa-Diez et al. 2015).

In summary, the present results indicate that pretreatment with glutamine reduced lesions in the intestine and liver, inhibited lipid peroxidation, levels of NO, expression of iNOS and ER stress. Also, glutamine regulated the cytoprotective capacity, with upregulation of the Nrf2/Keap1 pathway and increased expression of antioxidant enzymes SOD, NQO1 and HSP70 in liver and intestine. Other glutamine action mechanisms are being studied to further clarify the protective effect observed in this model.

Conflict of Interest:

The authors declare that they have no conflict of interest.

Ethical approval:

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Figure legends

Fig. 1: Effect of glutamine administration on levels of enzymes of liver cell integrity in the serum of animals subjected to ischemia and reperfusion. Values are expressed as mean ± mean standard error. * Significant difference between the I/R group as compared to the SO and G+SO. # Significant difference between the G+I/R as compared to the I/R group. AST (*, # $p<0.001$), ALT (* $p<0.001$, # $p<0.05$).

Sham-operated (SO), glutamine + sham-operated (G+SO), ischemia and reperfusion (I/R), glutamine + ischemia and reperfusion (G+I/R).

Fig. 2: Effect of glutamine on intestine injury of animals subjected to the intestinal ischemia-reperfusion model.

The I/R group showed loss of villi, presence of hemorrhage, edema in the submucosa layer and inflammatory infiltrate. In the G+I/R group we observed the presence of villi as well as the reduction of submucosal edema, inflammatory infiltrate and hemorrhage. In the SO and G+SO groups no tissue changes were observed.

Legend: edema (E), hemorrhage (H), inflammatory infiltrate (IF), Loss villi (LV), villi (V).

Sham operated (SO), Glutamine + Sham operated (G+SO), ischemia and reperfusion (I/R), Glutamine + ischemia and reperfusion (G+I/R).

Values are expressed as the mean ± standard error. * Significant difference between the I/R group and the SO and G+SO groups. # Significant difference between the G+I/R group and the I/R group.

Fig. 3: Effect of glutamine on liver injury of animals subjected to the intestinal ischemia-reperfusion model. Sham operated (SO), Glutamine + Sham operated (G+SO), ischemia and reperfusion (I/R), Glutamine + ischemia and reperfusion (G+I/R).

The I/R group presents loss of hepatic architecture with necrosis points and presence of hemorrhage and focus of inflammatory infiltrate. The group pretreated with

glutamine (G+SO) demonstrated a reduction of these damages and the preservation of the hepatic parenchyma. The SO and G+SO groups showed no tissue changes.

Legend: edema (E), hemorrhage (H), inflammatory infiltrate (IF), necrosis (N).

Values are expressed as the mean ± standard error. * Significant difference between the I/R group and the SO and G+SO groups. # Significant difference between the G+I/R group and the I/R group.

Fig. 4: Effect of glutamine on immunohistochemical detection and expression of IL-1β in the intestine and liver of animals subjected to intestinal ischemia-reperfusion.

Sham operated (SO), Glutamine + Sham operated (G+SO), ischemia and reperfusion (I/R), Glutamine + ischemia and reperfusion (G+I/R). Values are expressed as the mean ± standard error. * Significant difference between the I/R group as compared to the SO and G+SO. # Significant difference between the G+I/R as compared to the I/R group. (*,# $p < 0.05$).

Fig. 5: Effect of glutamine on immunohistochemical detection and expression of TNF-α in the intestine and liver of animals subjected to intestinal ischemia-reperfusion.

Sham operated (SO), Glutamine + Sham operated (G+SO), ischemia and reperfusion (I/R), Glutamine + ischemia and reperfusion (G+I/R). Values are expressed as the mean ± standard error. * Significant difference between the I/R group as compared to the SO and G+SO. # Significant difference between the G+I/R as compared to the I/R group. (*,# $p < 0.05$).

Fig. 6: Effect of glutamine on levels of nitric oxide metabolites (nitrite and nitrate) and iNOS expression in the intestine and liver of animals subjected to ischemia and reperfusion. Values are expressed as mean ± mean standard error. * Significant difference between the I/R group as compared to the SO and G+SO. # Significant difference between the G+I/R as compared to the I/R group. NO (*,# $p < 0.05$), iNOS (*,# $p < 0.001$).

Sham-operated (SO), glutamine + sham-operated (G+SO), ischemia and reperfusion (I/R), glutamine + ischemia and reperfusion (G+I/R).

Fig. 7: Effect of glutamine on expression of Nrf2, Keap1, NQO1 and SOD in the intestine and liver of animals subjected to ischemia and reperfusion. Values are expressed as mean ± mean standard error. * Significant difference between the I/R group as compared to the SO and G+SO. # Significant difference between the G+I/R as compared to the I/R group. Nrf2 (*,# $p<0.01$), Keap1 (*,# $p<0.05$), NQO1 and SOD (*,# $p<0.01$).

Sham-operated (SO), glutamine + sham-operated (G+SO), ischemia and reperfusion (I/R), glutamine + ischemia and reperfusion (G+I/R).

Fig. 8: Effect of glutamine on the expression of HSP 70 in the intestine and liver of animals subjected to ischemia and reperfusion. Values are expressed as mean ± mean standard error. * Significant difference between the I/R group as compared to the SO and G+SO. # Significant difference between the G+I/R as compared to the I/R group. HSP 70 (p*,# <0.01).

Sham-operated (SO), glutamine + sham-operated (G+SO), ischemia and reperfusion (I/R), glutamine + ischemia and reperfusion (G+I/R).

Fig. 9: Effect of glutamine on the expression of ATF-6 and GRP7 in the intestine and liver of animals subjected to ischemia and reperfusion. Values are expressed as mean ± mean standard error. * Significant difference between the I/R group as compared to the SO and G+SO. # Significant difference between the G+I/R as compared to the I/R group. ATF-6, GRP78 intestine p*,# <0.01, ATF-6, GRP78 liver p*,# <0.05).

Sham-operated (SO), glutamine + sham-operated (G+SO), ischemia and reperfusion (I/R), glutamine + ischemia and reperfusion (G+I/R).

Table 1: Effect of glutamine administration on the levels of lipid peroxidation in the intestine and liver of animals subjected to ischemia and reperfusion.

Groups	TBARS (Intestine)	TBARS (Liver)
SO	0.45±0.07	0.16±0.01
G+SO	0.40±0.2	0.20±0.2
IR	1.83±0.20[*]	0.45±0.03[*]
G+IR	0.79±0.04[#]	0.25±0.02[#]

Values are expressed as mean ± mean standard error. [*] Significant difference between the I/R group as compared to the SO and G+SO. [#] Significant difference between the G+I/R group as compared to the I/R group (p<0.01).

Sham-operated (SO), glutamine + sham-operated (G+SO), ischemia and reperfusion (I/R), glutamine + ischemia and reperfusion (G+I/R).

Figure 1
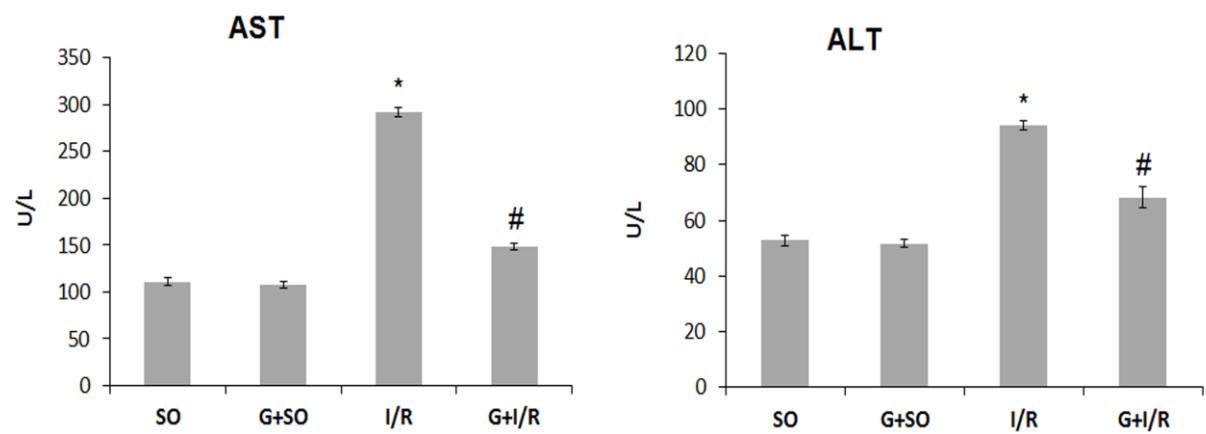
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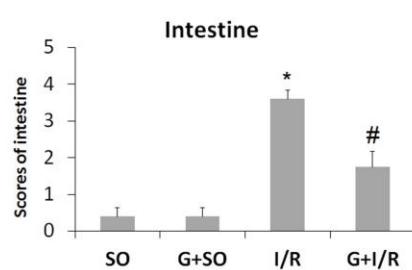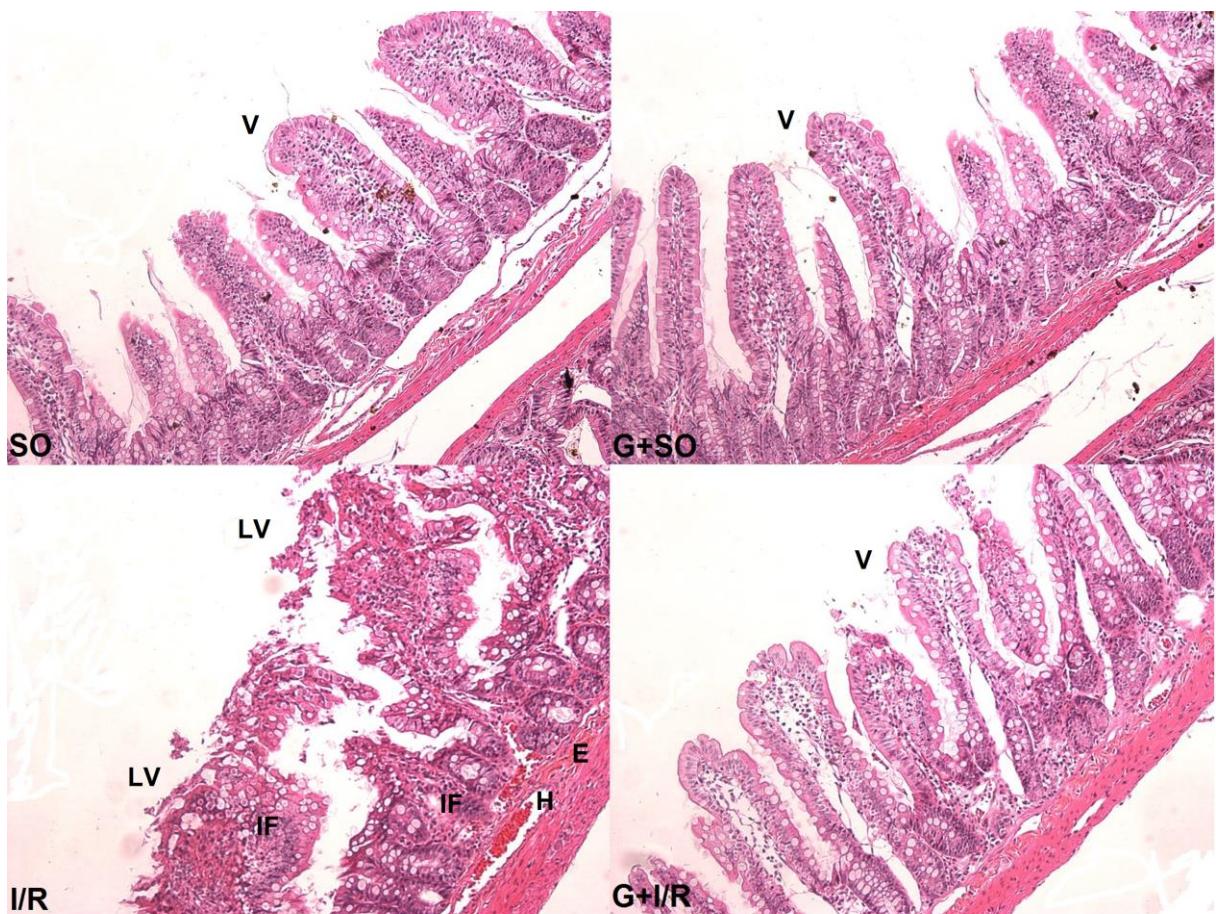
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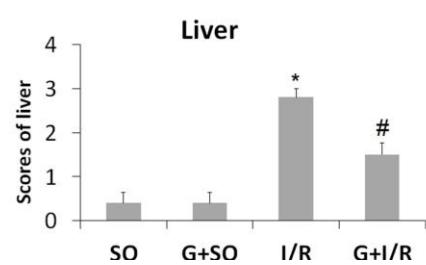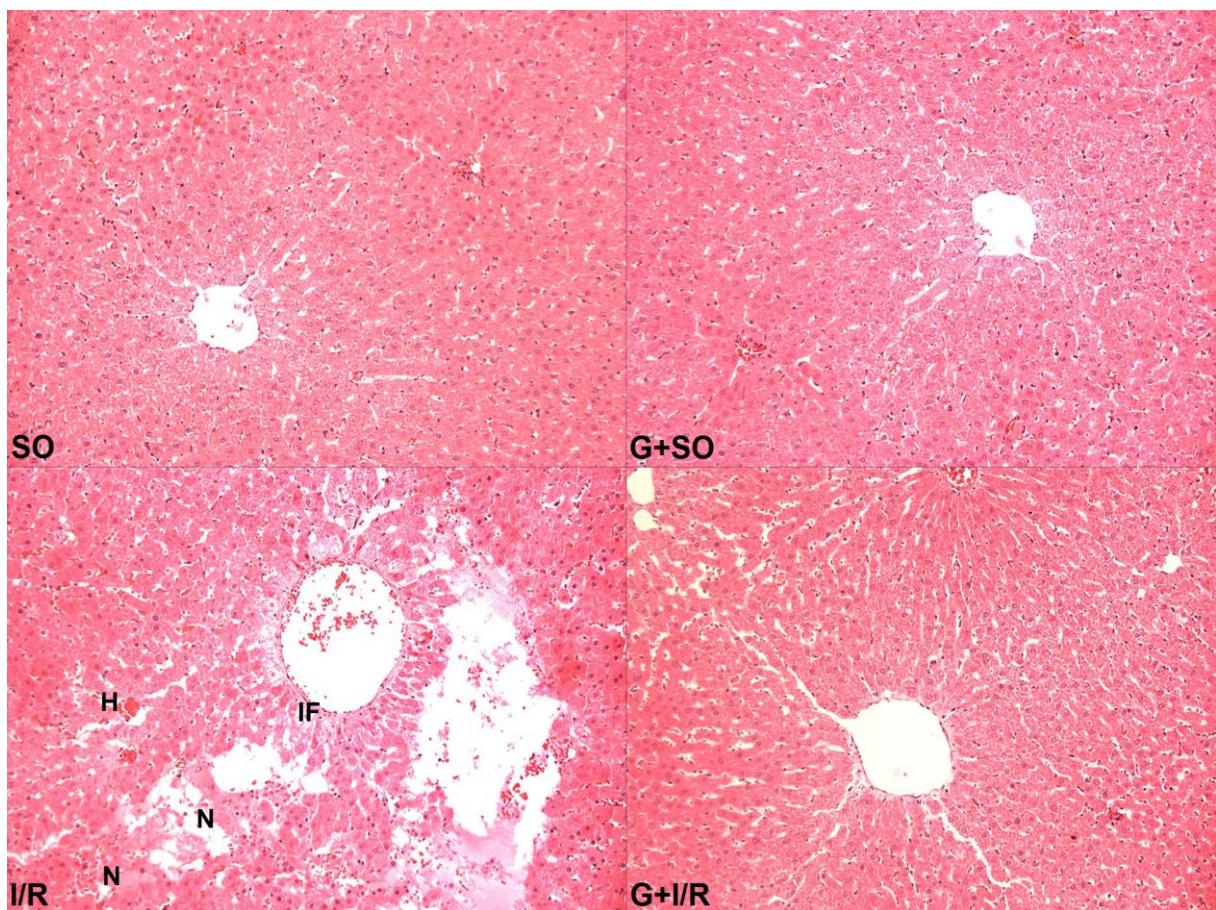
Figure 4

