UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO GONÇALO MONIZ

Curso de Pós-Graduação em Patologia Humana

TESE DE DOUTORADO

EFEITOS DO HEME E PROPRIEDADES TERAPÊUTICAS DA HIDROXIUREIA NO TRATAMENTO DA ANEMIA FALCIFORME

SÂNZIO SILVA SANTANA

Salvador – Bahia

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Tese apresentada ao Curso de Pós-Graduação em Patologia Humana para obtenção do grau de Doutor.

Salvador – Bahia
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Título da Tese: " **EFEITOS DO HEME E PROPRIEDADES TERAPÊUTICAS DA HIDROXIUREIA NO TRATAMENTO DA ANEMIA FALCIFORME.**"

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FONTES DE FINANCIAMENTO

“O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código de Financiamento 001”

Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB)

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)

Instituto Gonçalo Moniz (IGM-FIOCRUZ-BA)

Dedico este trabalho ao meu *Deus* e à minha adorável esposa *Geisiane Souza Santana*, com todo amor.

AGRADECIMENTOS

A caminhada foi árdua até aqui. Na trajetória de pouco mais de trinta anos vividas até aqui, alguém sempre cuidou de mim. Alguém que é soberano cujo nome está acima de todo nome, Jesus! “Ele não escolhe os capacitados, Ele capacita os escolhidos”. Obrigado Senhor por ouvir cada oração e abençoar-me com o Teu amor, pela minha família, pela minha saúde, pelo alimento de cada dia, por “abrir cada porta” proporcionando novas oportunidades, e cada conquista. Que seja feita a Tua vontade, pois eu creio que os Teus planos são maiores do que os meus.

Meu muito obrigado...

À minha amada esposa por todo amor, apoio, carinho, cumplicidade, cuidado, confiança e pelo sorriso mais lindo deste mundo. Os adjetivos são poucos para expressar o meu amor por você. Não é nada fácil lidar com noites em claro e com as adversidades de um Pós-Graduando. Estamos começando a rabiscar algumas dezenas de folhas dentre as inúmeras que estão por vir. Te amo infinitamente!

À minha mãe “Cassinha” e aos meus irmãos Samir e Emille, pelo alicerce familiar, apoio e amor durante toda a minha vida. À tia Cacilda pelo apoio de sempre, e toda a minha família. Muito obrigado por acreditarem em mim. Que o Senhor continue abençoando a todos. Amo vocês!

À tia Cândida (sogra), aos meus cunhados Jarbas e Caliane, e Viviane, pelo carinho e apoio de sempre. Os dias são mais divertidos com vocês. Amo vocês, e muito obrigado por fazerem parte da minha vida. Vocês são bênçãos de Deus!

Aos nossos padrinhos Sérgio e Iranildes, pelo carinho e ensinamentos da parte de Deus. Que vocês continuem sendo bênçãos de Deus na vida de outras pessoas.

À professora orientadora Marilda Gonçalves, por contribuir com a consolidação desse sonho. Obrigado pelos conselhos, por confiar em meu trabalho, pelos ensinamentos durante esses anos, e por ter esse “coração enorme”. A responsabilidade de transferir conhecimento é grande, e é algo recompensador. Espero dar continuidade a este princípio, passando adiante o conhecimento adquirido e contribuindo com a formação de outros profissionais. Beijo grande!

Aos amigos Paulo Melo, Bruno Veloso, Wendell Vilas e Magda Seixas pela receptividade, apoio e troca de conhecimentos durante a minha chegada no Centro de Pesquisas Gonçalo Moniz (CPqGM), hoje Instituto Gonçalo Moniz (IGM).

Aos amigos do laboratório Thassila Pitanga, Corynne Adanho e Jeanne Santana, que estiveram mais próximos nessa empreitada auxiliando de diversas formas, desde a execução e discussão de experimentos, ao incentivo e força nos momentos mais difíceis. E aos demais amigos, Modeste Yahouédéhou, Luciana Fiuza, Camylla Vilas, Milena Aleluia, Samuel Uche, Batholomew Chukwu, Junia Dutra, Silvana Paz, Rayra Santiago, Caroline da Guarda, Karoline Caetano e Rifkath Marie, pela convivência e apoio. Que Deus abençoe todos vocês!

Aos pesquisadores Vitor Maffili e Dalila Zanette, pela convivência e apoio de diferentes formas e pela amizade sincera.

Aos amigos de outros laboratórios/setores: Jaqueline Góes, Fernanda Khouri, Ricardo Riccio, Yuri Tabajara, Kelvin Edson e Ronald dos Santos, do Laboratório de Patologia Experimental (LAPEX); Cássio Santana, Afrânio Evangelista e Diogo Lúcio, do Laboratório de Engenharial Tecidual (LETI); Jailton Azevedo, do Laboratório de Epidemiologia Molecular e Bioestatística (LEMB); Laiton Borja e Daiana Oliveira, do Laboratório de Patologia e Biologia Molecular (LPBM); Sayonara Melo, do Laboratório de Enfermidades Infecciosas Transmitidas por Vetores (LEITV); Priscila Guerra, do Laboratório de Interação Parasito-Hospedeiro e Epidemiologia (LAIPHE); e Jacqueline Silva do antigo Laboratório de Biomorfologia Parasitária (LBP); e Elze Leite (diretoria). São muitas individualidades para resumir em um parágrafo. Meu sincero obrigado por contribuírem com a concretização deste trabalho.

A Dra Valéria pela colaboração em parte deste trabalho.

Ao Programa de Pós-Graduação em Patologia Humana e Experimental (PGPAT-FIOCRUZ-UFBA) e aos professores e pesquisadores vinculados, pela oportunidade concedida e por contribuirem com o conhecimento e amadurecimento científico.

A FAPESB e CNPq pelo fomento à pesquisa, em especial, à FAPESB, pela concessão da bolsa durante os quatro anos de formação.

A toda a equipe da biblioteca do IGM-FIOCRUZ-BA pelo auxílio na formatação e catalogação deste trabalho.

Por fim, às pessoas que estiveram engajadas direta ou indiretamente com a realização deste tratalho, em especial, os pacientes com anemia falciforme. Espero que esses resultados possam contribuir futuramente para proporcionar uma melhor qualidade de vida para vocês.

“Minha graça é suficiente para você, pois o meu poder se aperfeiçoa na fraqueza (...) Por isso, por amor de Cristo, regozijo-me nas fraquezas, nos insultos, nas necessidades, nas perseguições, nas angústias. Pois, quando sou fraco é que sou forte”.

(2 Coríntios 12: 10-11 NVI)

SANTANA, Sânzio Silva. Efeitos do heme e propriedades terapêuticas da hidroxiureia no tratamento da anemia falciforme. 2018. 196 f. Tese (Doutorado em Patologia Humana) – Universidade Federal da Bahia. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2018.

RESUMO

INTRODUÇÃO: a fisiopatologia da anemia falciforme (AF) é marcada por crises hemolíticas e vasoclusivas intermitentes com aumento da condição redox no microambiente vascular que favorece a cronicidade inflamatória. **OBJETIVOS:** avaliar a associação do heme com marcadores clínico-laboratoriais em pacientes com AF; o papel de hemácias falciformes íntegras e lisadas na expressão gênica do inflamassoma NLRP3, e se o tratamento de pacientes com hidroxiureia (HU) interfere nesta expressão e na liberação de IL-1β e leucotrieno B_4 (LTB_4); e investigar as propriedades antioxidantes da HU. **MÉTODOS:** o heme plasmático de pacientes com AF (n = 80) foi dosado por ELISA, os marcadores laboratoriais foram determinados por métodos automatizados, e as análises genéticas por polimorfismo de comprimento de fragmentos de DNA (RFLP). O histórico clínico dos pacientes foi obtido através de prontuários. Os ensaios de expressão gênica de componentes do inflamassoma NLRP3 (*NRLP3*, *IL1B*, *CASP1* e *IL18*) por RT-qPCR foram realizados em células mononucleares de sangue periférico humano (PBMC) de voluntários sadios desafiados com hemácias falciformes íntegras e lisadas (n = 8), e hemácias normais (n = 10); e em leucócitos totais de pacientes com AF tratados (n = 13) ou não tratados com hidroxiureia (HU) (n = 15) e voluntários sadios (n = 20). As determinações de IL-1β e LTB_4 foram realizadas por ELISA. A atividade antioxidante foi avaliada por ensaios de varredura usando o radical 2,2-difenil-1-picrilhidrazila (DPPH). A expressão de superóxido dismutase 1 (*SOD1*), glutationa peroxidase (*GPx*), glutationa S-redutase (*GSR*) e heme oxigenase-1 (*HMOX1*) foi realizada por RT-qPCR em human umbilical vein endotelial cells (HUVEC) e PBMC. Análises de microarranjo foram realizadas em HUVEC tratadas com HU. **RESULTADOS:** o aumento no heme livre esteve associado ao aumento de hemoglobina S (HbS), contagem de monócitos, marcadores hepáticos, triglicérides e lipoproteína de densidade muito baixa (VLDL-C); e a diminuição dos níveis de hemoglobina fetal (HbF) e lipoproteína de alta densidade (HDL-C). O genótipo BEN/BEN esteve associado a níveis mais elevados de HbF que os CAR/CAR. A liberação de heme livre não apresentou associação com os haplótipos, mas foi associada ao histórico clínico de AVC. As hemácias falciformes íntegras e lisadas (SS-RBC) induziram a expressão de componentes do inflamassoma NLRP3 e a secreção de IL-1β e LTB_4. SS-RBC íntegras induziram expressão de *IL1B* com secreção de IL-1β e LTB_4, em comparação com SS-RBC lisadas ou hemácias de voluntários sadios (AA-RBC). A diminuição significativa na expressão de *NLRP3* e secreção de LTB_4 foram observadas em pacientes tratados com HU. A HU apresentou atividade antioxidante em concentrações equivalentes à encontrada no plasma de pacientes tratados com a droga (~200 μM). Os tratamentos com HU ou HU+hemina não induziram toxicidade em PBMC e HUVEC. O tratamento HU+hemina estimulou a produção de nitrato/nitrito em PBMC e HUVEC. A HU aumentou a expressão de *SOD1* e *GPx* em PBMC e HUVEC. O aumento na expressão de *GSR* foi observado em PBMC e HUVEC tratadas com HU+hemina. O tratamento com HU (ou em combinação com a hemina) não interferiu na expressão de *HMOX1* em PBMC quanto em HUVEC. A HU induziu a expressão de *SOD2*, *GSR*, *GST1* (glutationa S-transferase-1), *GSTM2*

(glutationa S-transferase mu 2), *MGST1* (glutationa S-transferase 1), *CBR1* (carbonil redutase 1), proteinas quinases phosphatidylinositol 3-phosphato C (*PRKCB*, *PRKCZ*, *PIK3C2B*) e sequestossomo-1 (*p62/SQSTM1*). Em contraste, foi observada a diminuição na expressão do fator transcricional *BACH1*. “Upstream analyses” demonstraram predição de ativação de Jun, miR-155 e mir-141-3p. **CONCLUSÕES:** a liberação excessiva de heme a partir de eventos hemolíticos recorrentes pode contribuir substancialmente para a gravidade clínica da AF. Hemácias íntegras e lisadas podem atuar como DAMPs induzindo diferentemente a expressão de componentes do inflamassoma e a produção de IL-1β e LTB_4, contribuindo para o estabelecimento da inflamação. O tratamento com HU parece diminuir a inflamação por vias dependentes de NLRP3 e LTB_4. A HU apresenta propriedades antioxidantes diretas eliminando radicais livres além de induzir o sistema enzimático de resposta antioxidante via Nrf2 sob regulação do sequestossomo-1. Esses achados podem auxiliar no desenvolvimento de novas estratégias terapêuticas que possam ser utilizadas em conjunto com a indução de HbF visando minimizar a cronicidade inflamatória da AF.

Palavras-chave: Anemia falciforme, Hidroxiureia, Estresse oxidativo, Inflamassoma.

SANTANA, Sânzio Silva. Effects of heme and therapeutic properties of hydroxyurea in the treatment of sickle cell anemia. 2018. 196 f. Tese (Doutorado em Patologia Humana) – Universidade Federal da Bahia. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2018.