A
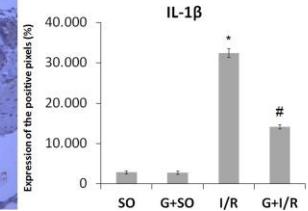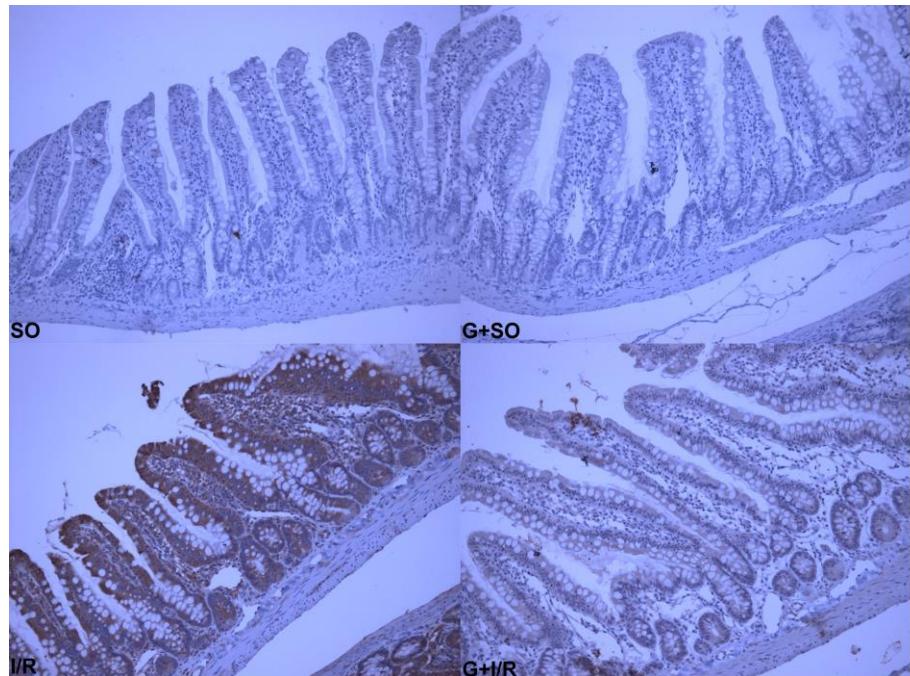
B
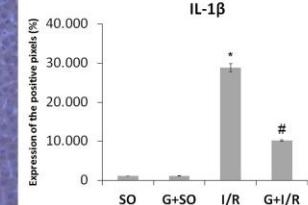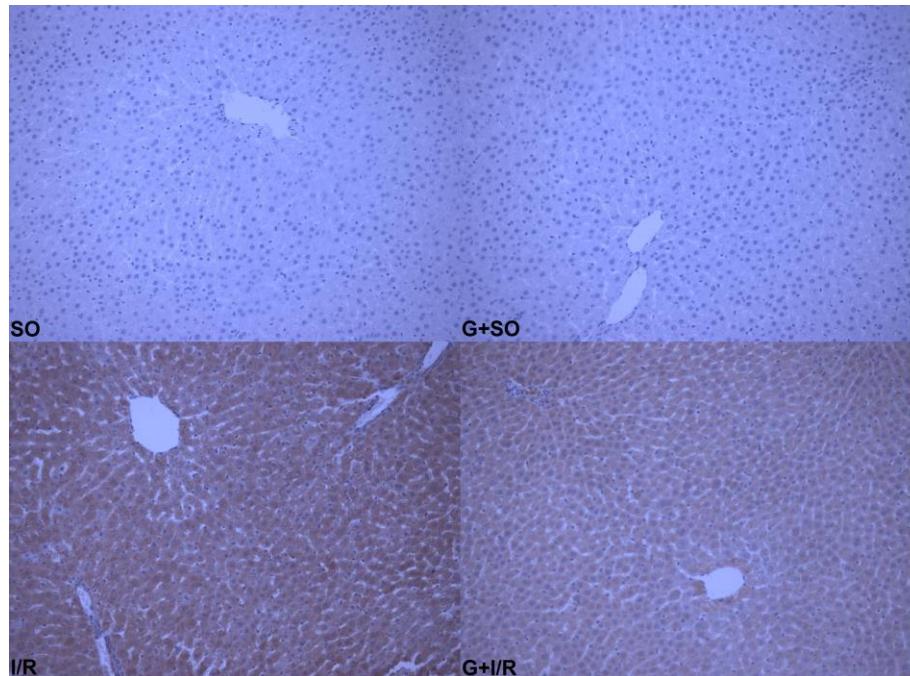
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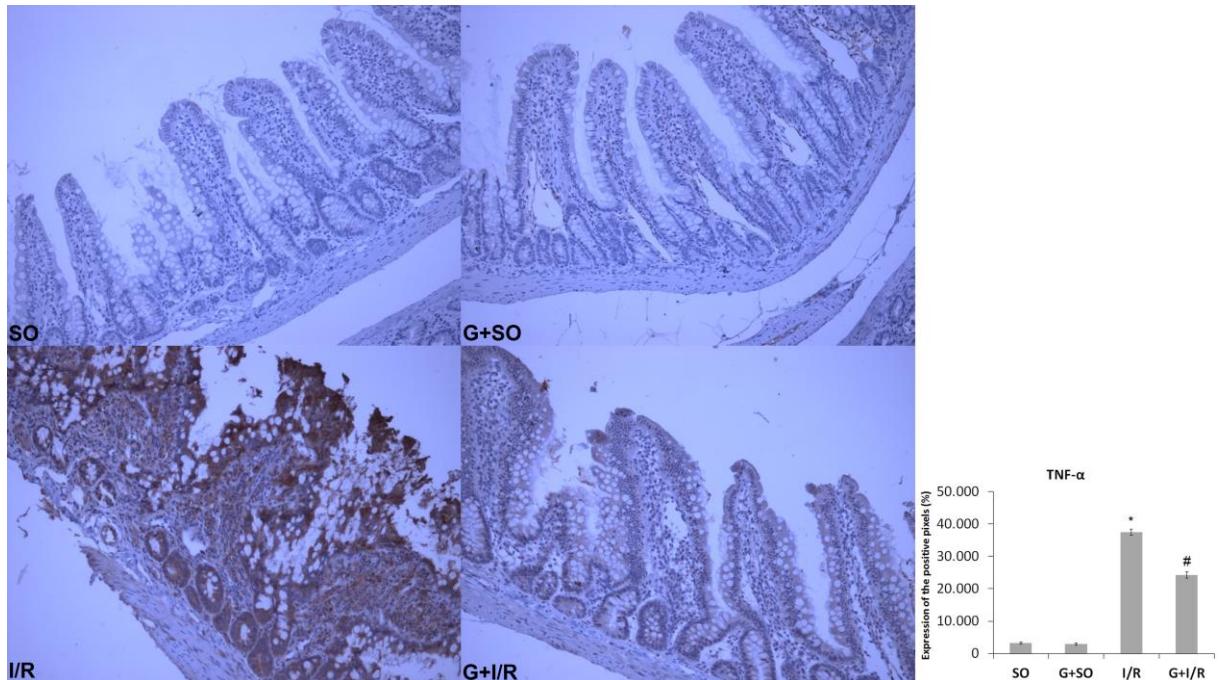
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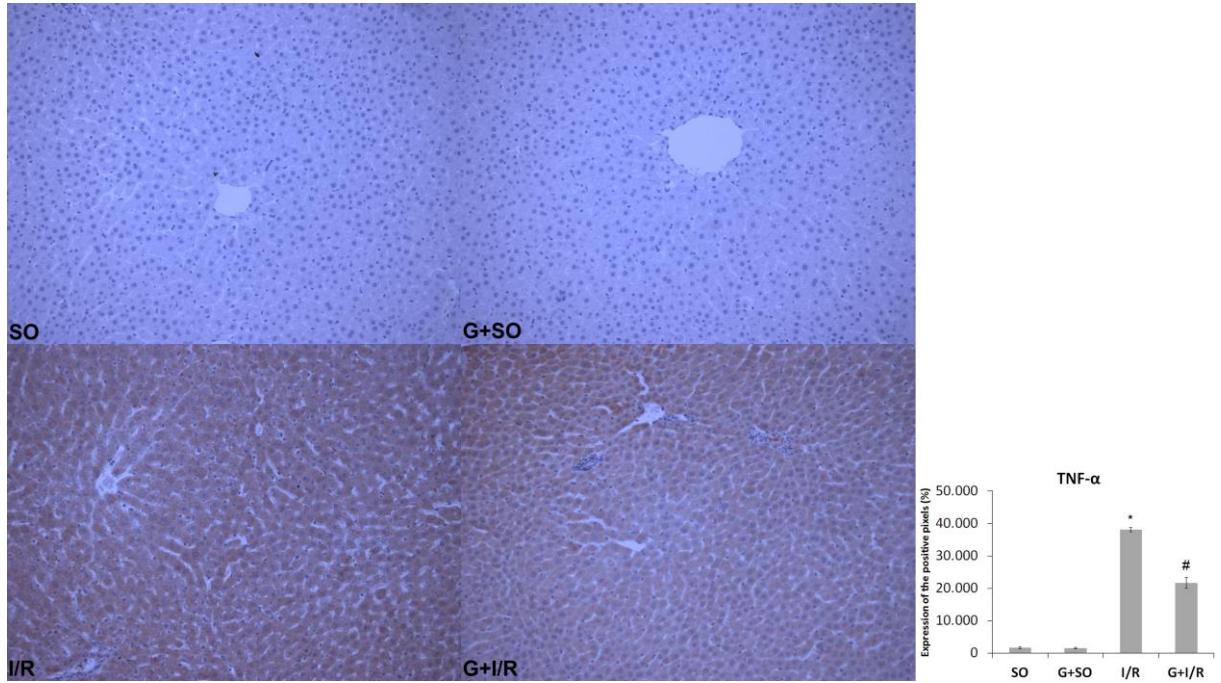
Figure 6