ABSTRACT

INTRODUCTION: the pathophysiology of sickle cell anemia (SCA) is marked by intermittent hemolytic and vasoconstrictive seizures with increased redox status in the vascular microenvironment that favors inflammatory chronicity. **OBJECTIVES:** to evaluate the association of heme with clinical-laboratory markers in patients with SCA; the role of intact and lysed erythrocytes in the gene expression of NLRP3 inflammation, and whether the treatment of patients with hydroxyurea (HU) interferes with this expression and the release of IL-1β and leukotriene B4 (LTB_4); and investigate the antioxidant properties of HU. **METHODS:** plasma heme of patients with SCA (n = 80) was dosed by ELISA, laboratory markers were determined by automated methods, and genetic analyzes by DNA fragment length polymorphism (RFLP). The clinical history of the patients was obtained through medical records. The gene expression assays of NLRP3 (*NRLP3*, *IL1B*, *CASP1* and *IL18*) components of RT-qPCR were performed on human peripheral blood mononuclear cells (PBMCs) of healthy volunteers challenged with whole and lysed sickle cells (n = 8), and normal erythrocytes (n = 10); and total leukocytes from treated (n = 13) or not treated with HU (n = 15) and and healthy volunteers (n = 20). The determinations of IL-1β and LTB_4 were performed by ELISA. The antioxidant activity was assessed by sweep tests using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). Expression of superoxide dismutase 1 (*SOD1*), glutathione peroxidase (*GPx*), glutathione S-reductase (*GSR*) and heme oxygenase-1 (*HMOX1*) was performed by RT-qPCR in human umbilical vein endothelial cells (HUVEC) and PBMC. Microarray analyzes were performed on HUVEC treated with HU. **RESULTS:** increased free heme was associated with increased hemoglobin S (HbS), monocyte count, hepatic markers, triglycerides and very low density lipoprotein (VLDL-C); and decreased levels of fetal hemoglobin (HbF) and high-density lipoprotein (HDL-C). The BEN/BEN genotype was associated with higher levels of HbF than the CAR/CAR. The release of free heme was not associated with haplotypes, but was associated with the clinical history of stroke. Intact and lysed red blood cells (SS-RBC) induced the expression of NLRP3 inflammation components and secretion of IL-1β and LTB_4. SS-RBC induced *IL1B* expression with secretion of IL-1β and LTB_4, compared to lysed SS-RBC or erythrocytes of healthy volunteers (AA-RBC). Significant decrease in NLRP3 expression and LTB_4 secretion were observed in patients treated with HU. HU presented antioxidant activity at concentrations equivalent to that found in the plasma of patients treated with the drug (~200 μM). Treatments with HU or HU+hemin did not induce toxicity in PBMC and HUVEC. The HU+hemin treatment stimulated nitrate/nitrite production in PBMC and HUVEC. HU increased expression of *SOD1* and GPx in PBMC and HUVEC. The increase in *GSR* expression was observed in PBMC and HUVEC treated with HU+hemin. Treatment with HU (or in combination with hemin) did not interfere in the expression of *HMOX1* in PBMC as in HUVEC. HU induced expression of *SOD2*, *GSR*, *GST1* (glutathione S-transferase-1), *GSTM2* (glutathione S-transferase mu 2), *MGST1* (glutathione S-transferase 1), *CBR1* (carbonyl reductase 1), protein kinases phosphatidylinositol 3 -phosphate C (*PRKCB*, *PRKCZ*, *PIK3C2B*) and sequestosome-1 (*p62/SQSTM1*). In contrast, the decrease in

transcriptional factor *BACH1* expression was observed. Upstream analyzes demonstrated prediction of activation of Jun, miR-155 and mir-141-3p. **CONCLUSIONS:** excessive heme release from recurrent hemolytic events may contribute substantially to the clinical severity of SCA. Whole and lysed blood cells can act as DAMPs by inducing the expression of inflammatory components and the production of IL-1β and LTB_4, contributing to the establishment of inflammation. Treatment with HU appears to decrease inflammation by NLRP3 and LTB_4 dependent pathways. HU has direct antioxidant properties by eliminating free radicals and inducing the enzymatic system of antioxidant response via Nrf2 under sequestosome-1 regulation. These findings may aid in the development of new therapeutic strategies that can be used in conjunction with the induction of HbF in order to minimize the inflammatory chronicity of SCA.

Keywords: Sickle cell anemia, Hydroxyurea, Oxidative stress, Inflammasome

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LISTA DE ABREVIATURAS E SIGLAS

AA-PBMC	Células mononucleares de sangue periférico de voluntários sadios (com hemoglobina AA)
AA-RBC	Eritrócitos de voluntários sadios (hemoglobina AA)
ADP	Adenosina difosfato (do inglês, *adenosine diphosphate*)
AF	Anemia falciforme
APAE-BA	Associação de Pais e Amigos dos Excepcionais de Salvador-Bahia
ARE/EpRE	Elemento de resposta antioxidante/Elemento de resposta ao eletrófilo (do inglês, *antioxidant response element/electrophile response element*)
AS	Ácido siálico
ASC	Proteína associada a apoptose (do inglês, *apoptosis-associated speck-like protein containing a CARD*)
ATP	Trifosfato de adenosina (do inglês, *adenosine triphosphate*)
AVC	Acidente vacular cerebral
BACH1	Proteína reguladora da transcrição BACH1 (do inglês, *BTB and CNC Homology 1, Basic Leucine Zipper Transcription Factor 1*)
BCAM/Lu	Molécula de adesão celular basal/Lutheran (do inglês, *basal cell adhesion molecule/Lutheran*)
BEN	Haplótipo Benin ligado ao *cluster* de genes da β-globina
CAT	Catalase
CD	Grupamento de diferenciação (do inglês, *cluster differentiation*)
CAR	Haplótipo CAR ligado ao *cluster* de genes da β-globina
CAMER	Haplótipo Camarões ligado ao cluster de genes da β-globina
cDNA	DNA complementar de fita simples
Células Ta/Th	Células T auxiliares (do inglês, *T helper*)
cGMP	Monofosfato cíclico de guanosina (do inglês, *ciclic* guanosine monophosphate)
CO	Monóxido de carbono
CVOs	Crises vasoclusivas

DAMPs	Padrões moleculares associados ao dano (do inglês, *damage-associated molecular patterns*)
DF	Doença falciforme
DPPH	2,2-Difenil-1-picrilhidrazil (do inglês, *2,2-diphenyl-1-picrylhydrazyl*)
eNOS	Óxido nítrico sintase endotelial (do inglês, *endothelial nitric oxide synthase*)
ERN	Espécies reativas do nitrogênio
EROs	Espécies reativas do oxigênio
ESEL-1	E-selectina 1
FBN	Fibronectina
FDA	Do inglês, *Food and Drug Administration*
FT	Fator tecidual
GADPH	Gluceraldeído-3-fosfato desidrogenase (do inglês, *glyceraldehyde 3-phosphate dehydrogenase*)
GCs	Guanilato cilase solúvel
GCS	γ-Glutamato cisteína sintetase (do inglês, *γ-glutamate-cysteine synthetase*)
GPx	Glutationa peroxidase
GSH	Glutationa reduzida
GSR	Glutationa S-redutase
GTP	Trifosfato de guanosina (do inglês, *guanosine triphosphate*)
H_2O_2	Peróxido de hidrogênio
Hb	Hemoglobina
HbF	Hemoglobina fetal
HbS	Hemoglobina S
HDL-C	Lipoproteína de alta densidade (do inglês, *high-density lipoprotein-cholesterol*)
HMGB1	Proteína box-1 do grupo de alta mobilidade (do inglês *high-mobility group box 1*)
HMOX1	Gene da heme oxigenase-1
$^{\bullet}OH$	Radical hidroxila
HO-1	Heme oxigenase-1

HU	Hidroxiureia
ICAM-1	Molécula de adesão intercelular-1 (do inglês, *intercellular Adhesion Molecule-1*)
ICAM-4	Molécula de adesão intercelular 4 (do inglês, *intercellular Adhesion Molecule 4 – Landsteiner-Wiener Blood Group*)
IL	Interleucina
IL18Bp	Proteína ligante de IL-18 (do Inglês, *IL18 bind protein*)
Keap1	*Kelch-like ECH-associated protein1*
LAM	Laminina
LDL	Lipoproteína de baixa densidade (do inglês, *low-density lipoprotein-cholesterol*)
LRR	Repetições ricas em leucina (do inglês, *leucine-rich repeat*)
LTB_4	Leucotrieno B_4
LW	Proteína do grupo sanguíneo Landsteiner-Wiener
Mac-1 ou αMβ2	Antígeno de macrófago-1 (do inglês, *macrophage-1 antigen*)
MAPK	Proteína quinase ativada por mitógeno (do inglês*, mitogen-activated protein kinase)*
metHb	Metemoglobina
MyD88	Resposta primária de diferenciação mieloide 88 (do inglês, *Myeloid differentiation primary response 88*)
NACH	Do inglês, *NAIP (neuronal apoptosis inhibitor protein), C2TA (class 2 transcription activator, do MHC), HET-E (heterokaryon incompatibility) e TP1 (telomerase-associated protein 1)*
$NADP^+$	Fosfato de dinucleótido de nicotinamida e adenina (do inglês, *nicotinamide adenine dinucleotide phosphate*)
NALP3 ou NLRP3	Do inglês, *NACHT, LRR and PYD domains-containing protein 3* ou *NLR family, pyrin domain containing protein 3*
NETs	Armadilhas extracelulares de neutrófilos (do inglês, *neutrophil extracellular traps*)
NF-kB	Fator nuclear kappa B (do inglês, *factor nuclear kappa B*)
NLR	Receptor do tipo NOD (do inglês, *NOD-like receptor*)
NO_3^-	Nitrato

NOD	Receptor do tipo NOD (do inglês, *NOD-like receptor*)
NOS	Óxido nítrico sintase (do inglês: *Nitric oxide synthase*)
NQO1	NAD(P)H:quinona oxidoredutase 1
NQO2	NRH:quinona oxidoredutase 2
Nrf2 ou NFE2L2	Fator respiratório nuclear 2 [do inglês, *nuclear factor (erythroid-derived 2)-like 2*]
$O_2^{\bullet-}$	Ânion superóxido
NO	Óxido nítrico (do inglês, *Nitric oxide*)
$ONOO^-$	Peroxinitrito
OxLDL	Lipoproteína de baixa densidade oxidada (do inglês, *oxidized low-density lipoprotein*)
p62/SQSTM1	Sequestossomo1
PAMPs	Padrão molecular associado ao dano (do inglês, *pathogen-associated molecular*)
PBMC	Células mononucleares do sangue periférico (do inglês, *peripheral blood mononuclear cells*)
PGE_2	Prostaglandina E_2
p-HDL	Lipoproteína de alta densidade pró-inflamatória (do inglês, *pro-inflammatory high-density lipoprotein*)
PHHF	Persistência hereditária da HbF
PKC	Proteína quinase C (do inglês, *Protein kinase C*)
PMA	Forbol 12-mirisato 13-acetato (do inglês, *phorbol 12-myristate 13-acetate*)
PNTN	Programa Nacional de Triagem Neonatal
PSGL-1	Ligante-1 da glicoproteína P-selectina (do inglês, *P-selectin glycoprotein ligand-1*)
PYD	Domínio pirina (do inglês, PYD, *Pyrin domain*)
RNS	Espécies reativas de nitrogênio (do inglês, *reactive nitrogen species*)
ROS	Espécies reativas de oxigênio (do inglês, *reactive oxygen stress*)
SA	Ácido siálico (AS; do inglês, sialic acid)
SAUD	Haplótipo Arábia Saudita e Índia ligado ao cluster de genes da β-globina

SCA	Anemia falciforme (do inglês, *sickle cell anemia*)
SCD	Doença falciforme (do inglês, *sickle cell disease*)
SEN	Haplótipo Senegal ao cluster de genes da β-globina
SOD-1	Superóxido dismutase-1
SRTN	Serviço de Referência em Triagem Neonatal
Syk	Tirosina quinase do baço (do inglês, *spleen tyrosine kinase*)
TCD4+	Receptor CD4 ativado
TCD8+	Receptor CD8 ativado
TLR	Receptor do tipo Toll (do inglês, *Toll-like receptor*)
TNF-α	Fator de necrose tumoral-α (do inglês, *Tumor necrosis factor-α)*
TSP	Trombospondina
ULvWF	Fator von Willebrand ultra-large (do inglês, *ultra-large von Willebrand fator*)
VCAM-1	Molécula de adesão celular vascular 1 (do inglês, *vascular cell adhesion protein*)
VLDL-C	Lipoproteína de muito baixa densidade (do inglês, *very low-density lipoprotein-cholesterol*)
UPS	Sistema de proteassoma ubiquitina (do inglês, *ubiquitin proteasome system*)
XO	Xantina oxidase

SUMÁRIO

1. INTRODUÇÃO

A doença falciforme (DF) é uma doença grave, reconhecida pela Organização Mundial de Saúde (OMS), que repercute em grande mobimortalidade dos pacientes (PIEL et al., 2013). A anemia falciforme (AF) é a manifestação mais grave da doença, em que indivíduos apresentam a mutação em ambos genes codificantes da cadeia β da hemoglobina que promovem a codificação de uma hemoglobina (Hb) variante denominada HbS (ZAGO e PINTO, 2007; STEINBERG, 2008). Quando se apresenta em estado desoxigenado (desoxi-HbS), a HbS polimeriza resultando no fenômeno de falcização. A interação da HbS com outras variantes de Hb ou talassemia caracterizam a DF. Além disso, indivíduos com AF apresentam grande heterogeneidade clínica, sendo as complicações associadas às crises vasoclusivas (CVOs) e hemolíticas frequentes na doença (ODENHEIMER et al., 1983; NOURAIE et al., 2013).

Durante as crises hemolíticas na AF muitos produtos são liberados no microambiente vascular, como a Hb e o heme, que contribuem para a inflamação crônica e o desenvolvimento das CVOs. Esses produtos de hemólise atuam em diferentes mecanismos, por exemplo, ativando células do sistema imune via receptor do tipo Toll 4 (TLR4, *Toll-like receptor 4*) e via receptor do tipo NOD contendo a proteína 3 (*NOD-like receptor* contendo a proteína 3 (NLRP3) ativando o inflamassoma, dentre outros (DUTRA et al., 2014; DUTRA e BOZZA, 2014; GLADWIN e OFORI-ACQUAH, 2014). Deste modo, a liberação contínua dos produtos de hemólise na AF e arginase, promovem a diminuição da biodisponibilidade de óxido nítrico (NO, *nitric oxide*) repercutindo em disfunção endotelial (ROTHER et al., 2005; STEINBERG, 2008). Além disso, a ativação endotelial observada na AF contribui para o aumento das interações adesivas de leucócitos, plaquetas e hemácias gerando vasoclusão (SETTY e STUART, 1996; SPACE et al., 2000; SOLOVEY et al., 2001; MORRIS et al., 2005; MACK e KATO, 2006; WOOD et al., 2006; VILAS-BOAS et al., 2010).

As CVOs e hemolíticas seguidas por lesões por isquemia-reperfusão aumentam o estresse oxidativo no microambiente vascular gerando inflamação crônica (STUART e NAGEL, 2004; BELCHER et al., 2010a; CHIRICO e PIALOUX, 2012). Deste modo, sistemas enzimáticos antioxidantes como superóxido dismutase (SOD), catalase (CAT) e peroxidase (GPx) assumem importante papel para o

estabelecimento da homeostasia oxidativa (IGHODARO e AKINLOYE, 2017). A heme-oxigenase 1 (HO-1) também possui papel fundamental em resposta ao estresse oxidativo induzido pela hemólise, atuando na degradação do heme liberado a partir da Hb livre (NATH et al., 2001; OWUSU et al., 2013).

A hidroxiureia (HU) é a principal droga utilizada no tratamento da DF (WARE, 2010; NZOUAKOU et al., 2011; AGRAWAL et al., 2014; WONG et al., 2014). Em linhas gerais, a HU atua na indução de hemoglobina fetal (HbF), aumentando a biodisponibilidade de NO, e diminuindo a liberação de leucócitos e monócitos na circulação sanguínea (COKIC et al., 2003).

Embora os benefícios terapêuticos sejam claros, poucos estudos têm dado um foco distinto à ação da HU em mecanismos alternativos que ampliem o campo do conhecimento sobre a sua ação e os seus efeitos sistêmicos em doenças hemolíticas, especialmente na AF. Deste modo, tendo em vista a cronicidade do processo inflamatório e a ampla utilização da HU como modalidade terapêutica da AF, novos estudos são importantes para ampliar a compreensão acerca dos mecanismos e efeitos da HU em diferentes vias, visando a elucidação de novos alvos e perspectivas terapêuticas.

2. REFERENCIAL TEÓRICO

2.1 EPIDEMIOLOGIA

Estima-se cerca de 300.000 nascimentos anuais com AF, sendo a Nigéria, República Democrática do Congo e Índia, os países com maior incidência da doença (PIEL et al., 2013). A prevalência da doença é alta em muitas áreas da África Subsaariana, bacia do Mediterrâneo, Oriente Médio e Índia (FIGURA 1). Além disso, acredita-se que a DF está presente em cerca de 7% da população mundial, com prevalência elevada nos países da África, América do Sul, América Central, Arábia Saudita e Índia (WEATHERALL e CLEGG, 2001). No entanto, a grande dispersão intercontinental do alelo β^S se deu em virtude do intenso tráfico de escravos no passado, e pelos intensos movimentos populacionais contemporâneos (PIEL et al., 2014). Além disso, acredita-se que o alelo β^S em heterozigose (traço) se estabeleceu por conferir nível de proteção contra a malária, muito comum nessas regiões, embora essa hipótese seja controversa (HASSELL, 2010; PIEL et al., 2014).

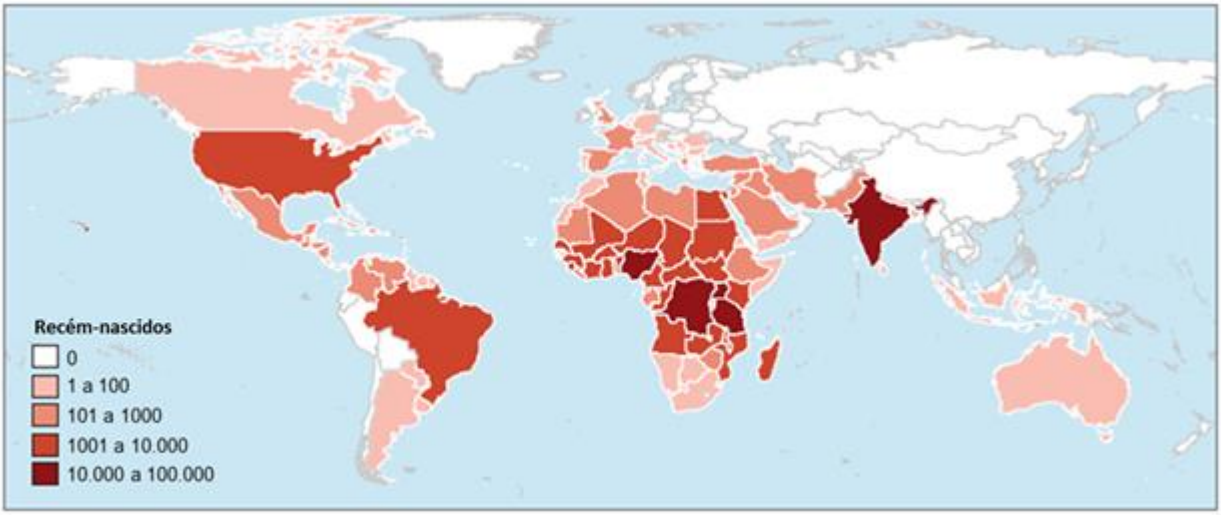

FIGURA 1. Distribuição mundial de recém-nascidos com anemia falciforme em 2015. Os dados são baseados em estimativas de Piel e colaboradores (2013) [Adaptado de PIEL et al., (2017)].

Devido a preocupação decorrente da grande morbimortalidade da DF, o Ministério da Saúde (MS) instituiu em 2001 o Programa Nacional de Triagem Neonatal (PNTN) – Portaria GM/MS nº 822, de 6 de junho de 2001 – onde a DF passou a fazer parte das doenças investigadas no “teste do pezinho”. O objetivo consiste em identificar precocemente distúrbios e doenças do recém-nascido,

proporcionando intervenção adequada e acompanhamento contínuo, visando a redução de mobimortalidade e a melhoria da qualidade de vida desses pacientes (BRASIL, 2001).

No Brasil, os dados epidemiológicos para DF não são recentes, mas estima-se que cerca de dois milhões de pessoas são portadoras do alelo β^S. Além disso, a distribuição deste alelo é bastante heterogênea (TABELA 1), apresentando maior prevalência nas regiões Norte e Nordeste (CANÇADO e JESUS, 2007; SIMÕES et al., 2010), sendo a Bahia o estado brasileiro com a maior incidência da doença (1:650 nascidos vivos).

TABELA 1. Proporção de nascidos vivos diagnosticados com doença falciforme e traço falciforme pelo Programa Nacional de Triagem Neonatal (PNTN)

Estados	Doença falciforme	Traço falciforme
	Proporção/ Nascidos Vivos	Proporção/ Nascidos Vivos
Bahia	1: 650	1: 17
Rio de Janeiro	1: 1.200	1: 21
Pernambuco	1: 1.400	1: 23
Maranhão	1: 1.400	1: 23
Minas Gerais	1: 1.400	1: 23
Goiás	1: 1.400	1: 25
Espírito Santo	1: 1.800	1: 25
São Paulo	1: 4.000	1: 35
Mato Grosso do Sul	1: 5.850	----
Rio Grande do Sul	1: 11.000	1: 65
Santa Catarina	1: 13.500	1: 65
Paraná	1: 13.500	1: 65

Fonte: Adaptado de Simões et al., (2010)

Em estudo retrospectivo realizado por Amorim e colaboradores (2010), foram analisados os resultados de exames do banco de dados do Serviço de Referência em Triagem Neonatal (SRTN) da Associação de Pais e Amigos dos Excepcionais de Salvador-Bahia (APAE-Salvador-Bahia) entre 2007 e 2009, com identificação de casos com DF. Os dados mostraram a incidência de 1:601 para DF e de 1:1435 para AF, sendo que 41,9% possuíam o fenótipo HbSS (AF), e 34,7% o fenótipo HbSC. Os maiores números de casos foram encontrados em Salvador (23,2%), seguidos de Feira de Santana (4,6%), Lauro de Freitas (2,2%), Camaçari (1,9%) e Valença (1,8%), que tornaram a Bahia o estado com a maior prevalência da DF falciforme no país.

2.2 ANEMIA FALCIFORME: MUTAÇÃO DE PONTO SIMPLES ASSOCIADA A PATOLOGIA HEREDITÁRIA COMPLEXA

A AF possui herança autossômica recessiva, cuja alteração está associada à presença de Hb variante S (HbS ou Hbβ^S), decorrente da mutação de ponto G<u>A</u>G>G<u>T</u>G, localizada na sexta posição do gene que codifica a cadeia beta da globina (*HBB*). Esta mutação promove a substituição do ácido glutâmico pela valina (glu6val). A AF é considerada a manifestação mais grave da doença por apresentar mutação em ambos genes codificantes β (HbSS) (ZAGO e PINTO, 2007; STEINBERG, 2008). Quando submetida a condições de hipoxemia, a HbS torna-se insolúvel e sofre polimerização resultando no fenômeno de falcização que está diretamente relacionado a concentração de HbS no microambiente intraeritrocitário. A HbS também pode interagir com outros tipos variantes de Hb (HbSC, HbSD e HbSE) ou talassemias (HbS/β^0, HbS/β^+ ou HbS/α), caracterizando outros tipos de DF (STEINBERG e RODGERS, 2001; ZAGO e PINTO, 2007).

Os cinco principais haplótipos ligados ao grupo de genes da globina β^s têm sido descritos como modificadores genéticos de risco de gravidade da DF. Eles foram inicialmente descritos em populações provenientes de países ou áreas do continente africano, ou próximos a este; por esse motivo recebem a nomeação de acordo com os seus locais de origem: Benin (BEN), República Centro-Africana (CAR), Senegal (SEN), Camarões (CAMER), Arábia Saudita e Índia (SAUD) (NAGEL e RANNEY, 1990; GALIZA NETO e PITOMBEIRA, 2003). A gravidade desses haplótipos está diretamente associada à expressão da HbF, cuja expressão está elevada no período embrionário. A HbF apresenta como particularidade a afinidade alta por oxigênio, impedindo a iniciação da cascata de polimerização da HbS e, consequentemente, as CVOs/hemolíticas subsequentes. Embora o processo natural de desenvolvimento da Hb curse com a substituição de HbF por HbA, concentrações residuais de HbF (até 2%) podem ser encontradas em indivíduos normais. Além disso, a persistência hereditária da HbF (PHHF) pode ocorrer, sendo caracterizada pela síntese contínua da HbF (até 30%) na vida adulta, conferindo aos pacientes grandes benefícios e um prognóstico clínico mais favorável (STEINBERG, 2005; SANKARAN; ORKIN, 2013). Deste modo, os haplótipos podem atuar como moduladores genéticos do gene codificante da globina gama (γ^G) (*HBG*) resultando em aumento na expressão de HbF, além de regular negativamente a substituição da

HbF por HbS. Essa regulação contribui, pelo menos em parte, para a persistência de HbF observada nos haplótipos SEN e SAUD. Por outro lado, os haplótipos CAR e BEN têm sido associados à gravidade fenotípica da doença, onde indivíduos com o genótipo CAR/CAR apresentam pior prognóstico clínico por apresentar níveis reduzidos de HbF (NAGEL, 1984; NAGEL et al., 1987; GREEN et al., 1993; POWARS e HITI, 1993; GALIZA NETO e PITOMBEIRA, 2003; PIEL; STEINBERG; REES, 2017). A Figura 2 apresenta a mudança no perfil de expressão das cadeias de globina com substituição gradual da γ^G ao longo do desenvolvimento embrionário pela globina β, dando origem a HbA da fase adulta.

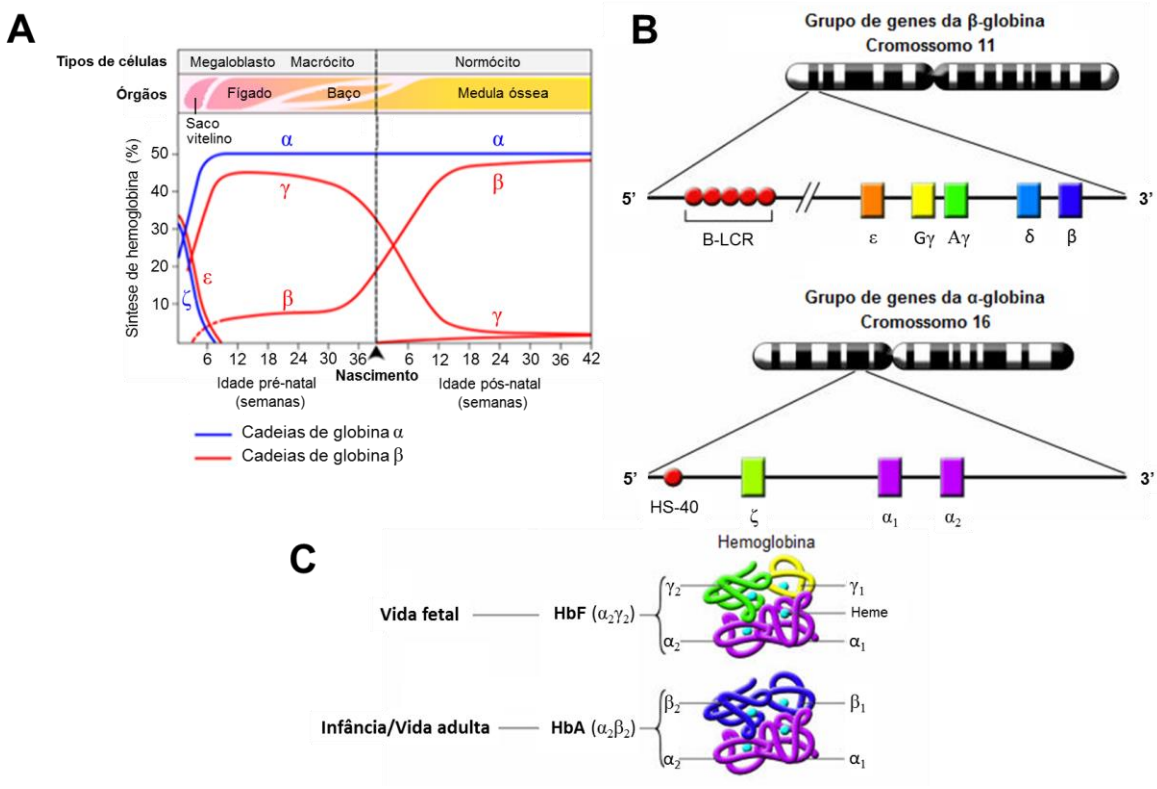

FIGURA 2. Modificação do perfil de hemoglobinas embrionárias para a hemoglobina adulta. A) A expressão de cadeias embrionárias relacionadas a cadeia de globina α, decrescem exponencialmente ao longo das oito primeiras semanas do período gestacional, sendo substituídas pela globina α adulta [Adaptado de Wood (1976)]. Em contraste, moduladores genéticos promovem mais tardiamente a substituição das cadeias globina γ por β. **B)** Representação da região estimuladora dos *loci* das globinas α e β, conhecida como a região de controle de *locus* (LCR), e os sítios de hipersensibilidade (HS) correspondentes. **C)** Representação da estrutura quaternária de HbA e HbF. As ilustrações "B" e "C" foram adaptadas de Bank (2005).