A - Intestine
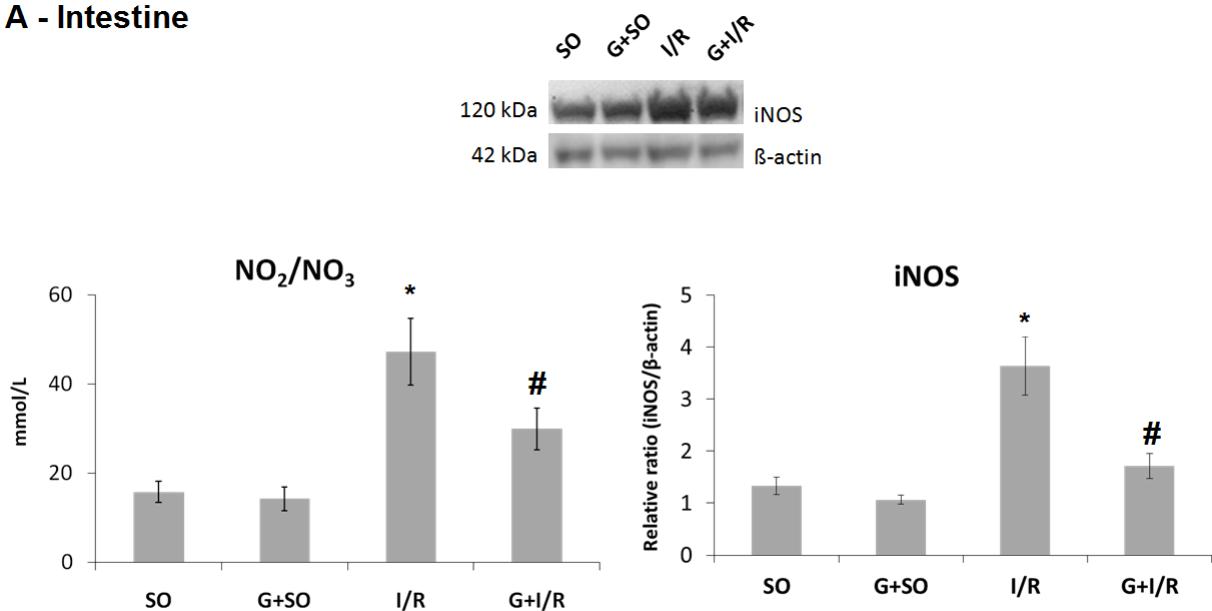
B - Liver
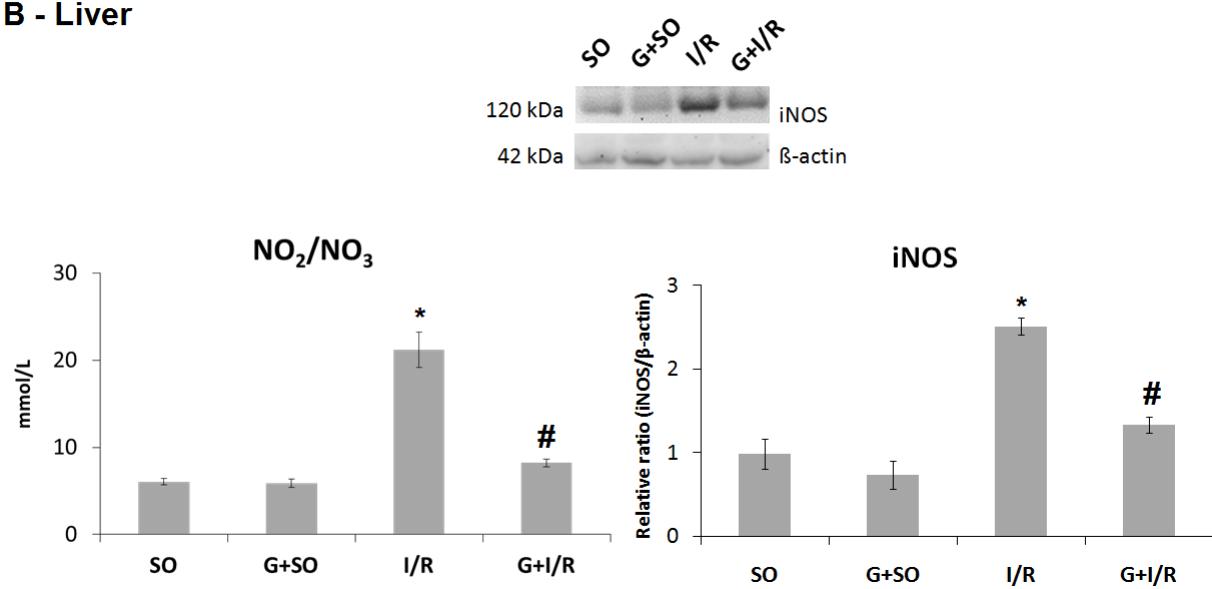
Figure 7

A
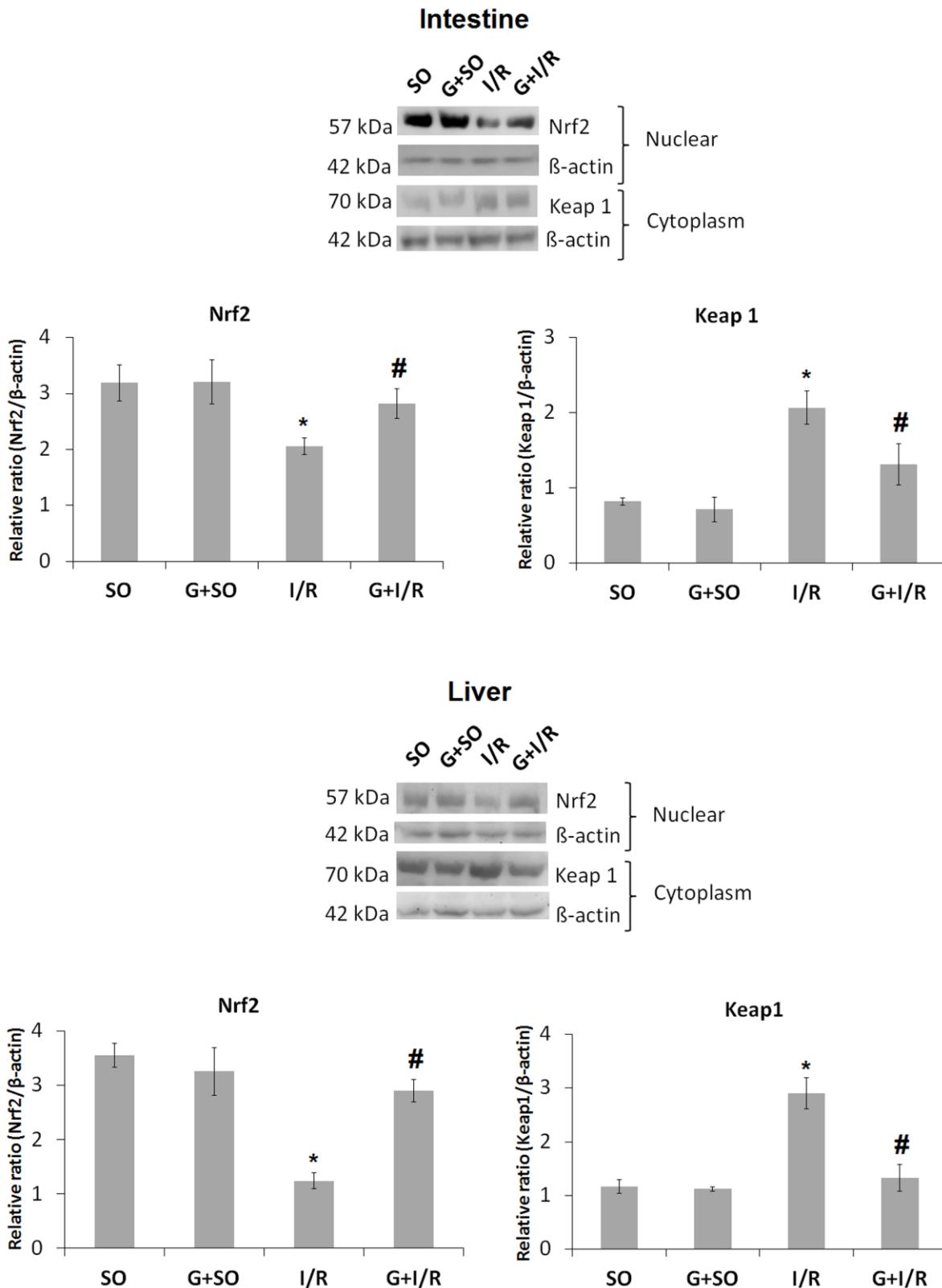
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B
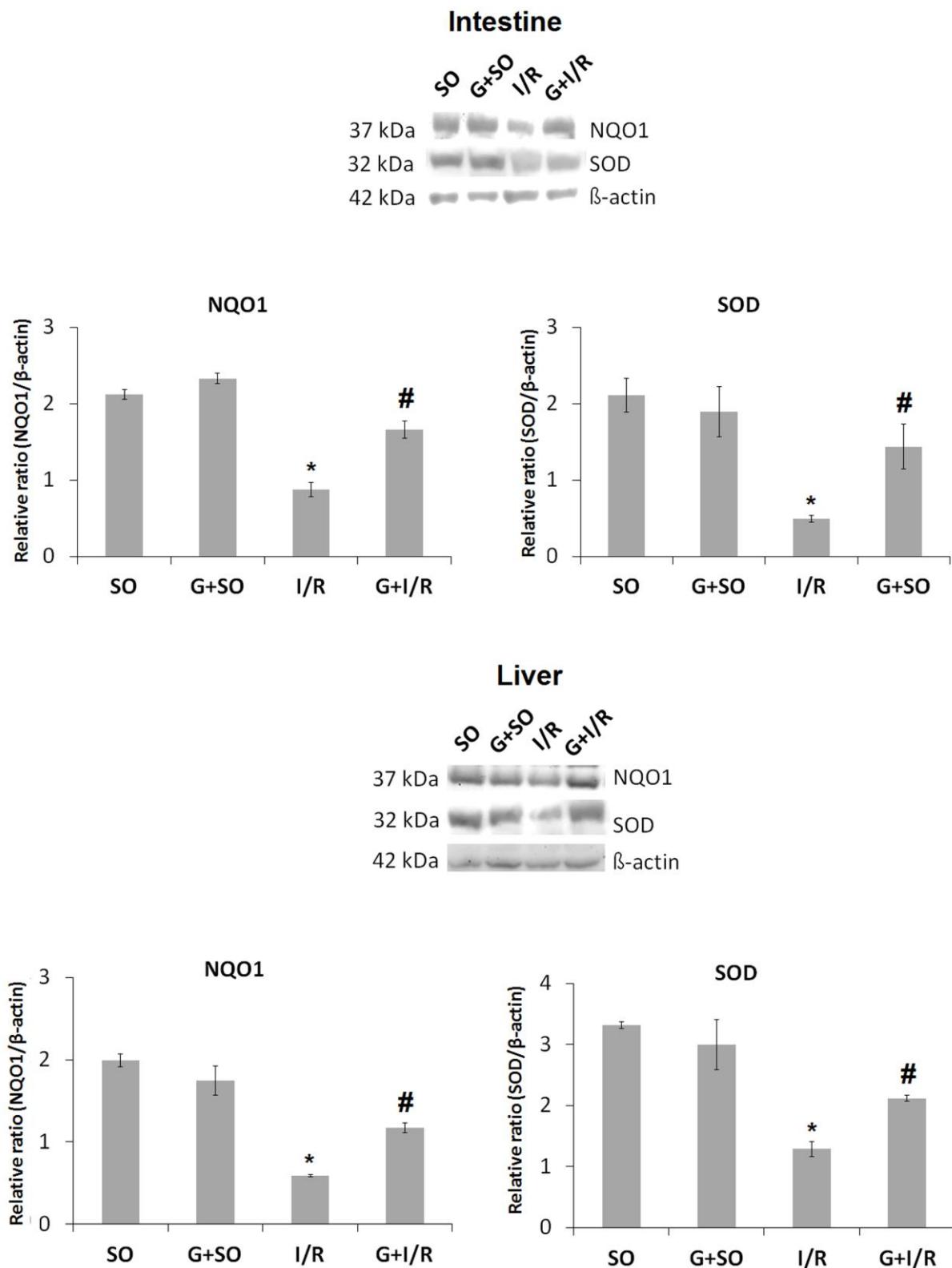
Figure 8