Quando o eritrócito é submetido a condições de baixa tensão de oxigênio (hipóxia) a $Hb\beta^s$ (desoxi-HbS) torna-se insolúvel e sofre polimerização que resulta no alongamento e enrijecimento eritrócitário, um processo denominado de falcização. Esta instabilidade molecular apresentada pela HbS é decorrente da substituição de

um aminoácido de natureza polar (ácido glutâmico, glu) por um aminoácido de natureza apolar (valina, val) diminuindo a solubilidade da Hb no ambiente intraeritrocitário, e aumentando a formação de agregados com a Hb adjacente [fenilalanina (β-85) e leucina (β-88)] (FIGURA 3). O processo de iniciação da cascata de polimerização é denominado nucleação ou gelificação (*gelation*) (EATON; HOFRICHTER, 1987; MOZZARELLI; HOFRICHTER; EATON, 1987; BALLAS e MOHANDAS, 1996). Essas interações hidrofóbicas entre os filamentos de Hb favorecem o aumento da viscosidade citosólica com formação de estruturas multipoliméricas no interior dos eritrócitos e promovem deformação, enrijecimento e diminuição da flexibilidade, tornando-os mais suceptíveis à destruição mecânica dentro do sistema vascular (EVANS e MOHANDAS, 1987; MESSMANN et al., 1990). Por outro lado, na presença do oxigênio a HbS (oxi-HbS) encontra-se solúvel no citosol eritrocitário, apresentando certa estabilidade. Deste modo, a falcização consiste em um fenômeno transitório onde o eritrócito alterna períodos de polimerização (estado desoxi-HbS) e despolimerização de HbS (oxi-HbS).

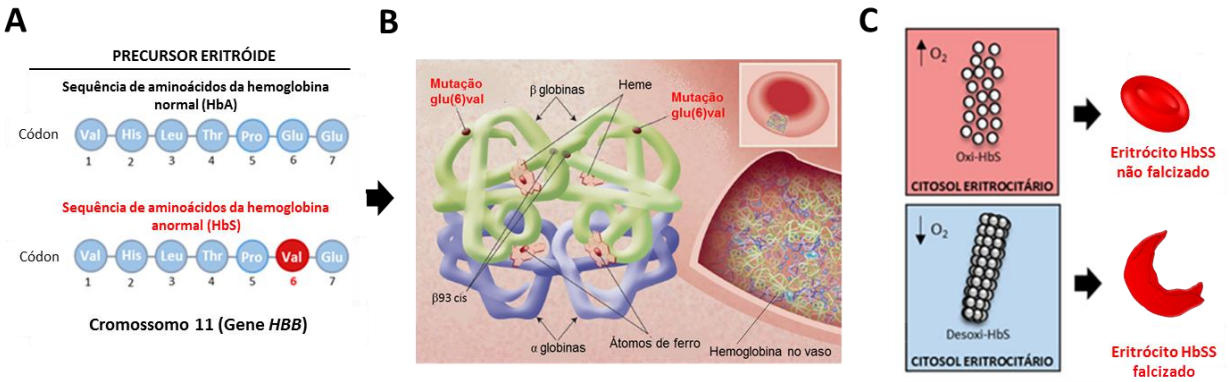

FIGURA 3. Representação esquemática do fenômeno de falcização da hemoglobina em indivíduos com hemoglobina HbS. A) A mutação no sexto códon do gene beta localizado no cromossomo 11, é responsável pela codificação da hemoglogina S (HbS) por substituição do ácido glutâmico por valina (glu6val). **B)** Representação da estrutura tetramérica de HbS proveniente da mutação na cadeia de β-globinas. A molécula é composta por duas cadeias de α (α_2) e β (β_2) globinas e quatro grupos prostéticos heme, responsáveis pela fixação de moléculas de oxigênio (O_2). A ilustração da Hb contida em "B" foi adaptada de Schechter (2008). **C)** Em condições de oxigenação, a HbS é estabilizada pelo oxigênio (oxi-HbS) e não inicia a cascata de polimerização. Por outro lado, em condições de hipóxia (desoxi-HbS), a hemoglobina apresenta alta instabilidade, perde a solubilidade no microambiente intraeritrocitário e inicia o processo de nucleação (ou gelificação) dando origem ao eritrócito falcizado. As ilustrações "A" e "C" são de autoria própria.

Eritrócitos falcizados apresentam desidratação e acidose citosólica intensas, mediadas pelo efluxo anormal de íons potássio (K^+) por duas vias: *(i)* co-transporte de cloreto de potássio (K^+-Cl^-); *(ii)* e pelo canal de efluxo de potássio ativado por cálcio (canal de Gardos) (BALLAS e MOHANDAS, 1996; FRANCO et al., 2000). A elevação das concentrações intracelulares de cálcio (Ca^{2+}) observada na DF

promove a inativação da bomba cálcio/ATPase, repercutindo no aumento da concentração da Hb corpuscular média (CHCM) da desoxi-HbS (FIGURA 3) (NOGUCHI e SCHECHTER, 1981; FRENETTE e ATWEH, 2007). Os eventos de falcização/desfalcização consecutivos aliados ao intenso dano oxidativo existente, provocam lesões graduais e cumulativas na membrana eritrocitária, aumentam a sua fragilidade, e impossibilitam o retorno do eritrócito para a sua morfologia normal (discóide) (PADILLA; BROMBERG; JENSEN, 1973; RODGERS e NOGUCHI; SCHECHTER, 1985; FRENETTE e ATWEH, 2007).

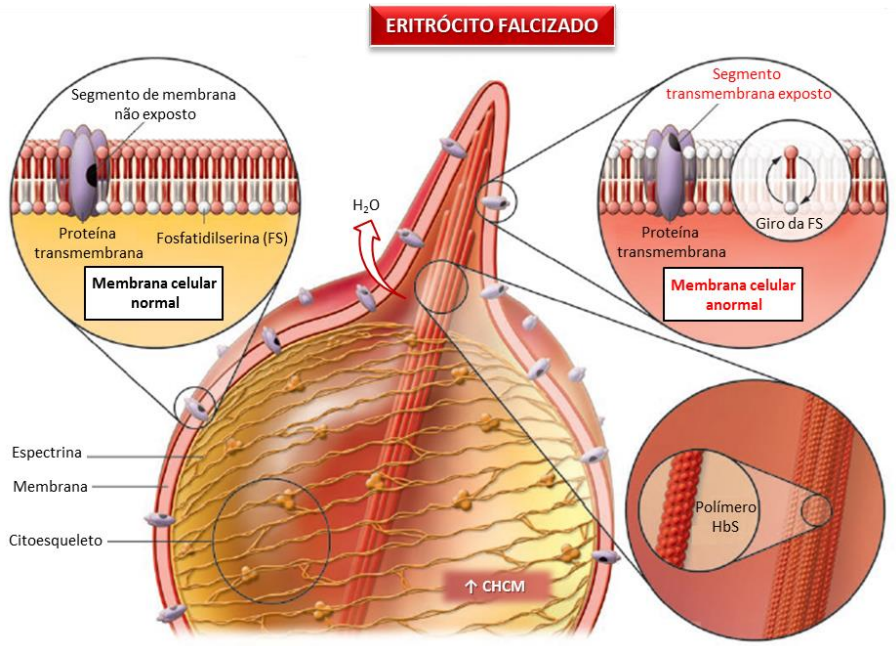

FIGURA 4. Alterações estruturais dos eritrócitos por polímeros de HbS. A desoxigenação da HbS induz uma alteração na conformação do eritrócito, na qual a cadeia mutante se liga a um sítio hidrofóbico complementar resultante da substituição do ácido glutâmico pela valina (Glu6Val), levando à formação de um polímero de hemoglobina (polímero de Hb). Os polímeros de Hb interferem na organização do citoesqueleto eritrocitário dando origem à morfologia característica de foice. Essas alterações resultam na exposição de epítopos de proteínas transmembrana e alterações em segmentos lipídicos, como a fosfatidilserina (FS). A exposição de glicolipídios carregados negativamente contribui para o estado pró-inflamatório e pró-trombótico na AF [Adaptado de Frenette e Atweh (2007)].

A taxa de polimerização está correlacionada com a concentração de HbS e com o heme livre, liberados dos eritrócitos após sua auto-oxidação (BUNN et al., 1982; UZUNOVA et al., 2010). Além disso, a fase de reoxigenação dos eritrócitos é uma das principais fontes de produção de radicais livres na DF mas, durante este período, os eritrócitos normais também podem gerar uma quantidade significativa de superóxido ($O_2^{\bullet-}$) devido às reações de transferência de elétrons entre o ferro ferroso (Fe^{2+}) do heme e o oxigênio. Na presença de oxigênio, o heme auto-oxida formando metemoglobina[1] (metHb-Fe^{3+}) e $O_2^{\bullet-}$. Alguns estudos mostram que a HbS pode se

[1] Metemoglobina – é uma forma da Hb em que o ferro no grupo prostético heme está no estado Fe^{3+} e não no Fe^{2+} da Hb normal. A metemoglobina é incapaz de se ligar ao oxigênio.

auto-oxidar 1,7 vezes mais rápido que a HbA, enquanto outros mostram uma taxa equivalente (HEBBEL et al., 1988; SHENG; SHARIFF; HEBBEL, 1998; WOOD; HSU; GLADWIN, 2008). A formação de peróxido de hidrogênio (H_2O_2), quando exposta à metHb, decompõe a Hb e libera ferro férrico (Fe^{3+}). Este ferro pode então reagir com o H_2O_2 remanescente para produzir mais radicais hidroxila ($^{\bullet}OH$), o mais reativo e prejudicial das espécies reativas de oxigênio (EROs) (ASLAN; THORNLEY-BROWN; FREEMAN, 2000). Os eritrócitos falciformes acabam gerando cerca de duas vezes mais $O_2^{\bullet-}$, H_2O_2 e $^{\bullet}OH$ do que os eritrócitos normais (AMER et al., 2006). Essa grande perturbação na fisiologia normal do eritrócito gerada pela presença da HbS, reduz cerca de cinco vezes a sua meia-vida em comparação com eritrócitos normais (em média 120 dias) (MCCURDY e SHERMAN, 1978; WOOD; HSU; GLADWIN, 2008). Além disso, a meia-vida reduzida estimula eritropoiese em um mecanismo compensatório e faz com que reticulócitos imaturos sejam liberados (reticulocitose) da medula para suprir o déficit de eritrócitos na circulação.

O controle de destruição de eritrócitos envelhecidos e não-funcionais ocorre, em sua maioria, por sequestro esplênico, embora também possa ocorrer em outros sítios (p. ex., fígado) por intermédio das células do sistema fagocitário mononuclear (ou sistema reticulo-endotelial – SRE). Sucessivos eventos de falcização acarretam em danos irreversíveis à membrana eritrocitária com exposição de proteínas estruturais importantes como a banda-3, espectrina, fosfatidilserina e a glicoproteína IV (CD36). Deste modo, cerca de 2/3 dos eritrócitos falciformes são retirados da circulação pela via esplênica (hemólise extravascular) por sistema de reconhecimento autólogo; e 1/3 por hemólise intravascular, liberando grande quantidade de arginase, Hb/heme e ferro livre (MORRIS et al., 2008; WOOD; HSU; GLADWIN, 2008). A hemólise excessiva gera grande sobrecarga esplênica provocando lesões repetitivas por hipóxia/isquemia que conduzem à fibrose seguida de destruição do baço (auto-esplenectomia) (ZAGO e PINTO, 2007). Este microambiente crítico é determinante para o desenvolvimento da disfunção endotelial que será tratada mais adiante. A figura 4 resume o que foi tratado até aqui, ilustrando a importância da HbS na falcização como evento primário desencadeador da doença, apresentando como desfechos críticos as CVOs e hemolíticas. Note que estas vias fisiopatológicas estão estreitamente relacionadas.