**A - Intestine**
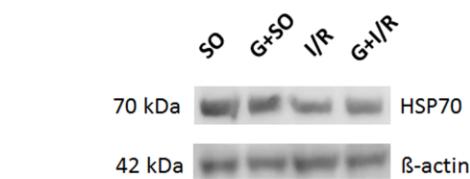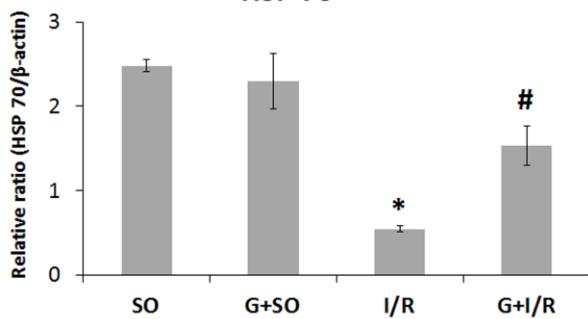
B - Liver
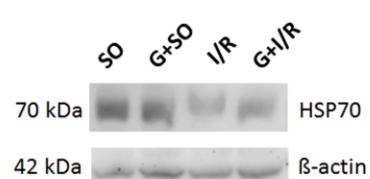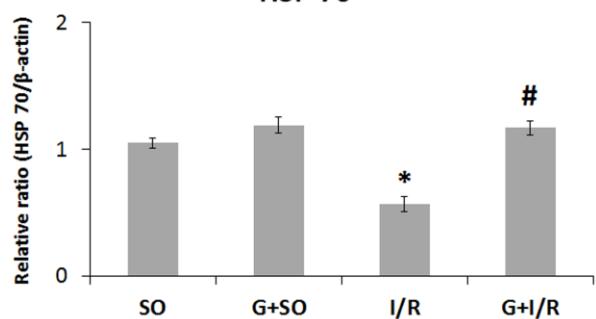
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A
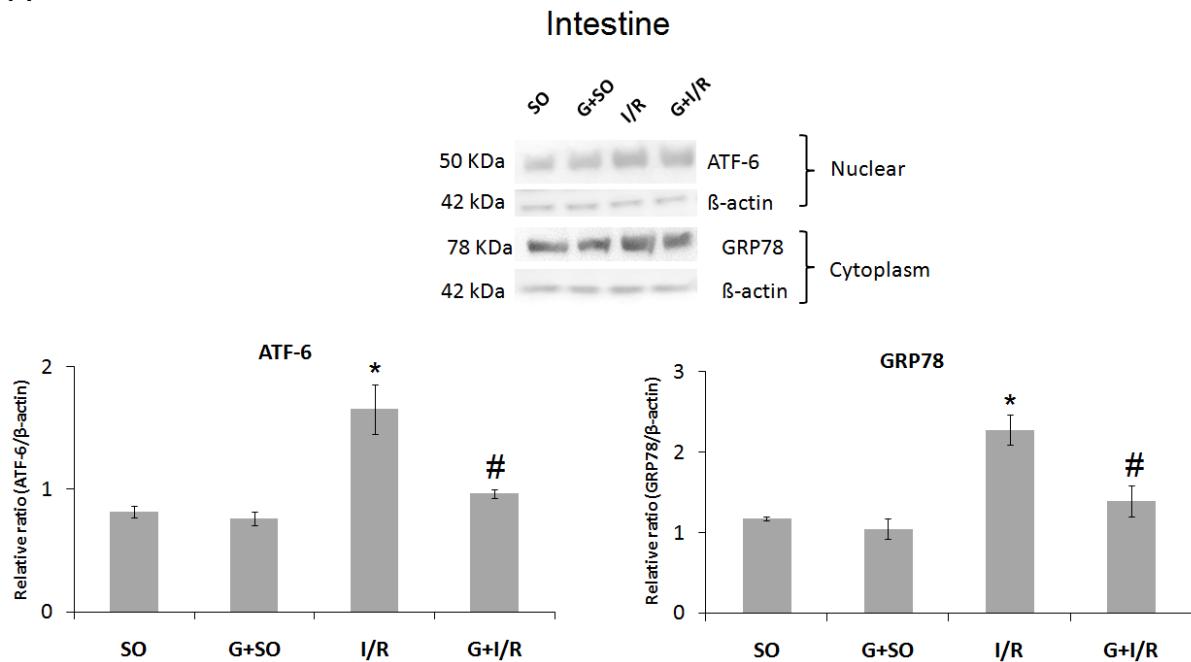
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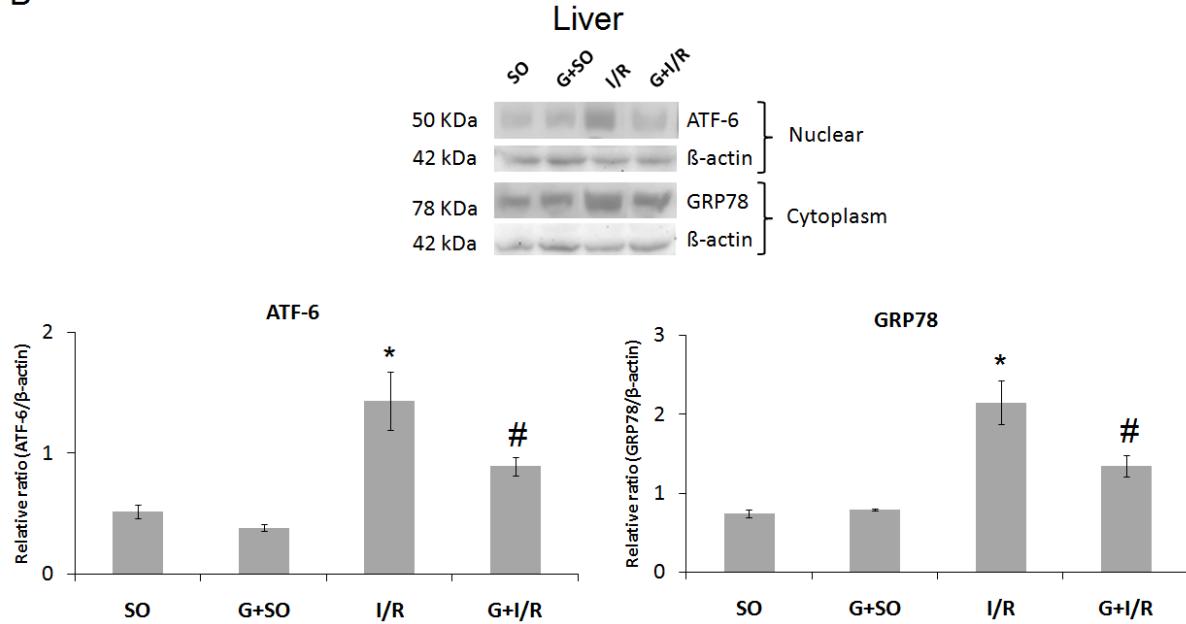
8 CONSIDERAÇÕES FINAIS

A I/R intestinal está associada com elevada taxa de mortalidade devido às suas complicações como a resposta inflamatória sistêmica que pode lesar diferentes órgãos como o fígado. Alguns fatores podem estar associados com essas lesões como a geração de ERO, óxido nítrico, liberação de citocinas pró-inflamatórias, NF-κB e estresse do RE. Os estudos que estão sendo desenvolvidos nessa área demonstram que o uso de antioxidantes pode reduzir as lesões locais e em órgãos adjacentes a I/R intestinal. Evidências indicam que Gln desempenha um papel benéfico na manutenção da função da barreira intestinal, reduzindo a liberação de substâncias tóxicas para a circulação sistêmica. E relatos indicam que a Gln torna-se uma fonte essencial nos pacientes críticos reduzindo a taxa de mortalidade nesses casos.

Neste estudo, demonstramos que a Gln protegeu o intestino e fígado dos animais submetidos a I/R intestinal.

8.1 Conclusões do artigo I
"Effect of glutamine on liver injuries induced by intestinal ischemia-reperfusion in rats"
- Diminuiu os níveis séricos das enzimas séricas hepáticas AST, ALT e FA;
- Reduziu dano ao DNA;
- Diminuiu os níveis de LPO;
- Restabeleceu a atividade das enzimas antioxidantes CAT, SOD e GPx;
- Aumentou os níveis de GSH;
- Reduziu a expressão das proteínas relacionadas a processos inflamatórios (IL-6 e NF-κB);

8.2 Conclusões do artigo II
**"*Protective effect of glutamine on the main and adjacent organs damaged by ischemia/reperfusion in rats*"
- Diminuiu os níveis séricos das enzimas séricas hepáticas AST, ALT;
- Reduziu as alterações histológicas no intestino e fígado;
- Diminuiu os níveis dos metabólitos do NO e a expressão da iNOS;

- Reduziu a expressão das proteínas relacionadas a processos inflamatórios (IL-1β e do TNF-α);

- Ativou a expressão do Nrf2 diminuiu a expressão de seu inibidor o Keap1 e aumentou a expressão da SOD e NQO1;

- Diminuiu o dano celular pela HSP70;

- Reduziu a expressão das proteínas marcadoras do estresse do RE (GRP78 e ATF-6).

Os resultados obtidos nessa tese nos demonstraram que a Gln foi capaz de reduzir os danos intestinais e hepáticos no modelo experimental de I/R intestinal devido à sua ação antioxidante que reduziu a geração exacerbada de ERO e, consequentemente, inibiu a ativação de fatores como o NF-κB, impedindo a liberação de citocinas pró-inflamatórias, bem como reduziu o estresse do RE e demonstrou eficácia no equilíbrio do sistema antioxidante.

9 PERSPECTIVAS FUTURAS

A continuidade deste estudo está na importância de conhecer mais profundamente outros mecanismos que podem ser bloqueados ou ativados impedindo o dano local e sistêmico nesse modelo. Assim, pretendemos avaliar algumas vias como de autofagia, marcadores de apoptose e outras rotas inflamatória como a da COX-2 e dos receptores *toll-like*.

Seguiremos também com outros projetos paralelos envolvendo os modelos estudados no nosso grupo de pesquisa como o projeto intitulado "Efeito da quercetina no modelo de experimental de colite induzida por TNBS" aprovado pelo FIPE/HCPA sob o número 17-0067.

10 ANEXOS

10.1 Anexo A

Resultados do projeto piloto foram publicados em *abstract section* na *Annals of Hepatology 2014;13(5):621-711.*

Parte desses resultados originou o artigo em anexo (ANEXO B) e o artigo I dessa tese.

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the liver architecture, signs of chronics damage and lymphocytic infiltrate. We also found nodular formations with homogeneous pattern, similar to cihrrosis, confirmed by picrosirius. However, group IV showed improvement in this subject. **Conclusion.** The use of melatonin as antioxidant was effective in reducing liver damage caused by increased production of free radicals.

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THE ROLE OF GLUTAMINE IN EXPERIMENTAL MODEL OF INTESTINAL ISCHEMIA AND REPERFUSION

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Introduction. The intestinal ischemia-reperfusion (I/R-i) can cause cellular damage to the tissue and in distant organs such as the liver. Some aggressor agents are involved in these processes, such as: the generation of free radicals and the release of pro-inflammatory cytokines. Due to the involvement of free radicals in the lesions of I/R-i, some therapeutic antioxidants options are being studied and tested in I/R-i lesions. The aim of the study was to evaluate effects of glutamine in an animal model of I/R. **Material and methods.** Twenty male Wistar rats were divided into four experimental groups: sham operated (SO), glutamine + sham operated (G+SO), ischemia-reperfusion (I/R), glutamine+ischemia-reperfusion-i (G+I/R). The rats were subjected to occlusion of the superior mesenteric artery for 30 min followed by 15 min of reperfusion. The glutamine (25 mg/kg/day) was administered 24 and 48 h before I/R. Local and systemic injuries were determined by evaluating intestinal and liver segments for oxidative stress using lipid peroxidation (LPO), activity of superoxide dismutase (SOD) and immunohistochemical assays of interleukin-6 (IL-6) and nuclear factor kappa beta (NF-κB) in tissues. The statistical analysis used was ANOVA followed by Student-Newman-Keuls (mean ± SEM) significant at $p < 0.05$. **Results.** The animals treated with glutamine showed a significant reduced the expression of IL-6 and NF-κB and levels of LPO-Gut (SO: 0.45 ± 0.07, G + SO: 0.40 ± 0.02, I/R: 1.83 ± 0.20; G + I/R: 0.78 ± 0.04) and liver (SO: 0.16 ± 0.01, G + SO: 0.20 ± 0.02, I/R: 0.45 ± 0.03; G + I/R: 0.24 ± 0.02) compared to animals in the I/R group. The SOD activity showed a significant increase in G + I/R group compared to the I/R group - Gut (SO: 72.3 ± 6.4, G + SO: 77.9 ± 3.2, I/R: 53.18 ± 1.73; G + I/R: 74.02 ± 5.99) and liver (SO: 36.63 ± 1.52, G + SO: 33.13 ± 2.75, I/R: 26.64 ± 0.46; G + I/R: 33.21 ± 0.53). **Conclusion.** These results suggest that pretreatment with glutamine prevents mucosal injury and improves gut and liver recovery after I/R injury in rats.

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EXPERIMENTAL MODEL OF PORTAL HYPERTENSION POSSIBLY INDUCES HEMODYNAMIC CHANGES ON WISTAR RATS IN 1H, 3H, 6H, 24 H AND 5 DAYS AFTER SURGERY-PRELIMINARY DATA

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Background. Portal hypertension (PH) is a clinical syndrome associated with the development of a hyperdynamic circulation. This hyperdymanic disturb is associated with hemodynamic changes on the circulatory system. The aim of the study was to evaluate these hemodynamic changes in animals submitted to an experimental model of partial portal vein ligation (PPVL), at different times after surgery. **Material and methods.** Ten male Wistar rats were divided into 2 groups: 1. Sham-operated (SO), 2. PPVL. Rats were anesthetized with ketamine hydrochloride (100 mg/kg ip) and xylazine hydrochloride (50 mg/kg ip) and the procedure of PPVL was performed. After a medium incision in the abdomen, bowels were gently withdrawn on a humidified gauze with saline and the portal vein was isolated. A 20 g needle was placed on the portal vein and both were tied up using a 3.0 silk yarn, the needle being gently withdrawn after ligation. The sham-operated group was submitted to the same procedure, although their portal veins did not undergo partial portal vein ligation. After these procedures, rats were catheterized in mesenteric vein and femoral artery in order to perform the posterior hemodynamic measures. We awaited 1 h and performed the measures. The same was done in 3 h, 6 h, 24 h and 5 days after surgery. **Results.** Systolic blood pressure showed an increase on PPVL group after 1 h (SO 122 ± 4; PPVL 132.5 ± 4: p = 0.02), 3 h (S 125.5 ± 5, PPVL 136.6 ±7: p = 0.01) and 6h after surgery (SO: 123.1 ± 4, PPVL 132.6 ± 6: p = 0.01). In 24 h, values of the groups tended to be equal. 5 days after surgery, an increase on SO group and a decrease on PPVL group was observed (SO 134 ± 8, PPVL 124.2 ± 9: p < 0.001). We also recorded simultaneously the pressure on mesenteric vein and femoral artery by 24 h and 5 days after surgery. In 24 h, portal pressure was higher in PPVL group (SO 5 ± 1, PPVL 9 ± 2: p = 0.02) and arterial pressure was also higher (SO 104.5 ± 4 PPVL 110 ± 7: p = 0.04). In 5 days, portal pressure remained higher, and we observed a tendency on arterial pressure increase. **Conclusion.** By these previous results, we come to believe that the partial portal vein ligation procedure leads to a hemodynamic disbalance on the circulatory system since the first hour after surgery, leading to probable cardiocirculatory disturbs. Further investigations are being conducted in order to understand the pathophysiology of this process.

Table I: Values of superoxide dismutase activity in the intestine and liver.

	SOD (USOD/mgprot) Intestine	SOD (USOD/mgprot) Liver
SO	72.3±6.43	36.63±1.52
G+SO	77.9±3.21	33.13±2.75
I/R	53.2±1.74*	26.64±0.46*
G+I/R	74.0±6.02#	33.21±0.54#

The results of enzymes activity of SOD showed a significantly decrease in intestine and liver the I/R group in relation to groups SO/G+SO and a significant increase in the group G+I/R with respect to the I/R group.

SOD - Intestine *($p < 0.05$), #($p < 0.05$); Liver *($p < 0.01$), #($p < 0.05$).

Figure 1

A

TBARS - Intestine

B

TBARS - Liver

Figure 2

A

IL-6 - Intestine

B

IL-6 - Liver

Figure 3

A

NF-kB - Intestine

B
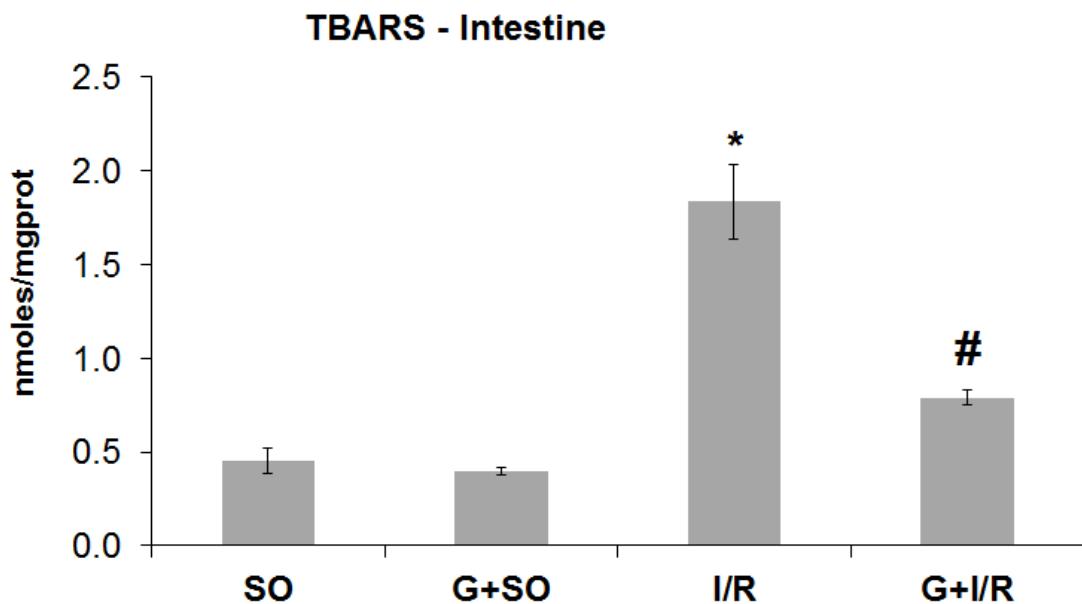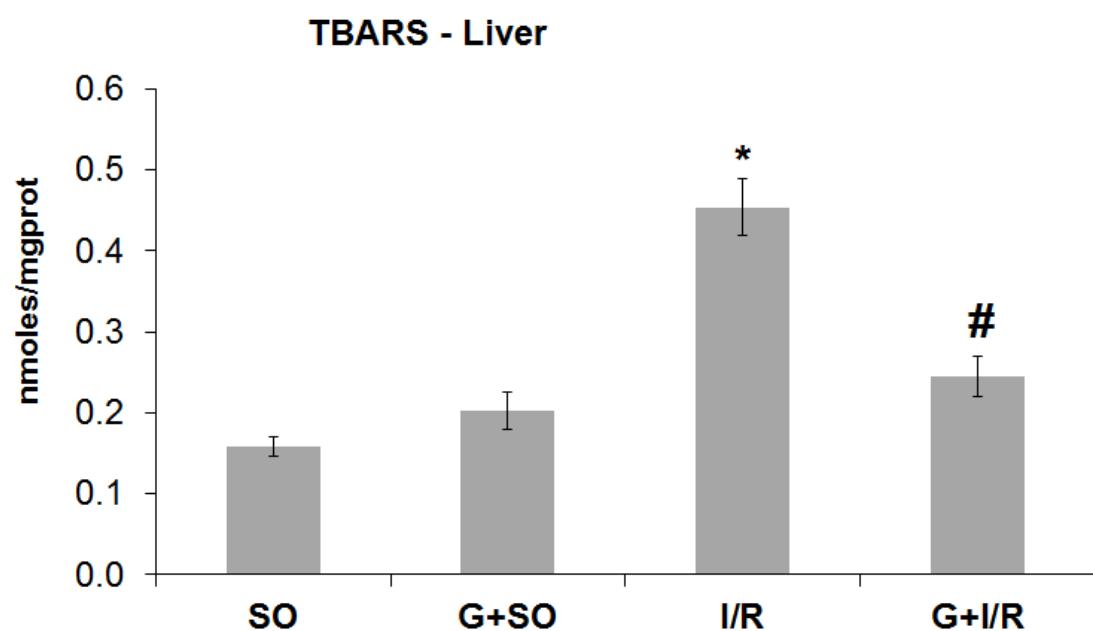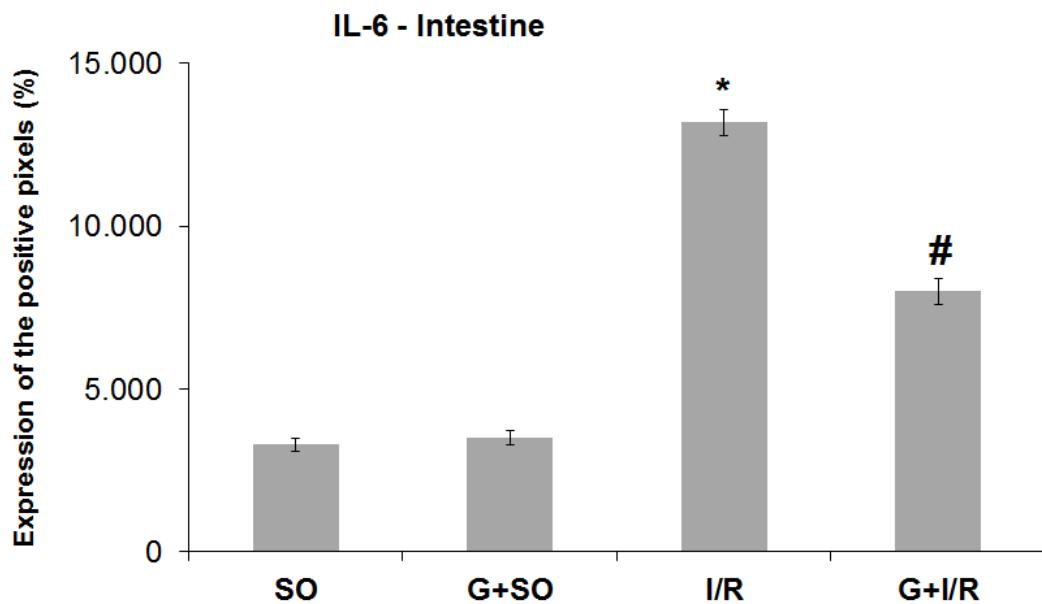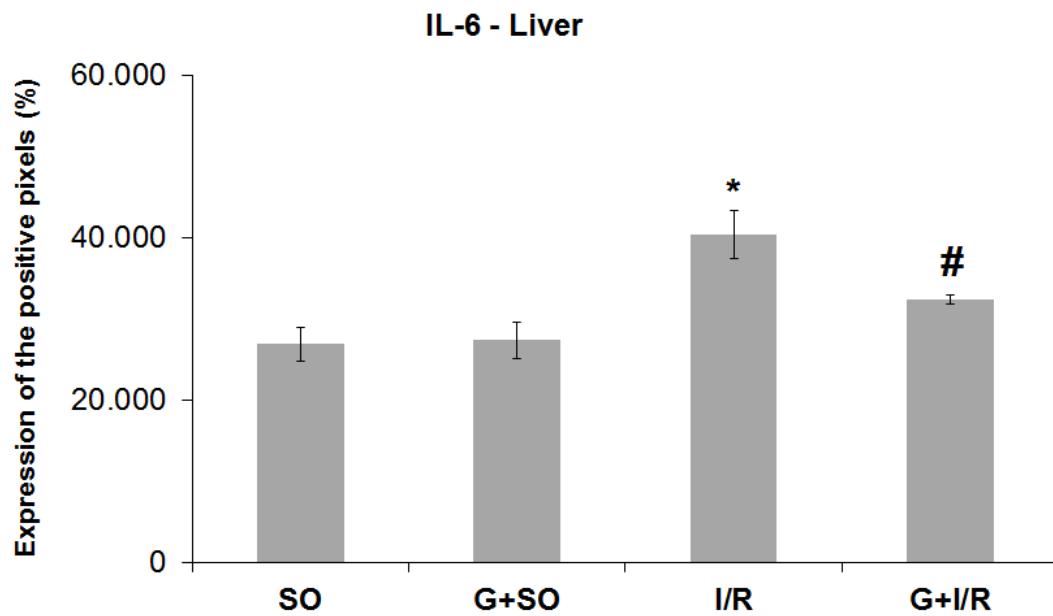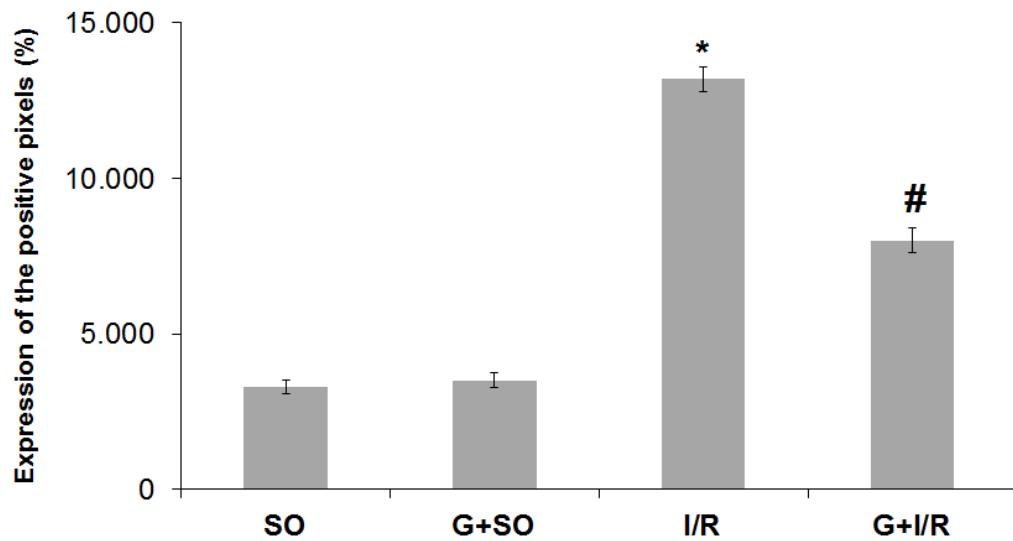
NF-kB - Liver
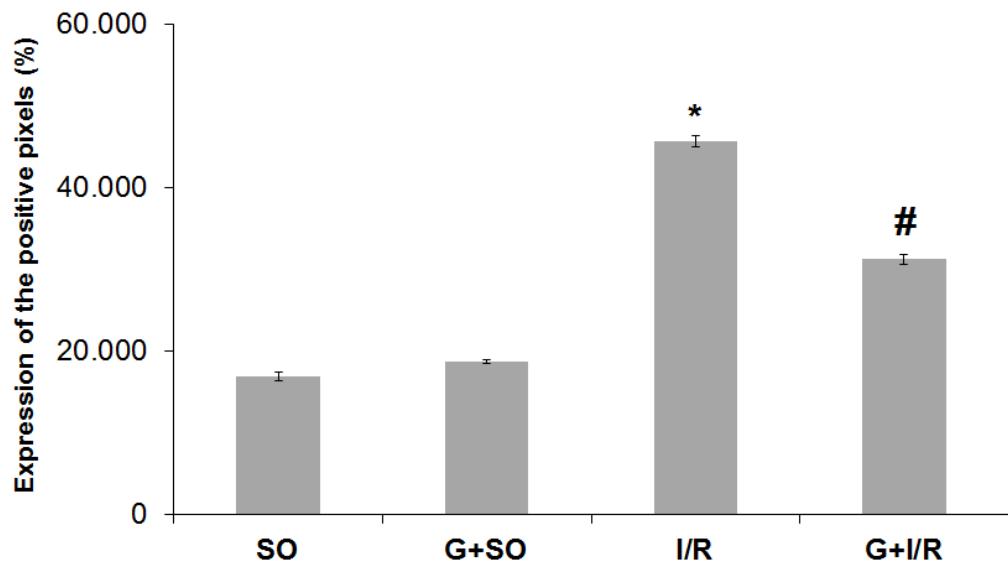
10.2 Anexo B

Artigo Publicado pelo nosso grupo de pesquisa com modelo de isquemia e reperfusão intestinal.