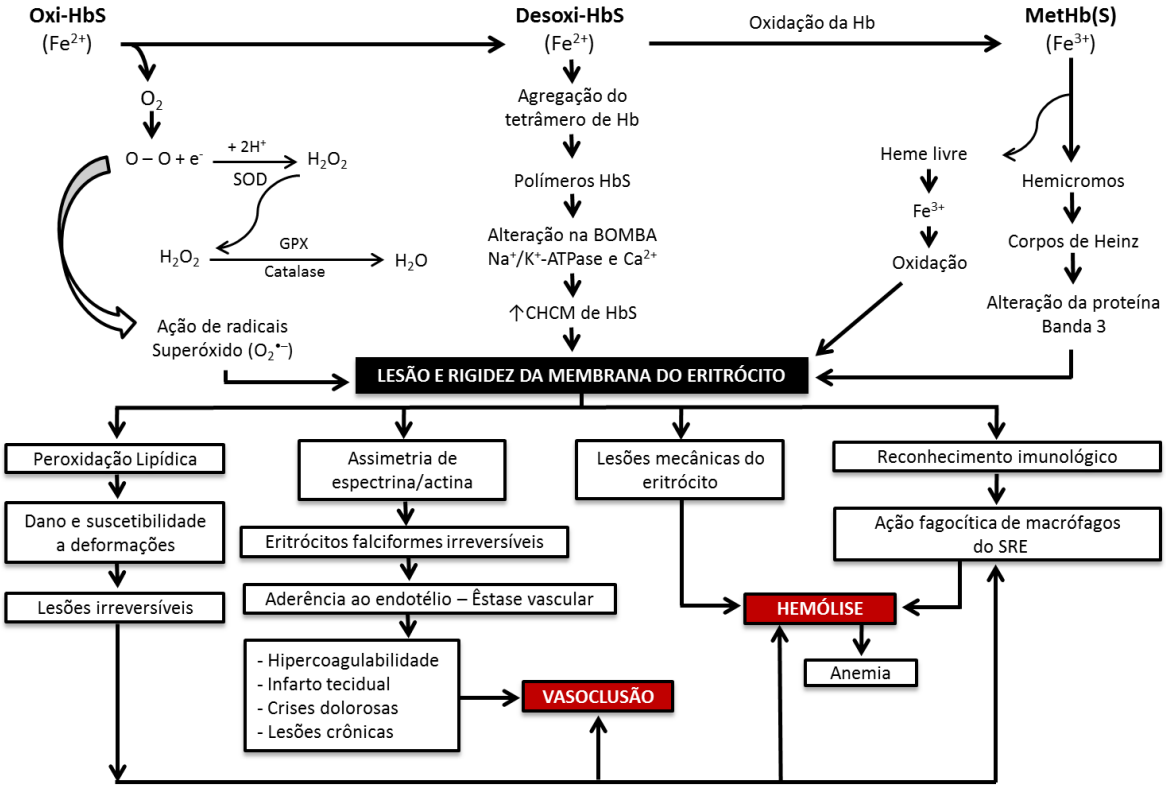

FIGURA 5. Vias fisiopatológicas da anemia falciforme. A geração de espécies reativas de oxigênio (EROs) ocorre após as reações de oxido-redução entre a hemoglobina e oxigênio durante a oxigenação dos tecidos. A liberação do O_2 nos tecidos torna-o suscetível ao ataque de elétrons livres (e^-) com formação de íons superóxido ($O_2^{\bullet-}$) e peróxido de hidrogênio (H_2O_2), estimulação de resposta enzimática antioxidante por reações específicas altamente coordenadas. A superóxido dismutase (SOD) atua na dismutação de $O_2^{\bullet-}$ em O_2 e H_2O_2; e as peroxidases catalase (CAT) e glutationa peroxidase (GPx) atuam na decomposição do H_2O_2 em água (H_2O) e O_2. Outras enzimas contribuem indiretamente para o homeostase dos sistemas antioxidantes, mas não estão demonstradas aqui. Após a liberação do O_2, a HbS desoxigenada (desoxi-HbS) pode sofrer auto-oxidação formando metemoglobina S (MetHbS) liberando heme livre e ferro férrico (Fe^{3+}), participando de diversas reações oxidativas como a reação de Fenton, desnaturação de hemoglobina (corpos de Heinz) e alterações em proteínas estruturais de membrana como a banda 3. Além disso, essas alterações promovem aumento da abertura de canais de sódio e potássio-ATPase (Na^+/K^+-ATPase) dependentes (canais de Gardos) ou não dependentes de cálcio (Ca^{2+}), gerando acidose intraeritrocitária, desidratação e aumento da concentração da hemoglobina corpuscular média (CHCM). O aumento da concentração de HbS intefere no arranjo de proteínas do citoesqueleto, gerando danos na membrana, enrijecimento e alteração da morfologia discóide típica para a forma de foice. Como consequência, esses eritrócitos tornam-se sucetíveis a lise por *(i)* lipoperoxidação, *(ii)* lesões mecânicas ao percorrer leitos vasculares, e *(iii)* autofagia por ação do sistema reticuloendotelial (SRE) ou sistema mononuclear fagocitário no baço ou fígado, removendo eritrócitos não-funcionais da circulação. Além disso, eritrócitos falciformes podem contribuir para a êstase vascular gerando vasoclusão com alterações da hemostasia sanguínea, crises álgicas agudas e lesões por hipóxia e/ou isquemia [Adaptado de Naoum e Naoum (2004)].

A fisiopatologia da DF apresenta grande heterogeneidade clínica (ODENHEIMER et al., 1983; NOURAIE et al., 2013). Dentre as complicações pode-se destacar (FIGURA 6): anemia crônica, complicações hepatobiliares e renais, episódios dolorosos agudos, propensão à infecções (devido ao comprometimento esplênico e redução da capacidade de opsonização de bactérias), complicações

neurológicas, pulmonares, oftálmicas, ósseas (como osteonecrose), retinopatia, ulceração de membros inferiores, e alterações do sistema reprodutor masculino (decorrente do priapismo) (STEINBERG, 2008; MINNITI et al., 2010; BRODERICK, 2012; DA SILVA JUNIOR; DAHER; DA ROCHA, 2012; LIONNET et al., 2012; MILLER e GLADWIN, 2012; BONANOMI e LAVEZZO, 2013; TALAHMA; STRBIAN e SUNDARARAJAN, 2014; PIEL; STEINBERG; REES, 2017).

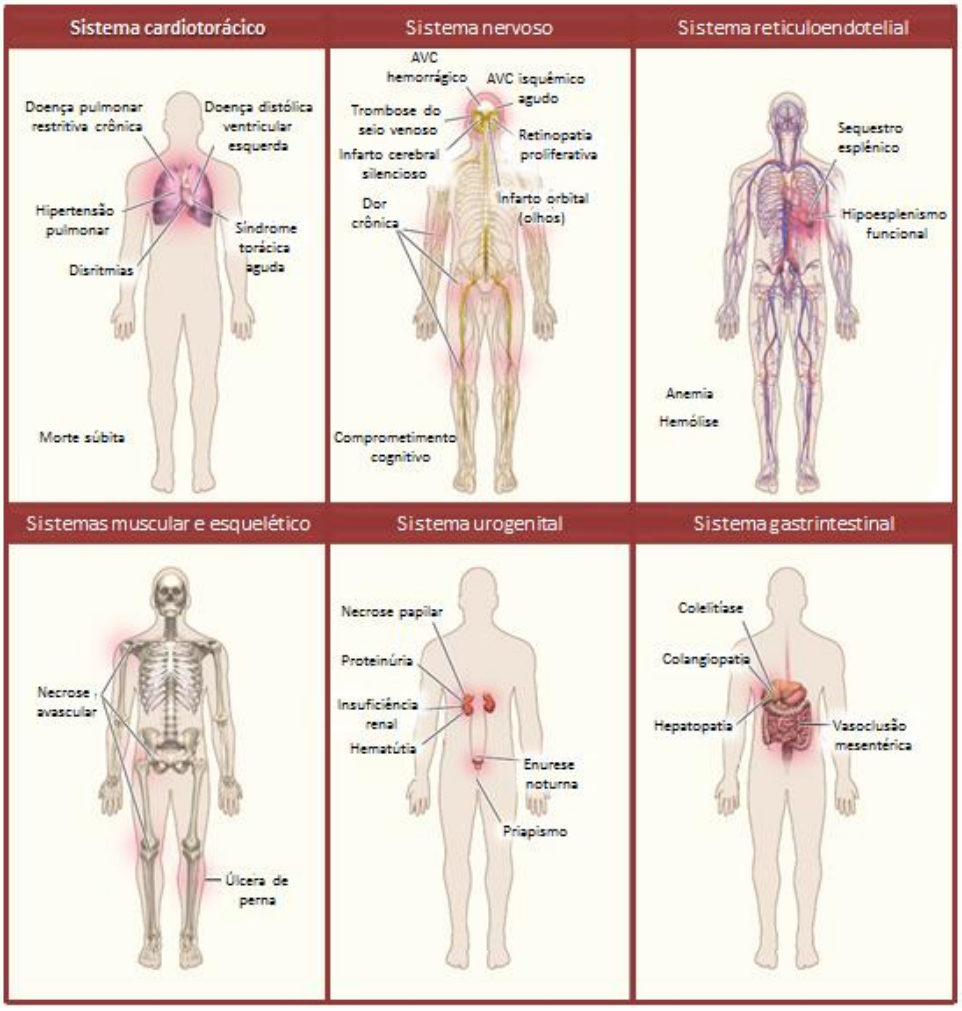

FIGURA 6. Complicações clínicas comuns da doença falciforme. As complicações são provenientes dos eventos hemolíticos e/ou vasoclusivos da doença acometendo os sistemas: *(i)* cardiotorácico – complicações cardíacas e pulmonares como hipertensão pulmonar e síndrome torácica aguda (STA) que pode levar os pacientes a óbito; *(ii)* nervoso central – danos ao sistema nervoso central e transtornos cognitivos como o acidente vascular cerebral (AVC) e a retinopatia; *(iii)* reticuloendotelial – provenientes dos frequentes eventos hemolíticos, gerando comprometimento imunológico e susceptibilidade aumentada a infecções; *(iv)* muscular e esquelético – proveniente dos eventos obstrutivos hipoxêmicos/isquêmicos; *(v)* urogenital – gerando danos renais e ao sistema reprodutor masculino; *(vi)* gastrintestinal – gerando danos hepáticos e obstrução de vasos mesentéricos no intestino [Adaptado de Piel et al., (2017)].

2.3 HEMÓLISE, VASOCLUSÃO E AS SUAS REPERCUSSÕES NA AF

2.3.1 **A importância da hemólise e do heme livre na fisiopatologia da AF**

A Hb livre e o heme são produtos liberados constantemente nas crises hemolíticas e exercem papel central no desencadeamento dos eventos vasoclusivos e da inflamação crônica observados na doença. No microambiente vascular a Hb livre apresenta-se em conformações tetraméricas ($\alpha_2\beta_2$) ou diméricas ($\alpha\beta$), sendo esta última a menos abundante. Devido ao seu tamanho e baixo peso molecular (34 kDa), os dímeros de Hb são capazes de permear pequenos vasos gerando dano tecidual em órgãos mais suceptíveis (p. ex., os rins) (SCHAER et al., 2013). A Hb no seu estado ferroso (Hb-Fe^{2+}) pode sofrer reações de auto-oxidação por EROs (p. ex., H_2O_2) formando Hb-férrica (Hb-Fe^{3+}) ou Hb-ferril (Hb-Fe^{4+}) que são altamente oxidantes (MILLER; ALTAMENTOVA; SHAKLAI, 1997; JIA et al., 2007; GLADWIN; KANIAS; KIM-SHAPIRO, 2012). Além disso, a Hb pode atuar diminuindo a biodisponibilidade de NO no sistema vascular de duas formas: *(i)* por interação direta da desoxi-Hb com o NO com formação de Hb-Fe^{3+}NO; e *(ii)* pela interação da oxi-Hb com NO após reação de dioxigenação gerando Hb-Fe^{3+} e nitrato (NO_3^-) (STEINBERG, 2008; CHIRICO e PIALOUX, 2012).

Na hemólise, também ocorre oxidação da Hb(Fe^{2+}) com formação de metHb-Fe^{3+} promovendo a liberação de heme (UMBREIT, 2007; UZUNOVA et al., 2010). A molécula de heme é formada por um grupamento de seis aneis heterocíclicos (sendo quatro pirrólicos), denominado protoporfirina IX, contendo uma molécula de ferro central no estado ferroso (II) (KUMAR e BANDYOPADHYAY, 2005). O anel porfirínico confere propriedades apolares a molécula, possibilitando que o heme atue diretamente como intercalante na bicamada lipídica gerando lipotoxicidade, oxidando proteínas e danificando DNA (AFT e MUELLER, 1983; VINCENT, 1989; BALLA et al., 1993; CARLSEN; MØLLER; SKIBSTED, 2005). Além disso, a presença do Fe^{3+} confere toxicidade adicional ao heme aumentando a suscetibilidade desses lipídios a danos oxidativos mediados por reações de Fenton e Haber-Weiss, que envolvem Fe^{2+} e H_2O_2 provenientes de células ativadas com formação de $^{\bullet}OH$ (VINCENT, 1989; BALLA et al., 1991; BALLA et al., 1993; CHIRICO e PIALOUX, 2012; GLADWIN; KANIAS; KIM-SHAPIRO, 2012). Essas reações promovem dano tecidual

e amplificam a inflamação (BALLA et al., 1991; BALLA et al., 2005; LUGRIN et al., 2014).

Por outro lado, algumas proteínas sintetizadas no fígado desempenham papel importante na captação de Hb, heme e Fe^{3+} livres, diminuindo os efeitos oxidantes e inflamatórios dessas moléculas no microambiente vascular. A haptoglobina (Hp) exerce papel importante de defesa após eventos de hemólise intravascular, captando a Hb e dirigindo-a para degradação e reciclagem do ferro (KRISTIANSEN et al., 2001; BELCHER et al., 2010a; ALAM; DEVALARAJA e HALDAR, 2017). O complexo Hb-Hp é reconhecido pelo receptor CD163, de alta afinidade, que está presente na superfície de monócitos ou macrófagos situados no fígado ou baço (KRISTIANSEN et al., 2001; ROTHER et al., 2005). Estes fenômenos constituem-se em via importante de internalização e reciclagem de ferro em macrófagos teciduais, e também contribuem para a indução da hemeoxigenase (HO-1) (SCHAER et al., 2006; BUEHLER et al., 2009).