"Glutamine prevents oxidative stress in a model of mesenteric ischemia and reperfusion".
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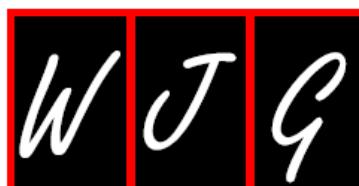
RANDOMIZED CONTROLLED TRIAL

Glutamine prevents oxidative stress in a model of mesenteric ischemia and reperfusion

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Abstract

AIM: To evaluate preventative effects of glutamine in an animal model of gut ischemia/reperfusion (I/R).

METHODS: Male Wistar rats were housed in a controlled environment and allowed access to food and water *ad libitum*. Twenty male Wistar rats were divided into four experimental groups: (1) control group (control) - rats underwent exploratory laparotomy; (2) control + glutamine group (control-GLU) - rats were subjected to laparotomy and treated intraperitoneally with glutamine 24 and 48 h prior to surgery; (3) I/R group - rats were subjected to occlusion of the superior mesenteric artery for 30 min followed by 15 min of reperfusion; and (4) ischemia/reperfusion + glutamine group (G + I/R) - rats were treated intraperitoneally with glutamine 24 and 48 h before I/R. Local and systemic injuries were determined by evaluating intestinal and lung segments for oxidative stress using lipid peroxidation and the activity of superoxide dismutase (SOD), interleukin-6 (IL-6) and nuclear factor kappa beta (NF-κB) after mesenteric I/R.

RESULTS: Lipid peroxidation of the membrane was increased in the animals subjected to I/R ($P < 0.05$). However, the group that received glutamine 24 and 48 h before the I/R procedure showed levels of lipid peroxidation similar to the control groups ($P < 0.05$). The activity of the antioxidant enzyme SOD was decreased in the gut of animals subjected to I/R when compared with the control group of animals not subjected to I/R ($P < 0.05$). However, the group that received glutamine 24 and 48 h before I/R showed similar SOD activity to both control groups not subjected to I/R ($P < 0.05$). The mean area of NF-κB staining for each of the control groups was similar. The I/R group showed the largest area of staining for NF-κB. The G + I/R group had the second highest amount of staining, but the mean value was much lower than that of the I/R group ($P < 0.05$). For IL-6, control and control-GLU groups showed similar areas of staining. The I/R group contained the largest area of IL-6 staining, followed by the G + I/R animals; however, this area was significantly lower than that of the group that underwent I/R without glutamine ($P < 0.05$).

CONCLUSION: These results demonstrate that pretreatment with glutamine prevents mucosal injury and improves gut and lung recovery after I/R injury in rats.

Core tip: Ischemia-reperfusion (I/R) leads to oxidative stress, with local and systemic consequences. Many enzymes and interleukins have been implicated in this process, among them interleukin-6 (IL-6) and nuclear factor kappa beta (NF-κB). The exact role of these enzymes is still not clear. Some substances, such as glutamine, have been studied as protective agents against oxidative stress. In an animal experimental model of intestinal I/R we have found that glutamine reduced lipid peroxidation, preserved superoxide dismutase activity, and decreased the expression of IL-6 and NF-κB in both lung and intestine, suggesting a protective role of this amino acid in the setting of intestinal I/R.

INTRODUCTION

Ischemic conditions such as arterial occlusions, transplants, mesenteric ischemia and shock occur commonly in medical practice and affect a growing number of individuals of various ages, leading to high morbidity and mortality. However, unlike ischemic injuries, reperfusion injuries alter not only the affected areas but also produce systemic changes, so that the reestablishment of the blood flow to ischemic areas may result in damage to the entire body. The damage to remote organs is termed post-traumatic multiple organ failure (MOF)[1].

Gut ischemia usually results from occlusion of the celiac trunk and/or the superior mesenteric artery by thrombi or emboli and, more frequently, from non-occlusive processes, such as in the case of decreased mesenteric blood flow that occurs in heart failure and sepsis[2]. In the gut, ischemia followed by reperfusion frequently results in MOF, with the gut being the organ that triggers the injury process in distant organs. A systemic inflammatory reaction is initiated from pro-inflammatory substances released by the gut into the lymphatic circulation, with the inflammation mainly affecting lungs, liver and kidneys[3].

Although the details about the molecular mechanisms that determine injuries in ischemic events are not yet well defined, it is known that reactive oxygen species (ROS) play an important role in the pathogenesis of gut injury after an ischemia/reperfusion (I/R) event[4]. Parks and Granger reported that the tissue damage that occurs during reperfusion is greater than the injury that occurs during ischemia. Rupture of the mucosal barrier, bacte-

rial translocation and activation of the inflammatory response, as well as acid-base balance and electrolyte disorders, are observed[5]. Superoxide and hydrogen peroxide are thought to be the main free radicals that contribute to I/R injury. Under normal conditions ROS are neutralized by endogenous antioxidant enzymes, but an excess of free radicals is observed during reperfusion, which results in oxidative stress[6]. Those free radicals originate when oxygen (O_2) is reintroduced into the ischemic tissue during reperfusion. Superoxide dismutase (SOD) is an antioxidant enzyme highly specific for superoxide elimination, thus reducing gastrointestinal lesions caused by I/R[7].

Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) is a transcription factor that plays a crucial role not only in normal states but also in the coordination of adaptive immune responses by regulating the expression of many cell mediators[8]. This factor, which was first described in 1986 by Sen and Baltimore[9], binds to specific kappa binding sites in the immunoglobulins of B cells. It is now well recognized that NF-κB is expressed in most cell types and that NF-κB consists of a dimer composed of members of the Relish (Rel) family. The NF-κB/Rel family contains five subunits, p50, p52, p65 (RelA), c-Rel and Rel-B. These subunits form homo- and heterodimers in several combinations. Generally, NF-κB is composed of two polypeptides, one of 50-kDa (p50) and one of *65-kDa* (p65). In homeostatic cells, NF-κB remains in the cytoplasm in its inactive form, associated with proteins that inhibit the kB site called kB inhibitors (IkB). Seven IkB isoforms have been described: IkBα, IkBβ, IkBγ, IkBε, Bcl-3, p100 and p105. NF-κB is activated by a variety of signals relevant to the etiology and pathophysiology of inflammation[8]. Intracellular and/or extracellular stimuli such as bacterial products (endotoxins, peptidoglycans), viruses and viral components, protozoa, cytokines (tumor necrosis factor-alpha (TNF-α), interleukins), free radicals and/or oxidants are needed to activate NF-κB[8]. In 2002, Haddad[10] suggested that NF-κB activation controls the oxidant/antioxidant balance.

Interleukin (IL)-6 and TNF-α levels are elevated in I/R as well as in MOF. Measurements of plasma levels of these endotoxins are important to assess the systemic effects of gut I/R. ROS promote oxidative stress as a result of the production of inflammatory cytokines, such as IL-6 and TNF-α, in addition to promoting the activation of neutrophils. IL-6 and TNF-α not only directly induce tissue damage but are also potent neutrophil activators. When sequestered in the intestinal tissue, these mediators and their enzymatic products promote increased micro vascular permeability, interstitial and perivascular edema, MOF and pulmonary edema[11].

The damage and loss of mucosal barrier integrity promotes bacterial translocation and the production of cytokines. The next stage is the transport of inflammatory mediators through the intestinal lymphatic system. The lung is the first exposed organ[12]. After resuscitation from hemorrhagic shock, lymph duct li-

gation prevents remote lung injury, the so-called "gut-lymph hypothesis"[13]. Lymphatic thoracic duct ligation prior to mesenteric I/R protects against lung injuries and modulates serum levels of endotoxins, D-lactate, diamine oxidase and cytokines. MOF causes acute lung injury (ALI) through the production of inflammatory mediators drained through the circulatory system. The gastrointestinal tract has the largest lymphatic system of the body. Mediators released by activated inflammatory cells during an acute event reach the interstitium, which is predominantly drained by lymphatics[13].

Several substances have been used for the treatment and/or prevention of experimental colitis. The experiments aimed to evaluate new drugs for the treatment of inflammatory processes or combinations of drugs to achieve better results[14]. Substances that inhibit or minimize the inflammatory process caused by aggressive agents, such as glutamine, have been used for prevention purposes. Glutamine is an uncharged, polar amino acid that is non-essential or occasionally essential, hydrophilic, and found on the surface of proteins where it interacts with water. Glutamine is the most abundant amino acid in peripheral blood[15]. This substance was initially used prophylactically in patients undergoing radiation therapy, leading to a reduction in the incidence and severity of actinic enteritis[16]. Glutamine also has a major role in the immune defense of the intestinal mucosal barrier due to its participation in the formation of immunoglobulins, especially IgA. Glutamine decreases the inflammatory effects of methotrexate-induced enterocolitis and reduces bacterial translocation in animals with abdominal sepsis[17]. This amino acid acts on macrophage activity, interfering with phagocytosis at inflammatory sites. In addition to the direct protective effects mentioned above, glutamine plays an important role in intestinal inflammatory processes by acting on ROS[14]. Glutamine is a multifunctional amino acid used for the synthesis of urea in the liver, renal aminogenesis, gluconeogenesis, and as the main respiratory fuel for many cells. Low glutamine concentrations are found during catabolic stress and are associated with susceptibility to infections. Glutamine is not only an important energy source for mitochondria but is also a precursor of the brain neurotransmitter glutamate, which then participates in the synthesis of the antioxidant glutathione[15]. Glutamine is thus vital in the regulation of the intracellular oxidative balance[16]. Glutamine has been used as a nutritional supplement in severely debilitated patients to reduce the deleterious effects of oxidative stress[18]. It has been shown that preventing oxidative stress in patients with severe conditions or multiple traumas or undergoing major surgery is useful as a treatment adjunct. In this setting, antioxidant therapy improves patient prognosis and decreases the overall rate of complications[19].

Clinical observations have shown that patients receiving dietary glutamine supplementation had a better tolerance to colitis resulting from radiation therapy for prostate and cervical neoplasms[16]. The same substance

was then used in patients with Crohn's disease (granulomatous enterocolitis) and ulcerative rectocolitis. A clinical improvement was observed in these patients, namely decreased diarrhea, increased fistulae healing rates and decreased use of medications. Because of the importance of active oxygen species in the genesis of colitis, the relationship between oxidative stress and the supposed beneficial clinical effect of glutamine in colitis has become a subject of research. The mechanism by which glutamine exerts beneficial effects appears to be associated with the biosynthesis of glutathione, which causes a consequent reduction in lipid peroxidation of the intestinal membrane during mesenteric I/R[20].

The aim of our study was to investigate the effects of glutamine treatment in an animal model of mesenteric I/R analyzing parameters such as lipid peroxidation, SOD activity, and immunohistochemical expression of IL-6 and NF-κB.

MATERIALS AND METHODS

Ethics
Animal care was in compliance with the normative resolution 04/97 of the Research and Ethics Committee of the Health Research Group and Graduate Teaching Hospital of Porto Alegre (Hospital de Clínicas de Porto Alegre-HCPA)[21].

Animals
Male Wistar rats [250-300 g; State Foundation for Production and Health Research (Fundação Estadual de Produção e Pesquisa em Saúde-FEPPS)] were housed in a controlled environment and allowed access to food and water *ad libitum*.

Surgical procedures
After trichotomy, rats were anesthetized with ketamine and xylazine solution [45 mg/kg intraperitoneally (*ip*)]. After midline laparotomy, the celiac and superior mesenteric arteries were isolated near their aortic origins. During this procedure, the intestinal tract was placed between gauze pads soaked with warm 0.9% NaCl solution. The superior mesenteric artery and the celiac trunk were clamped, resulting in total occlusion of these arteries for 30 min to induce splanchnic artery occlusion injury. After occlusion, the clamps were removed, and after 15 min of reperfusion, intestinal segments (10 cm) and pieces of the lung were removed for histological examination and biochemical studies.

Experimental groups
Rats were randomly allocated into the following groups: (1) ischemia/reperfusion (I/R): rats were subjected to splanchnic artery occlusion injury (30 min) followed by reperfusion (15 min) (*n* = 5); (2) ischemia/reperfusion + glutamine group (G + I/R): identical to the ischemia/reperfusion group but were treated with glutamine (25 mg/kg *ip*) 24 and 48 h before I/R (*n* = 5); (3) control

group (control): rats were subjected to identical surgical procedures as the above groups, except the blood vessels were not occluded and the rats were maintained under anesthesia for the duration of the experiment (n = 5); and (4) control + glutamine group (control-GLU): identical to the Control group except for the administration of glutamine (25 mg/kg *ip*) 24 and 48 h before identical surgical procedures (n = 5). The glutamine treatment dose of 25mg/kg *ip* was chosen based on previous studies[22].

Assessment of lipid peroxidation

Thiobarbituric acid reactive substances: Tissue samples were placed in test tubes; solutions were added in the following order: 0.75 mL of 10% trichloroacetic acid (TCA), 0.25 mL of homogenate, 0.5 mL of 0.67% thiobarbituric acid (TBA), and 0.25 mL of distilled water.

Thiobarbituric acid reactive substances (TBARS) consists of heating the homogenate with thiobarbituric acid and measuring the consequent formation of a colored product in a spectrophotometer at 535 nm. The coloration is due to the presence of malondialdehyde and other substances from biological lipid peroxidation[23].

SOD activity

SOD was measured according to Misra and Fridovich. The rate of auto-oxidation of epinephrine, which is inhibited by SOD, is measured in the presence of progressively increasing doses of SOD with a spectrophotometer at 560 nm. The amount of enzyme that inhibits auto-oxidation of epinephrine at 50% of the maximum dose is defined as 1 U SOD[24].

Evaluation of NF-κB and IL-6

To prepare slides for subsequent immunohistochemical analysis, tissue was sectioned at 3-μm thickness using a microtome (Leica SM 2000R, Germany). The sections were placed on slides pretreated with HistoGrip (Zymed, United States) and incubated in an oven at 60 ℃ for 24 h.

Sections were deparaffinized by incubating in xylene three times for 10 min, followed by rehydration of the sections using decreasing concentrations of ethanol. Antigen exposure was performed using the pTLINK platform (DAKO) for 40 min at 98 ℃ with the Envision Flex antigen retrieval solution, high pH (DAKO). The slides were then immediately washed in phosphate-buffered saline (PBS), pH 7.2. The blocking of endogenous peroxidases was performed with two 15-min incubations in a 3% solution of H_2O_2 in methyl alcohol, which were followed by three washes with PBS, pH 7.2. Non-specific binding was blocked using the commercial solution Serum-Free Protein Block (Dako, United States) for 30 min at room temperature.

The sections were incubated using the immunostaining Sequenza station (Thermo Shandon, United States) overnight at 2 ℃ and 6 ℃ and with the following primary antibodies diluted in Antibody Diluent with Background Reducing Components (Dako, United States): anti-NF-κB (Santa Cruz Biotechnology, United States) at 1:100

and anti-IL-6 (Santa Cruz Biotechnology, United States) at 1:100. After incubation with the primary antibody, sections were washed three times in PBS, pH 7.2. To amplify the antigen-antibody reaction, the Advance system HRP was used for IL-6 (Dako, United States) according to the manufacturer's recommendations, and for NF-κB, goat anti-rabbit IgG-HRP secondary antibody was used at 1:300 in PBS for 30 min at room temperature. Next, the slides were washed with PBS and incubated with diaminobenzidine (Dako Liquid DAB Substrate Chromogen System, United States) for 5 min. After washing with distilled water, slides were counterstained with Harris hematoxylin for 1 min, washed with water until complete removal of the dye and incubated in a 37 mmol/L ammonia solution for 15 s. Finally, the slides were dehydrated in absolute ethanol (four incubations of 2 min) and two treatments with xylene for 5 min. The slides were mounted with Entellan synthetic medium (Merck, Germany)[25].

Analysis of digital images

We used a digital analysis system composed of a Zeiss Axioskop 40 microscope (Oberkochen, Germany) with Neofluar lenses connected by a Roper Scientific video camera (Media Cybernetics, Rockville, United States) to a computer with an Image Capture Pro kit (Media Cybernetics, Rockville, MD, United States) capture card. Image Pro Plus version 4.5 (Media Cybernetics, Rockville, United States) was used to analyze digital images. The images were captured in TIFF (True Image File Format) format without compression by the same examiner with a light intensity pattern for all photos. Images were captured of at least fifteen random, non overlapping fields for each histological slide at 200 × magnification (44 pixel = 1 μm). The hot spot method was used to select fields on slides with focal positivity for the markers. Color selection was performed interactively by three trained observers and was then applied to all samples by the automated digital image analysis system. The initial area considered was 0.01 cm.

Statistical analysis

Quantitative data were initially described by mean and standard deviation. To compare groups, we used analysis of variance. For categorical data, we used scores and comparisons based on Fisher's exact test.

Analysis of variance with robust standard errors (Welch) was used to verify NF-κB and IL-6 results between groups.

The significance level for the experiments was P < 0.05. Data were analyzed with SPSS version 21.0.

RESULTS

Evaluation of oxidative stress by analysis of lipid peroxidation

Lipid peroxidation of the membrane was increased in both the gut and the lung in the animals subjected to I/R (P < 0.05). However, the group that received glutamine

A TBARS	SO	SO + G	I/R	G + I/R
Media	0.45	0.4	1.8	0.8
E.P.	0.1	0.1	0.4	0.1

A SOD	SO	SO + G	I/R	G + I/R
Media	72.3	77.9	53.2	74.0
E.P.	2.5	1.8	1.3	2.4

B TBARS	SO	SO + G	I/R	G + I/R
Media	3.0	2.6	6.6	3.8
E.P.	0.7	0.7	1.0	0.6

| B SOD	SO	SO + G	I/R	G + I/R
Media	167.00	145.41	220.44	180.49
E.P.	2.77	3.52	3.31	2.55
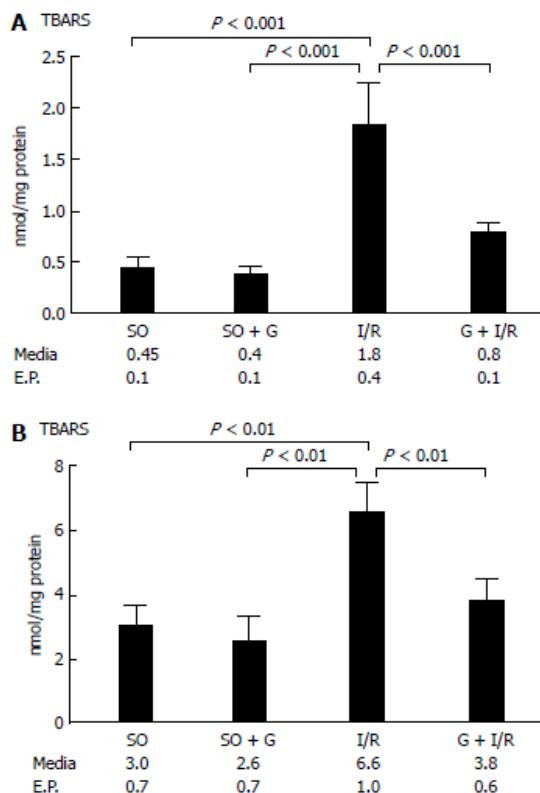
Figure 1 Lipid peroxidation (thiobarbituric acid reactive substances). A: In the gut; B: In the lungs. TBARS: Thiobarbituric acid reactive substances; SO: Control group means. I/R: Ischemia/reperfusion.

Figure 2 Levels of superoxide dismutase. A: In the gut; B: In the lungs. SOD: Superoxide dismutase; SO: Control group means. I/R: Ischemia/reperfusion.

24 and 48 h before the I/R procedure showed levels of lipid peroxidation similar to the control groups (animals not subjected to I/R and also the group receiving glutamine without I/R) that were significantly different from animals that only received I/R ($P < 0.05$). These results are shown in Figure 1.

SOD activity

Figure 2 shows that the activity of the antioxidant enzyme SOD was decreased in the gut of animals subjected to I/R. These findings were statistically significant ($P < 0.05$) when compared with the control group of animals not subjected to I/R. However, the group that received glutamine 24 and 48 h before I/R showed similar SOD activity to both control groups not subjected to I/R. There was a significant difference between the group of animals subjected to I/R and the group that received glutamine before I/R, suggesting that glutamine is a protective factor for mesenteric I/R.

NF-κB transcription factor

We calculated the mean area of NF-κB staining for each of the groups. As shown in Figures 3 and 4, the control and control-GLU groups presented similar mean areas. The I/R group showed the largest area of staining. The

G + I/R group had the second highest amount of staining, but the mean value was much lower than that of the I/R group. The same differences were observed among groups in the large intestine and the lung. These findings were statistically significant ($P < 0.05$).

Evaluation of IL-6

Images of IL-6 staining were analyzed in the same method as those stained for NF-κB. As shown in Figures 5 and 6, the control and control-GLU groups showed similar areas of staining. The I/R group contained the largest area of staining, followed by the G + I/R animals; however, this area was significantly lower than that of the group that underwent I/R without glutamine ($P < 0.05$).

DISCUSSION

As glutamine is glutathione precursor, and glutathione is the main non-enzymatic cellular antioxidant, is vital in the regulation of the intracellular oxidative balance[26].

This study demonstrates that glutamine treatment exerts important protective effects against splanchnic artery occlusion injury in a murine model. Our data provide evidence that glutamine attenuates: (1) the lipid peroxidation of gut mucosa; (2) the decrease in SOD activity; (3) the
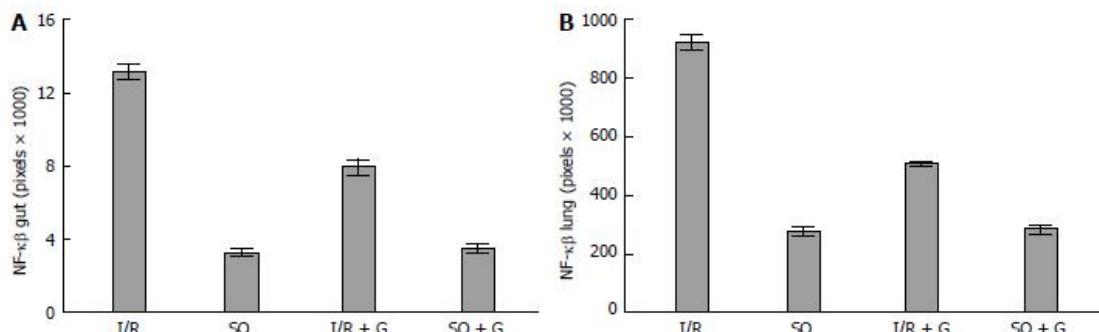
Figure 3 Immunohistochemical expression of nuclear factor kappa beta. A: In the gut; B: In the lungs. SO: Control group means; I/R: Ischemia/reperfusion; NF-κB: Nuclear factor kappa beta.
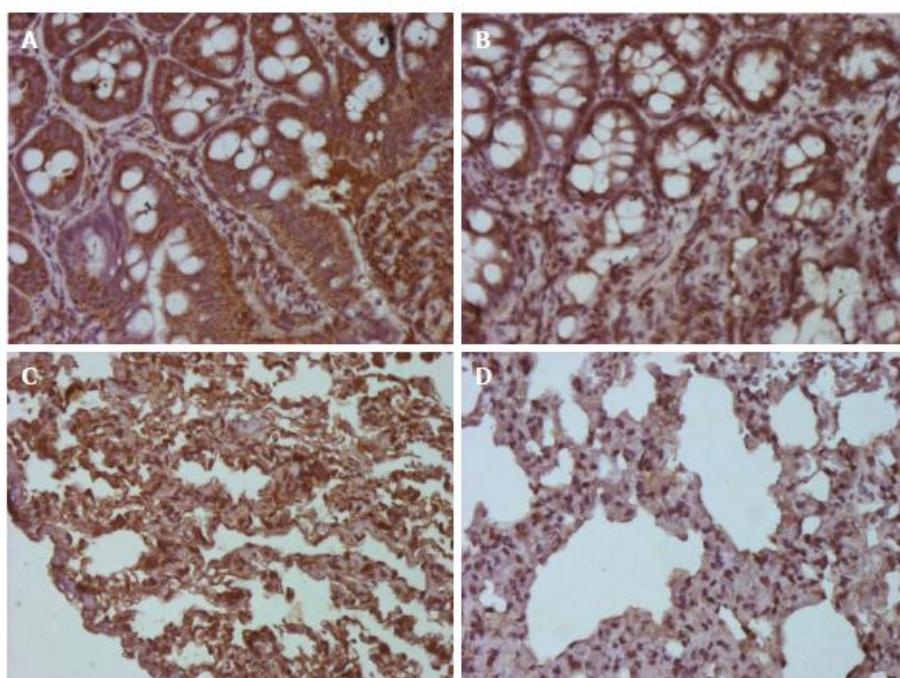

Figure 4 Digital images of the immunohistochemical expression of nuclear factor kappa beta (photomicrography, × 200). A: Ischemia/reperfusion (I/R) gut; B: G + I/R gut; C: I/R lung; D: G + I/R lung.

increases in NF-κB expression; and (4) IL-6 expression that occur after I/R.