A HO-1 é a isoforma induzível pelo heme sendo expressa nos tecidos em resposta ao estresse oxidativo, e está elevada na AF em decorrência da hemólise excessiva característica da doença (NATH et al., 2001; OWUSU et al., 2013). Atua degradando o heme da Hb livre para formar biliverdina/bilirrubina e monóxido de carbono (CO). A bilirrubina é um poderoso antioxidante celular; já o CO exerce efeitos anti-inflamatórios e anti-apoptóticos, dependentes da via de sinalização da proteína quinase ativada pelo mitógeno p38 (MAPK) (RYTER et al., 2002; WU; WANG, 2005; BAINS et al., 2010; ZIBERNA et al., 2016). A HO-1 possui papel protetor importante contra os efeitos deletérios decorrentes da sobrecarga do heme liberado na hemólise, minimizando os efeitos provocados pelos danos por isquemia-reperfusão nas CVOs (OTTERBEIN et al., 2003). Além disso, estudos em modelo murino para AF demonstraram que a HO-1 pode atuar inibindo NF-kB, VCAM-1 e ICAM-1, modulando negativamente a ativação endotelial e a interação de leucócitos com o endotélio, após a estase induzida pela hipóxia/reperfusão (BELCHER et al., 2010a; BELCHER et al., 2010b).

A hemopexina (Hpx) é uma glicoproteína de fase aguda que também possui alta afinidade pelo heme livre no plasma, atuando na sua captação após reconhecimento do complexo heme-Hpx pelo receptor CD91 presente nos hepatócitos (FOIDART et al., 1983; HVIDBERG et al., 2005). Após o transporte para o fígado, a hemopexina é degradada pela HO-1 microssomal e, o ferro liberado é

rapidamente e captado pela ferritina, cuja expressão é regulada pela presença do complexo heme-Hpx-CD91 (DAVIES et al., 1979; BALLA et al., 2005).

Outros mecanismos de defesa descritos são as lipoproteínas séricas de alta (HDL, *high-density lipoprotein*) e baixa densidade (LDL, *low-density lipoprotein*), que podem se ligar ao heme eliminando-o da circulação sanguínea (BALLA et al., 1991; MILLER; ALTAMENTOVA; SHAKLAI, 1997; FASANO et al., 2002; GRINSHTEIN et al., 2003). No entanto, em microambientes altamente oxidantes, essas lipoproteínas podem sofrer oxidação formando produtos lipotóxicos altamente reativos como LDL oxidado (oxLDL) e HDL pró-inflamatório (p-HDL), induzindo inflamação e dano vascular (BALLA et al., 1991; JENEY et al., 2002; NAGY et al., 2010; OZTURK et al., 2013; ATAGA et al., 2015; SOUPENE et al., 2016).

Embora essas proteínas desempenhem papel antioxidante e anti-inflamatório importante, a hemólise constante na AF repercute na diminuição dos níveis plasmáticos dessas proteínas (ROTHER et al., 2005). Diante dessa resposta insuficiente, os produtos de hemólise podem ser reconhecidos por células endoteliais e leucócitos contribuindo para a resposta inflamatória sistêmica.

Os receptores do tipo Toll (TLR, *Toll-like receptor*) fazem parte do grande repertório de receptores presentes em células do sistema imune inato, atuando no reconhecimento de padrões moleculares associados a patógenos (PAMPs*, Pathogen-associated molecular*) ou padrões moleculares associados ao dano (DAMPs, *Damage-associated molecular patterns*). Estes receptores podem estar distribuídos na superfície ou em compartimentos intracelulares como endossomos e lisossomos (TAKEUCHI e AKIRA, 2010). Na hemólise, vários produtos endógenos provenientes de hemácias, células endotelials, leucócitos, são liberados no microambiente vascular e reconhecidos por TLRs como sinal de dano (DAMPs) defragrando a resposta inflamatória. Alguns exemplos de DAMPs são: adenosina monofosfato (AMP, *adenosine monophosphate*), adenosina trifosfato (ATP, *adenosine triphosphate*), proteína nuclear do grupo de alta mobilidade 1 (HMGB1), oxLDL, DNA (cromossômico ou mitocondrial), componentes da matriz extracelular (tenascina-C, fibronectina e hialuronano), cristais de ácido úrico, cristais de colesterol, entre outros (MARIATHASAN et al., 2006; YU; WANG; CHEN, 2010; OKA et al., 2012).

O heme tem sido descrito como DAMP eritrocitário e a sua liberação excessiva pode promover a vasoclusão e lesão pulmonar aguda por ativação de

TLR4 presente na superfície celular (GHOSH et al., 2013; BELCHER et al., 2014). (GLADWIN e OFORI-ACQUAH, 2014). Estudos demonstam que o heme é capaz de induzir a ativação de macrófagos por *(i)* mecanismo dependente de ligação com TLR4 com ativação de MyD88 (*myeloid differentiation primary response 88*) e TRIF (*TIR-domain-containing adapter-inducing interferon-β*) que promovem a produção de TNF pela via de proteína quinase ativada por mitógeno (MAPK, *Mitogen-activated protein kinase*) (FIGUEIREDO et al., 2007; TAKEUCHI e AKIRA, 2010) ou *(ii)* por mecanismo independente de TLR4, gerando EROs via tirosina quinase do baço (Syk, *Spleen tyrosine kinase*) (NG et al., 2008).

Neutrófilos ativados por heme via TLR4 induzem a formação de EROs e a liberação de armadilhas extracelulares de neutrófilos (NETs, *neutrophil extracelular traps*), ICAM-1, VCAM-1 e E-selectinas, e secreção de citocinas pró-inflamatórias como o TNF (FIGURA 7) (FIGUEIREDO et al., 2007; CHEN et al., 2014). As NETs são formadas por cromatina não condensada com enzimas granulares (como a elastase, catepsina G e mieloperoxidase), proteínas antimicrobianas entre outras (BRINKMANN et al., 2004; URBAN et al., 2009). Em codições normais, NETs apresentam papel importante no combate contra patógenos devido às suas propriedades antimicrobianas (BRINKMANN et al., 2004; URBAN et al., 2009). Entretanto, em condições patológicas, a estimulação excessiva de NETs pode desempenhar papel importante na patogênese de doenças inflamatórias e trombóticas (FUCHS et al., 2010; BORISSOFF e TEN CATE, 2011; BRILL et al., 2012). Foi demonstrado que a produção de NETs por neutrófilos expostos ao TNF-α promove lesão pulmonar em modelos murinos humanizados de DF (CHEN et al., 2014). Adicionalmente, o heme estimula a expressão de fator tecidual (FT) em células endoteliais e monócitos, e os seus níveis elevados têm sido associados com o aumento da coagulabilidade, risco de síndrome torácica aguda (STA) e vasoclusão (SETTY et al., 2008; REHANI et al., 2013; DA GUARDA et al., 2016; DE SOUZA et al., 2017).

Interessantemente, outros estudos demonstraram que o heme inibe a morte celular de neutrófilos por apoptose por mecanismo dependente de fosfoinositídeo 3-quinase (PIK3), MAPK e NF-κB, com geração de EROs por indução de proteínas quinase-C (PKC, *protein kinase-C*) e aumento de secreção de IL-8 (GRACA-SOUZA et al., 2002; ARRUDA et al., 2004). Níveis aumentados de heme também têm sido relacionados com o aumento de citocinas inflamatórias e ativação leucocitária,

sugerindo que o heme contribui para o aumento da longevidade de células inflamatórias e manutenção da inflamação crônica em doenças hemolíticas (HÆGER et al., 1996; GONÇALVES et al., 2001; WUN, 2001; GRACA-SOUZA et al., 2002; CARVALHO et al., 2017).

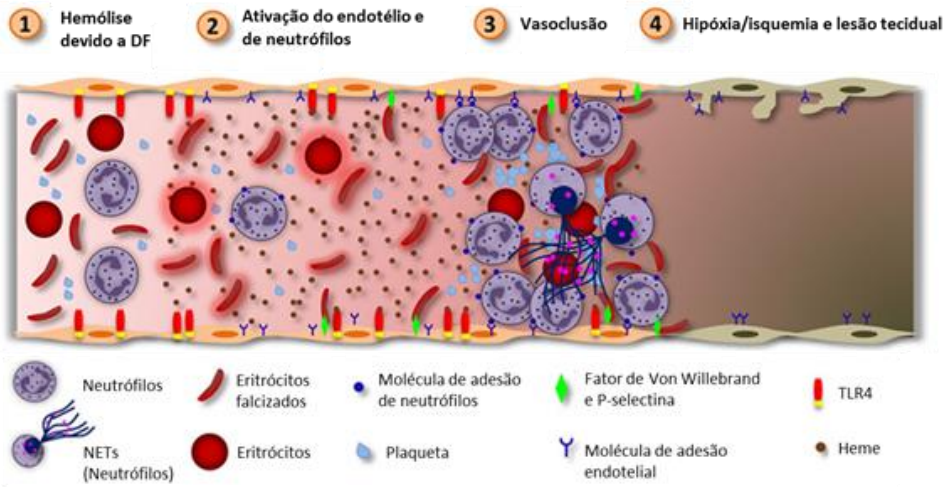

FIGURA 7. O heme induz a inflamação vascular via TLR4 na doença falciforme. (1) Eritrócitos falciformes são mais susceptíveis à lise devido a alterações estruturais no seu citoesqueleto e reações de auto-oxidação. Durante a hemólise intravascular, as proteínas séricas responsáveis pela remoção do heme ficam saturadas e o heme pode excercer seus efeitos inflamatórios. (2) O heme atua ativando neutrófilos e células endoteliais por geração de EROs, promovendo a expressão de moléculas de adesão e conferindo o estado pró-trombogênico ao endotélio vascular. Observa-se aumento da expressão de fator de von Willebrand (FvW), ICAM-1, VCAM-1 e E-selectinas. Os níveis elevados de heme livre também induzem a liberação de NETs por neutrófilos, via TLR4, gerando dano celular. (3) O aumento de moléculas de adesão, aliadas à condição oxidante, favorece a constrição vascular com aumento das interações entre eritrócitos, leucócitos e plaquetas, entre si e com o endotélio, promovendo estase sanguínea e vasoclusão. (4) A depender da extensão da oclusão pode haver comprometimento no aporte de oxigênio/substratos metabólicos (hipóxia/isquemia) gerando crises álgicas agudas e danos teciduais mais graves [Adaptado de Dutra e Bozza (2014)].

Outra via importante que tem sido descrita na AF é a da ativação do inflamassoma (DUTRA et al., 2014; GLADWIN e OFORI-ACQUAH, 2014; LI et al., 2014; KATO; STEINBERG e GLADWIN, 2017). Os DAMPs liberados na hemólise (p. ex., heme, ATP e cristais de ácido úrico) podem ser reconhecidos por um tipo distinto de receptor conhecido como NALP3 (ou NLRP3 – da família dos receptores do tipo NOD), composto por três domínios: domínio de oligomerização NACHT[1]; domínio de repetição rica em leucina (LRR, *leucine-rich repeat*); e um domínio pirina (PYD, *pyrin domain*) contendo a proteína 3 (NALP3) (TING et al., 2008). Após a ativação de NLRP3 ocorre a montagem da plataforma multienzimática, o inflamassoma. Essa plataforma é composta pelo domínio de reconhecimento e oligomerização NALP, uma proteína associada a apoptose (ASC, *apoptosis-associated speck-like protein*) e caspase-1 (CASP-1) (LATZ; XIAO; STUTZ, 2013).

[1] NACHT – NAIP (*neuronal apoptosis inhibitor protein*), C2TA (*class 2 transcription activator,* do MHC), HET-E (*heterokaryon incompatibility*) e TP1 (*telomerase-associated protein 1*).

No entanto, para que essa ativação ocorra duas etapas são requeridas: *(i)* ativação de NALP3 com indução de pró-IL-1β e pró-IL-18*; (ii)* montagem da plataforma inflamassoma com formação de CASP-1 ativa, catalisando a ativação das formas ativas de IL-1β e IL-18 a partir das suas formas inativas (zimógenos) (MARTINON; BURNS; TSCHOPP, 2002; LATZ; XIAO; STUTZ, 2013; WEN; MIAO; TING, 2013). Essas citocinas inflamatórias estão elevadas na DF e contribuem substancialmente para a manutenção dessa condição (PITANGA, T. N. et al., 2016; CARVALHO et al., 2017).

O inflamassoma NLRP3 (FIGURA 8) é o mais bem compreendido e algumas vias de ativação são descritas: *(i)* danos ou disfunção mitocondrial (formação de EROs mitocondrial, liberação de DNA mitocondrial ou cardiolipina); *(ii)* efluxo de K^+; e *(iii)* dano lisossomal (liberação de catepsina) (PETRILLI et al., 2007; HORNUNG et al., 2008; ZHOU et al., 2011; SHIMADA et al., 2012; IYER et al., 2013; DUTRA et al., 2014; GUO; CALLAWAY; TING, 2015).

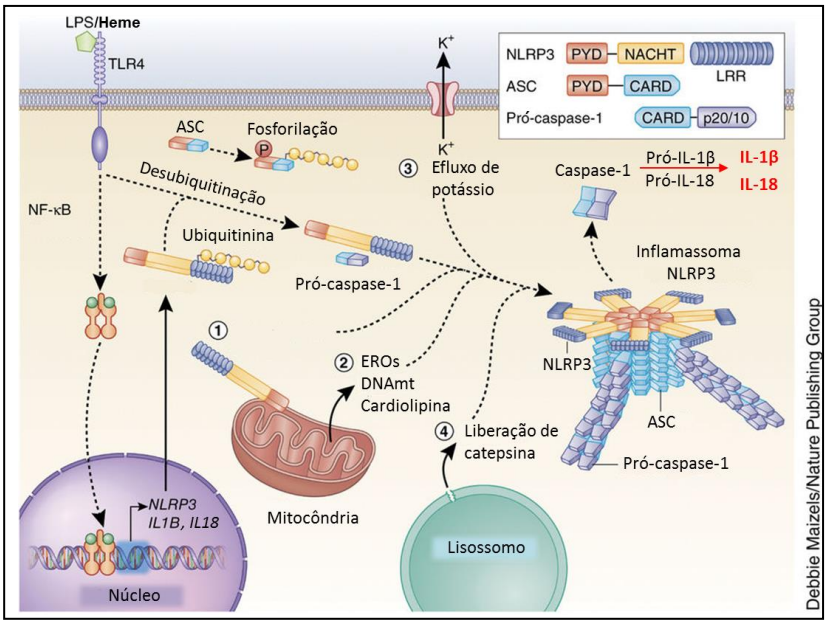

FIGURA 8. Componentes e ativação do inflamassoma NLRP3. A ativação do inflamassoma NLRP3 envolve dois passos. Inicialmente, o estímulo de ativação de NF-κB, como na ligação de LPS/heme ao TLR4, que induz a expressão elevada de *NLRP3,* assim como *IL1B e IL18* em suas formas inativas. O produto protéico NLRP3 sofre desubiquitinação e a proteína adaptadora ASC deve tornar-se linearmente ubiquitinada e fosforilada para a montagem do inflamassoma. Em seguida, um segundo sinal é requerido para ativação e formação da montagem do complexo inflamassoma NLRP3. O heme também pode atuar dessa forma, induzindo EROs independente de TLR4. Os principais estímulos são: fatores mitocondriais liberados no citosol (EROs mitocondrial, DNA mitocondrial ou cardiolipina), efluxo de K^+ através de canais iônicos, e liberação de catepsina após desestabilização de membranas lisossomais. O NLRP3 sofre oligomerização através de seus domínios *N*-terminais efetores PYD-PYD. As subunidades da pró-caspase-1 formam as subunidades ASC através das interações CARD-CARD, favorecendo a ativação autoproteolítica da pró-caspase-1 em caspase-1 que catalisa as reações de ativação da IL-1β e IL-18, a partir das suas formas inativas (pró-IL1β e pró-IL-18 [Adaptado de GUO et al., (2015)].

2.3.2 **Disfunção endotelial e vasoclusão**

A hemólise intravascular crônica na AF promove a saturação das proteínas envolvidas na captura da Hb livre, que se somando a liberação de arginase, constitui o ambiente favorável para o agravo da doença em virtude da diminuição da biodisponibilidade do NO (FIGURA 9) (ROTHER et al., 2005; STEINBERG, 2008). O NO é biossintetizado pela enzima óxido nítrico sintase endotelial (eNOS) a partir do seu substrato, a L-arginina. O NO ativa a guanilato ciclase solúvel (GCs), que converte a trifosfato de guanosina (GTP) em guanina monofosfato cíclica (GMPc), relaxando a musculatura vascular e promovendo vasodilatação (ROTHER et al., 2005). No entanto, durante a hemólise intravascular, a biodisponibilidade de NO é limitada por vários fatores. Já foi mencionado no tópico anterior (2.3.1) como a Hb livre contribui para isso. Somado-se à liberação excessiva de Hb, o aumento de $O_2^{\bullet-}$, a liberação da arginase eritrocitária e a diminuição da oferta do substrato L-arginina, contribuem para o desfecho da disfunção endotelial (STEINBERG, 2008; CHIRICO e PIALOUX, 2012). Deste modo, a disfunção endotelial constitui uma condição determinante para gravidade da AF devido a insuficiência da biodisponibilidade de NO, indispensável para regulação do tônus vascular pelas células endoteliais, repercutindo em estresse oxidativo, inflamação, lesão tecidual e vasoconstrição (HEBBEL, R. P.; OSAROGIAGBON, R.; KAUL, D., 2004; BLUM et al., 2005; CHIRICO; PIALOUX, 2012).

Como citado acima, as EROS/ERNs também contribuem para a disfunção endotelial. O $O_2^{\bullet-}$, por exemplo, pode ser gerado por várias vias, como a da xantina oxidase (XO), NAPDH oxidase, ou a partir de reações de desacoplamento da eNOS (CHIRICO e PIALOUX, 2012). Embora o $O_2^{\bullet-}$ seja extremamente prejudicial, os produtos formados através da sua reação com o NO são muito mais deletérios. No contexto da disfunção endotelial, o $O_2^{\bullet-}$ formado pode interagir com NO gerando NO_3^- e $ONOO^-$, este último, extremamente reativo com membranas celulares. Além disso, ao contrário do $O_2^{\bullet-}$, que pode sofrer inativação por reação de dismutação pela SOD, não existem sistemas enzimáticos que atuem na conversão de $ONOO^-$ em produtos inertes tornando ainda mais relevante a sua importância na propagação do processo inflamatório (ROTHER et al., 2005; STEINBERG, 2008; CHIRICO; PIALOUX, 2012).

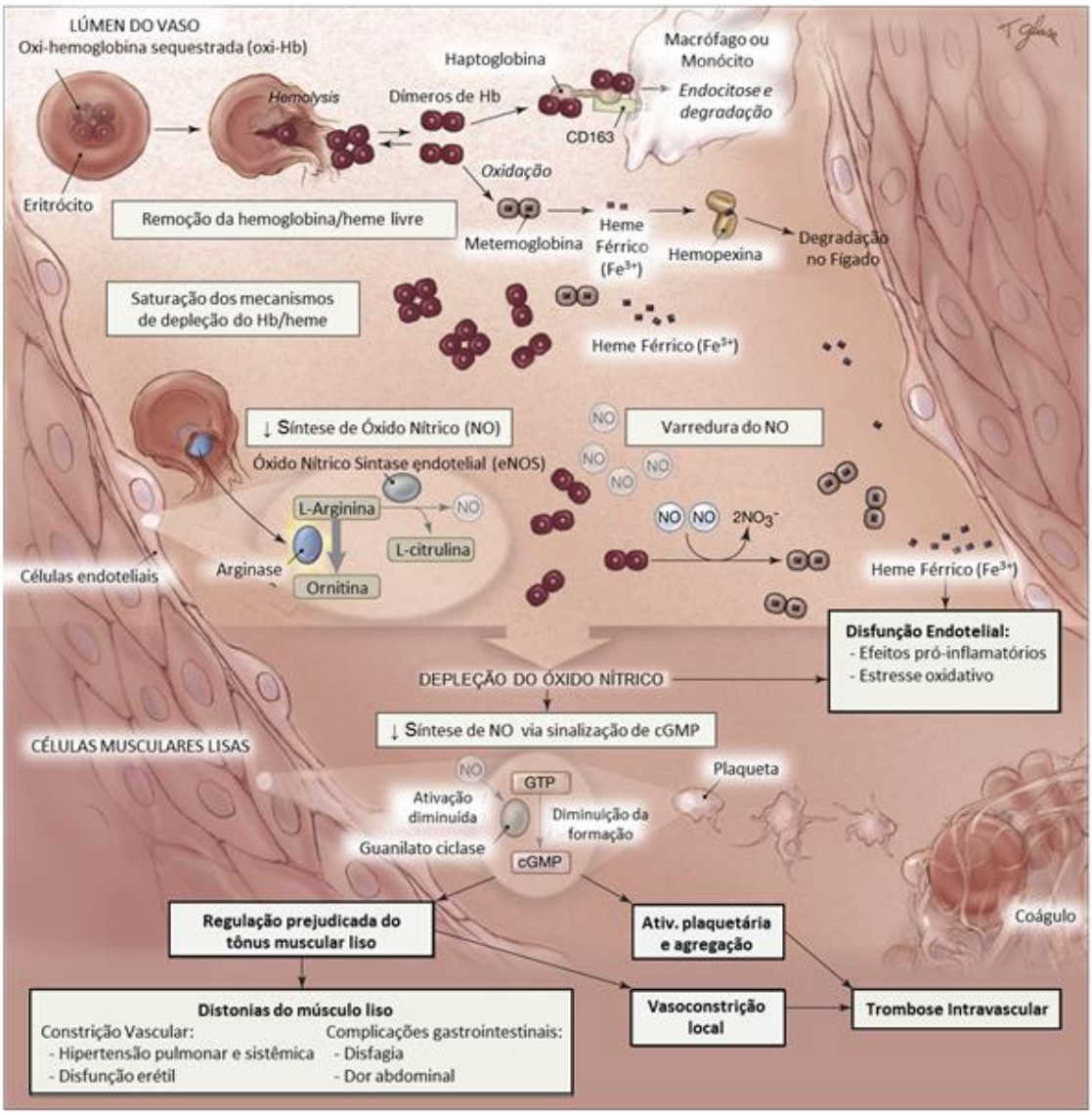

FIGURA 9. Diminuição da bioatividade do óxido nítrico na hemólise intravascular e disfunção endotelial na anemia falciforme. A hemólise intravascular libera hemoglobina (Hb) que é normalmente removida da circulação pela haptoglobina. O complexo haptoglobina-Hb (Hb-Hp) é reconhecido pelo receptor CD163 de alta afinidade localizado na superfície de monócitos/macrófagos para endocitose e degradação desse complexo. A Hb livre também pode sofrer oxidação liberando heme férrico (heme-Fe^{3+}), e o heme pode ser reconhecido pela hemopexina (heme-Hpx) via receptor CD91 de alta afinidade (não ilustrado), que promove o seu direcionamento para a degradação hepática realizada por macrófagos localizados nos hepatócitos. A hemólise excessiva na AF satura os sistemas de remoção promovendo o acúmulo de Hb e heme no plasma, orquestrando efeitos pró-inflamatórios e pró-oxidantes diretos nas células endoteliais dos vasos sanguíneos. O superóxido ($O_2^{\bullet-}$) produzido pelos sistemas oxidantes, como xantina oxidase (XO) e NAPDH oxidase (não ilustrados), também contribui para a depleção do NO, produzindo espécies reativas do nitrogênio (ERN), como nitrato (NO_3^-) e peroxinitrito ($ONOO^-$), este último altamente lipotóxico. Além disso, a reação do NO com a oxi-Hb e a degradação da L-arginina, substrato requerido pela óxido nítrico sintase endotelial (*nitric oxide synthase endothelial*, eNOS), contribuem para a redução drástica da oferta de NO, gerando a disfunção endotelial. A depleção de NO resulta em diminuição da ativação da guanilato ciclase, que utiliza trifosfato de guanosina (GTP) para a geração de monofosfato de guanina cíclico (GMPc). Níveis diminuídos de GMPc interferem na regulação do tônus muscular liso resultando em distonias, hipertensão sistêmica e pulmonar, disfunção erétil, disfagia e dor abdominal. A redução dos níveis de GMPc através da depleção de NO também pode levar a ativação e agregação plaquetária, promovendo a formação de trombos [Adaptado de Rother et al., (2005)].

A biodisponibilidade reduzida do NO contribui para a vasoclusão. Vários estudos demonstram que o endotélio de pacientes com AF apresenta adesividade aumentada para leucócitos, plaquetas e hemácias, favorecendo a condição pró-trombogênica constante. Dentre as moléculas participantes do processo pode-se destacar: ICAM-1, VCAM-1, P-selectina, E-selectina, FT, FvW e ET-1 (SETTY; STUART, 1996; SPACE et al., 2000; SOLOVEY et al., 2001; MORRIS et al., 2005; MACK; KATO, 2006; WOOD et al., 2006; VILAS-BOAS et al., 2010). Além disso, eritrócitos falciformes são 2,5 vezes mais propensos a aderir às células endoteliais quando comparados com eritrócitos de indivíduos sem a doença, e apresentam três vezes mais peroxidação lipídica (SULTANA et al., 1998; SPACE et al., 2000; KATO et al., 2005).

As CVOs são extremamente dolorosas e provocam danos teciduais sistêmicos, em que são observadas formações de trombos heterocelulares provenientes da agregação de eritrócitos falcizados, plaquetas e leucócitos ao endotélio ativado (FIGURA 10) (HEBBEL; OSAROGIAGBON; KAUL, 2004; CONRAN; FRANCO-PENTEADO; COSTA, 2009; MANWANI; FRENETTE, 2013; TELEN, 2016). Este ambiente pobre em oxigênio favorece ainda mais a falcização, e o fluxo sanguíneo lento possibilita o aumento das interações adesivas entre as células circulantes e o endotélio, exacerbando ainda mais a inflamação. A intensificação dessas interações adesivas pode acarretar em obstrução vascular comprometendo o aporte de oxigênio e/ou nutrientes (hipóxia/isquemia) aos tecidos (SETTY e STUART, 1996; OSAROGIAGBON et al., 2000; HEBBEL; OSAROGIAGBON; KAUL, 2004). Com a privação de oxigênio e/ou substratos metabólicos, ocorre geração intensa de EROs nas mitocôndrias e nos tecidos. Além disso, danos oxidativos consideráveis ocorrem também na fase de resolução da lesão após restabelecimento do fluxo sanguíneo (HEBBEL; OSAROGIAGBON; KAUL, 2004; MANWANI e FRENETTE, 2013). Nessas condições, a privação de ATP acarreta na produção de hipoxantina e XO no tecido hipoxêmico/isquêmico. Após a reperfusão tecidual, o oxigênio reage com a XO gerando $O_2^{\bullet -}$ que, além da disfunção endotelial, promove dano oxidativo ao tecido (OSAROGIAGBON et al., 2000; SZOCS, 2004).