In our study, the amount of lipid peroxidation was greater in the group of animals subjected to I/R. The addition of glutamine significantly decreased lipid peroxidation compared with animals that underwent I/R without glutamine treatment. Other authors, such as Mondello *et al*[27] and He *et al*[13] observed similar beneficial results of glutamine for I/R with different methodologies. Mondello *et al*[27] have induced intestinal ischemia in rats by clamping the superior mesenteric artery and the celiac trunk for 30 min, then releasing it and promoting reperfusion during 1 h. Glutamine was administered 15 min before reperfusion at the dose of 1.5 mg/kg, *iv* Their findings showed a reduction in: (1) the infiltration of

neutrophils in the ileum; (2) the formation of the pro-inflammatory cytokines; (3) the expression of the adhesion molecules ICAM-1 and P-selectin; (4) the IKB-α degradation and the nuclear translocation of NF-κB; and (5) the nitrotyrosine formation and PARP activation. He *et al*[13] utilized a rat model of I/R, but administering glutamine enterically before and after a 60 min ischemia; additionally, in one subgroup the lymphatic mesenteric duct was also ligated before the production of intestinal ischemia. They concluded that both the enteral administration of glutamine and the ligature of the lymphatic mesenteric duct prevented intestinal permeability, attenuating systemic inflammatory reactions and ALI. In contrast, Fukatsu *et al*[28] have shown that in a murine model of gut I/R, an *iv* glutamine was detrimental in terms of

Zabot GP *et al*. Glutamine prevents oxidative stress in ischemia-reperfusion
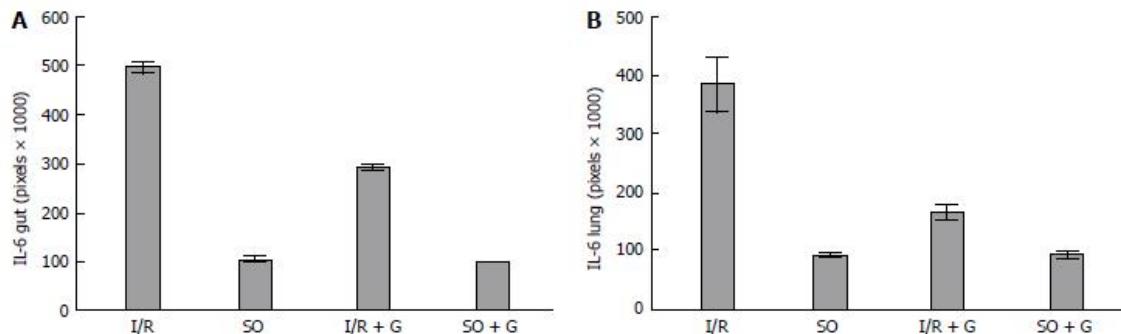
Figure 5 Immunohistochemical expression of interleukin-6. A: In the gut; B: In the lungs. SO: Control group means; I/R: Ischemia/reperfusion; IL: Interleukin.
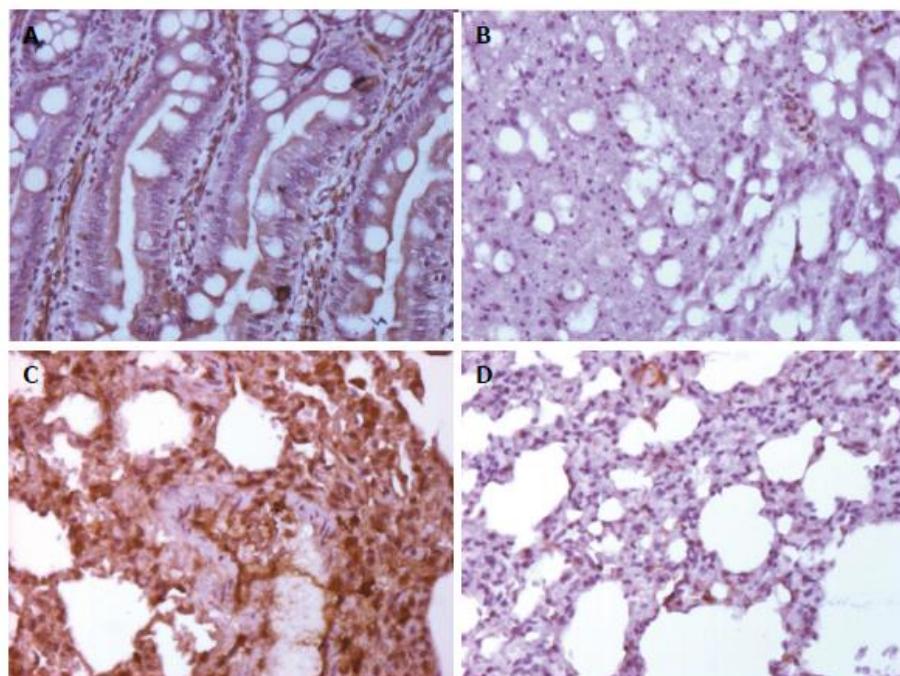

Figure 6 Digital images of the immunohistochemical expression of interleukin-6 (photomicrography, × 200). A: Ischemia/reperfusion (I/R) gut; B: G + I/R gut; C: I/R lung; D: G + I/R lung.

survival and organ injury due to the increased priming of circulating myeloid cells.

In our study, SOD activity was decreased in animals submitted to I/R. In the Control and Control-GLU groups, the decrease in SOD activity was much lower and similar between the two groups. The addition of glutamine to animals submitted to I/R produced a decrease that was not as significant but that was lower than that found in the I/R only group. The first authors to describe the role of SOD in oxidative stress were Misra and Fridovich[24]. In their pivotal study, SOD was prepared from bovine erythrocytes, being able to inhibit the autoxidation of epinephrine at a pH 10.2. Recently, Salman *et al*[29] administered glutamine by gavage to Sprague-Dawley rats, at a dose of 1 g/kg for 10 d prior to intestinal I/R, studying tissue damage in the intestines and lungs. These authors measured the intestinal and pulmonary levels of SOD, in addition to serum levels of TNF-α and IL-6, concluding that pre-treatment with a bolus dose of enteral glutamine was able to minimize the extent of ALI in rats.

Tissue I/R activates families of protein kinases that converge on specific transcription factors (protein activator-1 (PA-1) and NF-κB) that regulate the expression of pro-inflammatory genes. In our study, the activity of NF-κB was higher in both the intestines and the lungs in the group subjected to I/R. However, in the group of animals that received prophylactic glutamine *ip* 24 and 48 h before I/R, the levels of NF-κB were lower. This difference between groups was statistically significant. Sen and Baltimore[9] published the first report on NF-κB, suggesting its important role in cellular inflammatory response to injury. However, the exact role of this transcription factor re-

mains controversial. According to Haddad[10], NF-κB appears to perform an important function in the generation and resolution of intestinal I/R lesions, as a transcription factor that is directly influenced by reactive species and pro-inflammatory signs. Bowie *et al*[30], in a review article about oxidative stress and Nf-kB activation, determined that in most cases, the role of oxidative stress in NF-κB activation is at best facilitatory rather than causal, if a there exists a role at all. Ypsilantis *et al*[31] tested the hypothesis that the action of 2-mercaptoethane-sulfonate (mesna) is mediated by the inhibition of NF-κB, studying the oxidative stress on a rat model of I/R, analyzing glutathione, malondialdehyde concentration, SOD and NF-κB. These authors concluded that prophylaxis with mesna prevents oxidative stress induced by I/R in the intestine via inhibition of NF-κB activation.

ROS-mediated oxidative injury as a consequence of increased production of inflammatory cytokines such as IL-6 and TNF-α and the neutrophil activation play critical roles in the pathogenesis of I/R. IL-6 and TNF-α not only directly induce tissue damage but are also potent activators of neutrophils. The neutrophils and their enzymatic products cause increased microvascular permeability, perivascular and interstitial edema, and even promote distant organ injury such as pulmonary edema when sequestrated in intestinal tissue. Cuzzocrea *et al*[11] studied the inflammatory process secondary to I/R in a knockout mice model, verifying by immunohistochemistry that IL-6 plays an important role in I/R injury, suggesting that the inhibition of IL-6 may actually represent a novel and possible strategy in the prevention of I/R injuries.

In our study, similarly to NF-κB, the immunohistochemical expression of IL-6 was found to be high in animals that underwent I/R in both the intestines and the lungs. The control and control-GLU groups showed similar results for IL-6, with observed levels well below those of the I/R group. However, the group that received a potentially protective factor, glutamine, before I/R showed a higher expression of IL-6 than the control and control-GLU groups but at levels that were statistically inferior to the I/R group.

In conclusion, this study demonstrates that *ip* administration of glutamine at a dose of 25 mg/kg 24 and 48 h before animals are subjected to 30 min of mesenteric ischemia and 15 min of reperfusion effectively protected against lipid peroxidation and preserved SOD activity. The activity of NF-κB and IL-6 were also reduced upon *ip* administration of glutamine at 24 and 48 h prior to I/R in rats. This adds to previously published data on glutamine as a protective factor in mesenteric I/R states in rats. Further studies are necessary to test the role of glutamine as a potential protective agent against I/R lesions in humans.

COMMENTS

Background

Ischemia-reperfusion (I/R) leads to oxidative stress, with local and systemic consequences. Many enzymes and interleukins have been implicated in this process, among them interleukin-6 (IL-6) and nuclear factor kappa beta (NF-κB). The exact role of these enzymes is still not clear. Substances that inhibit or minimize the inflammatory process caused by aggressive agents, such as glutamine, have been used for prevention purposes.

Research frontiers

Glutamine is the most abundant amino acid in peripheral blood. That amino acid acts on macrophage activity, interfering with phagocytosis at inflammatory sites. Plays an important role in intestinal inflammatory processes by acting on reactive oxygen species (ROS). Glutamine is a multifunctional amino acid used for the synthesis of urea in the liver, renal *aminogenesis*, gluconeogenesis, and as the main respiratory fuel for many cells.

Innovations and breakthroughs

This substance was initially used prophylactically in patients undergoing radiation therapy, leading to a reduction in the incidence and severity of actinic enteritis. It is thus vital in the regulation of the intracellular oxidative balance. Glutamine has been used as a nutritional supplement in severely debilitated patients to reduce the deleterious effects of oxidative stress. The present study demonstrated that the pretreatment with glutamine prevents mucosal injury and improves gut and lung recovery after I/R injury in a rat model.

Applications

The study results suggest that the glutamine protected against lipid peroxidation and preserved superoxide dismutase (SOD) activity. The activity of NF-κB and IL-6 were also reduced upon *ip* administration of glutamine at 24 and 48 h prior to I/R in rats. This adds to previously published data on glutamine as a protective factor in mesenteric I/R states in rats.

Terminology

Ischemia/reperfusion (I/R): gut ischemia usually results from occlusion of the celiac trunk and/or the superior mesenteric artery by thrombi or emboli and, more frequently, from non-occlusive processes, such as in the case of decreased mesenteric blood flow that occurs in heart failure and sepsis. In the gut, ischemia followed by reperfusion frequently results in multiple organ failure (MOF), with the gut being the organ that triggers the injury process in distant organs; SOD: is an antioxidant enzyme highly specific for superoxide elimination, thus reducing gastrointestinal lesions caused by I/R; NF-κB: nuclear factor of kappa light polypeptide gene enhancer in B-cells is a transcription factor that plays a crucial role not only in normal states but also in the coordination of adaptive immune responses by regulating the expression of many cell mediators; IL-6: levels are elevated in I/R as well as in MOF; Glutamine: is a polar amino acid that is *non-essential* or occasionally essential, hydrophilic, and found on the surface of proteins where it interacts with water.

Peer review

This is an interesting article studying how the pretreatment with glutamine prevents mucosal injury and improves gut and lung recovery after I/R injury in a rat model. The manuscript includes six clear figures. This research is easy to follow and finds some valuable information for scientific community interested in both glutamine and ischemia/reperfusion, as well as in oxidative damage and ROS. To date, this is the first investigation to study glutamine effect on NF-κB and IL-6, as well as in SOD and TBARS in a model of mesenteric ischemia/reperfusion.

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10.3 Anexo C

Artigos aceitos referentes à tese durante a vigência do doutorado:

1. HARTMANN, R.M.; LICKS, F.; SCHEMITT, E.G.; COLARES, J.R.; SOARES, M.C.; ZABOT, G.P.; FILLMANN, H.S., MARRONI, N.P. Protective effect of glutamine on the main and adjacent organs damaged by ischemia/reperfusion in rats. Protoplasma, 2017.

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