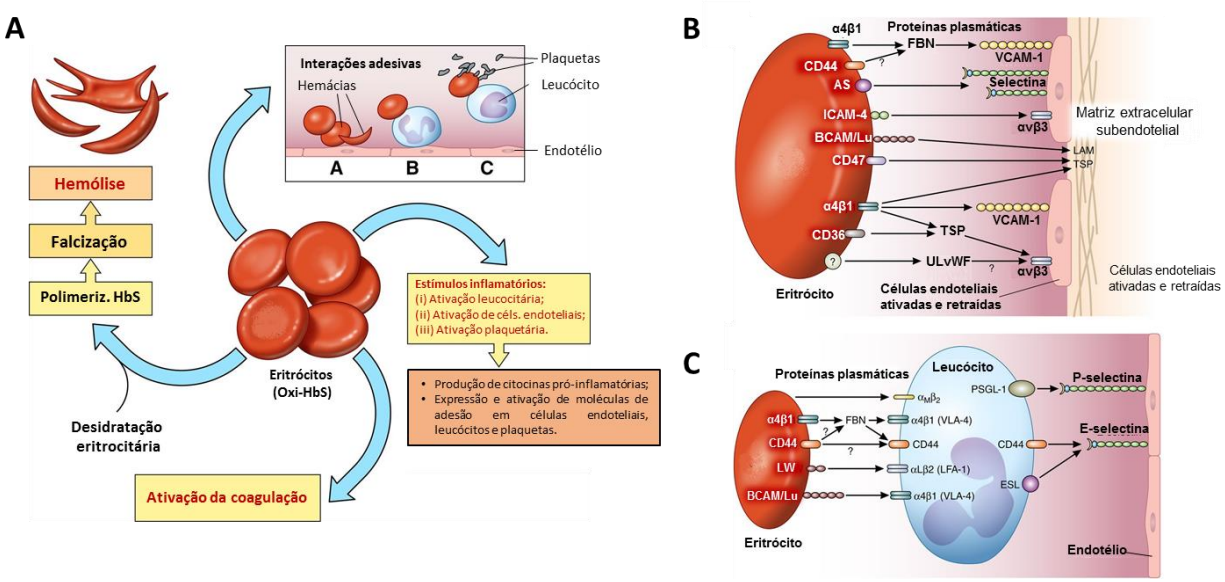

FIGURA 10. Interações adesivas e formação de agregados heterocelulares envolvendo eritrócitos HbS, leucócitos, plaquetas e endotélio. A) Representação sintética das interações envolvendo eritrócitos, leucócitos e plaquetas para o estabelecimento do processo inflamatório. **B)** Múltiplas interações entre eritrócitos SS e células endoteliais, matriz extracelular e proteínas plasmáticas. Os eritrócitos expressam múltiplas moléculas de adesão, que reconhecem ligantes específicos, como proteínas plasmáticas e proteínas de superfície das células endoteliais. Os receptores específicos de adesão nos eritrócitos que contribuem para a vasoclusão incluem ICAM-4 (proteína do grupo sanguíneo Landsteiner-Wiener, um receptor para várias integrinas), BCAM/Lu [proteína do grupo sanguíneo luterano, um receptor de lamininas (LAM) contendo cadeias α5 (lamininas-10 e -11)], e CD44 (que contém os antígenos do grupo sanguíneo indiano), que está envolvido na ligação à fibronectina (FBN) e E-selectina (ESL-1). AS: ácido siálico; FBN: fibronectina; TSP: trombospondina; LW: proteína do grupo sanguíneo Landsteiner-Wiener; ULvWF: *ultra-large von Willebrand fator*; PSGL-1: Ligante-1 da glicoproteína P-selectina (*P-selectin glycoprotein ligand-1*) [Adaptado de Telen (2016)].

2.3.3 **Envolvimento de leucócitos e plaquetas no estabelecimento e manutenção da cronicidade inflamatória**

A manutenção da cronicidade inflamatória observada em pacientes com AF é autoperpetuada pela ação conjunta do endotélio com leucócitos e plaquetas, e a interação entre eles (FIGURA 10) (CONRAN; FRANCO-PENTEADO; COSTA, 2009; MANWANI e FRENETTE, 2013). Nestes casos, observa-se aumento dos níveis de citocinas inflamatórias, como interleucinas (IL)-3, IL-6, IL-12, IL-1β, interferon (IFN)-γ e TNF-α (FRANCIS e HAYWOOD, 1992; MALAVE et al., 1993; DUITS et al., 1998; RODRIGUES et al., 2006; SWITZER et al., 2006; CONRAN et al., 2007; TORRES et al., 2016) mediadores inflamatórios/vasoativos, como ICAM-1, VCAM-1, FT, endotelina-1 (ET-1), prostaglandina E2 (PGE_2) e leucotrieno B_4 (LTB_4) (GRAIDO-GONZALEZ et al., 1998; GONÇALVES et al., 2001; CONRAN et al., 2004; CHANTRATHAMMACHART e PAWLINSKI, 2012; PITANGA, et al., 2016).

Os fenômenos hemolíticos geram disfunção endotelial e ativam leucócitos que aderem aos vasos sanguíneos, agregam-se a outras células sanguíneas bloqueando o lúmen, e estimulam o endotélio a aumentar ainda mais a expressão de moléculas de adesão contribuindo para a vasoclusão (LARD et al., 1999; OKPALA, 2004 e TELEN, 2016). Na AF observa-se aumento na contagem de leucócitos totais no sangue periférico, especialmente, neutrófilos (WONG et al., 1996; ANYAEGBU et al., 1998; NICKEL et al., 2015). Paradoxalmente, esses indivíduos apresentam suscetibilidade aumentada para infecções bacterianas secundárias, pois os leucócitos apresentam disfunções na quimiotaxia, opsonização, migração e mecanismos citolíticos/fagocíticos (ÇETİNER et al., 1989; HUMBERT et al., 1990; ANYAEGBU et al., 1998). Além disso, como tratado anteriormente, esses indivíduos costumam apresentar grande comprometimento esplênico em virtude do intenso sequestro de eritrócitos falcizados (BOOTH; INUSA; OBARO; 2010; BROUSSE; BUFFET; REES, 2014; REES, 2014). A depender da extensão da lesão o comprometimento pode ser parcial, provocando a sua atrofia (hipoesplenismo) ou pode levar ao aumento do órgão (esplenomegalia); ou destruição total (auto-esplenectomia) (HUMBERT et al., 1990; BOOTH; INUSA; OBARO; 2010). Em todas essas situações a remoção de patógenos é prejudicada, embora seja mais grave em indivíduos esplenectomizados. Por esse motivo, a administração de antibióticos (normalmente penicilina) é uma medida profilática importante empregada na clínica, para prevenir infecções que possam agravar a doença (BOOTH; INUSA; OBARO, 2010; BROUSSE; BUFFET; REES, 2014; KUCHAR; MISKIEWICZ; KARLIKOWSKA, 2015).

As plaquetas também desempenham grande contribuição na fisiopatologia da AF. Elas estão ativadas em indivíduos em estado estável, e ainda mais ativadas nas CVOs. Podem mediar a adesão de eritrócitos ao endotélio por meio da secreção de trombospondina, uma glicoproteína secretada na presença de trombina, constituindo por exemplo, uma condição importante para o desenvolvimento de trombose e hipertensão pulmonar (WUN et al., 1997; INWALD et al., 2000; VILLAGRA et al., 2007). Devido ao seu constante estado de ativação, promovem formação de agregados heterocelulares com eritrócitos, neutrófilos e monócitos, gerando obstrução de pequenos leitos vasculares. A ligação de plaquetas com outras células circulantes, requer a ação de ligantes glicoproteicos, como a P-selectina (CD62P) que interage com o antígeno de macrófago-1 (Mac-1, $\alpha_M\beta_2$ ou CD11b/CD18)

presente na superfície leucocitária. Além disso, as plaquetas podem modular a polarização de neutrófilos ao endotélio através de P-selectina e seu ligante (PSGL-1, *P-selectin glycoprotein ligand-1*), desencadeando a inflamação (SREERAMKUMAR et al., 2014; ZHANG et al., 2016).

A contribuição dos linfócitos T e B para a manutenção da inflamação crônica na AF não é muito bem compreendida. O número de linfócitos T circulantes é variável em indivíduos com AF em estado estável, embora muitos estudos apontem para a redução na proporção de células T CD4+ e T CD8+ circulantes e aumento de células T CD4+ e T CD8+ total, especialmente, o fenótipo de memória (BALANDYA et al., 2016). Também foi observada a redução de células T CD4+ supressoras. A redução desses subtipos de células T é mais pronunciada em pacientes que apresentam comprometimento esplênico e vasoclusão (KOFFI et al., 2003; SREERAMKUMAR et al., 2014; BALANDYA et al., 2016). Musa e colaboradores (2010) observaram que indivíduos com AF em estado estável e em CVOs, apresentam níveis aumentados de IL-2 em relação aos voluntários sadios; e que em CVOs há o aumento pronunciado de IL-4 e diminuição de IL-10 em comparação aos pacientes com AF em estado estável e voluntários sadios, além de uma proporção reduzida de TCD4+/TCD8+. Em conjunto, esses dados sugerem a coexistência de níveis de citocinas do tipo T auxiliar 1 (Ta1 ou *T helper 1, Th1*) e T auxiliar 2 (*Th2*), com diminuição dos subtipos de células T na vasoclusão.

Em relação aos linfócitos B, são descritas algumas alterações funcionais relacionadas às respostas humorais efetoras como a redução da resposta proliferativa específica ao antígeno, e a diminuição da secreção de imunoglobulinas do tipo M (IgM) por mecanismo independente de células T. Acredita-se que isso ocorra em consequência da destruição da zona marginal do baço, comum na AF (WANG et al., 1988; RAUTONEN et al., 1992; BOOTH; INUSA; OBARO, 2010; BROUSSE; BUFFET; REES, 2014).

Desta forma, as disfunções efetoras mediadas por células T e B previamente citadas podem explicar, pelo menos em parte, a susceptibilidade a infecções e a modulação deficiente da resposta imune que coopera para a cronicidade inflamatória presente em indivíduos com DF.

2.4 A RESPOSTA CELULAR ANTIOXIDANTE

O estresse oxidativo é o termo utilizado para descrever o desequilíbrio orgânico entre a produção de EROs e o mecanismo de defesa antioxidante. As EROs podem provocar peroxidação lipídica, danos ao DNA e afetar a função de proteínas celulares, contribuindo para o declínio geral das funções corporais e envolvimento na patogênese de diversas doenças (GIROTTI, 1985; BEETSCH et al., 1998; KATO et al., 2009). Dessa forma, a avaliação da capacidade total antioxidante do organismo pode constituir um sinal de alerta precoce e importante em diversas patologias (FASOLA et al., 2007; BELINI JUNIOR et al., 2012).

Tendo em vista a condição pró-oxidante proveniente dos distúrbios hemolíticos e vasoclusivos na DF, os mecanismos antioxidantes (FIGURA 11A) assumem papel crucial na manutenção do equilíbrio pró/antioxidante no combate de radicais livres como $O_2^{\bullet-}$ e H_2O_2. As principais defesas antioxidantes de primeira linha são: SOD, CAT e GPx (IGHODARO e AKINLOYE, 2017).

A SOD é uma metaloenzima, que requer um íon metálico como cofator [em humanos: manganês (Mn) ou cobre-zinco (Cu-Zn)] cobre ou magnésio) que eliminam radicais $O_2^{\bullet-}$ por dismutação em H_2O_2 e O_2 (FUKAI; USHIO-FUKAI, 2011; IGHODARO; AKINLOYE, 2017). Alguns estudos demonstraram o aumento da SOD em pacientes com DF, enquanto outros observaram o contrário (SCHACTER et al., 1985, (SCHACTER et al.,1988; AL-NAAMA; HASSAN; MEHDI, 2015). Schacter e colaboradores (1985) reportaram atividade diminuída da SOD-1 em indivíduos com AF em comparação com indivíduos saudáveis.

Em processos biológicos naturais ou devido a falcização, o H_2O_2 é depletado pela ação da GPx ou CAT (MATES; PEREZ-GOMEZ; NUNEZ DE CASTRO, 1999; VALKO et al., 2007; WILKING et al., 2013). A GPx é uma enzima localizada no citosol e na matriz mitocondrial que reduz o H_2O_2 utilizando a GSH (FERREIRA; MATSUBARA, 1997). A GSH atua como co-substrato da GPx, com propriedade doadora de elétrons, a qual poderá ser regenerada através da GSR com transferência de hidrogênios proveniente de NADPH. Estudos mostram que GPx, GSR e GSH são significativamente reduzidas na DF (CHIU e LUBIN, 1979; DAS e NAIR, 1980; VARMA et al., 1983; SAAD; SALLES; VELHO, 1991; REN et al., 2008; GIZI et al., 2011).

A CAT também age sobre o H_2O_2 transformando-o em água e oxigênio (IGHODARO e AKINLOYE, 2017) e a diminuição nos seus níveis tem sido descrita em eritrócitos falciformes, tornando a eliminação de H_2O_2 deficiente (DAS e NAIR, 1980; AL-NAAMA; HASSAN; MEHDI, 2015).

Outras proteínas atuam direta ou indiretamente na biotransformação/desintoxicação de xenobióticos ou drogas eletrofílicas como as enzimas NAD(P)H:quinona oxidoredutase 1 (NQO1), NRH:quinona oxidoredutase 2 (NQO2), glutationa S-transferase (GST), γ-glutamato-cisteína sintetase (GCS) e HO-1, glutationa reduzida (GSH) e metaloproteínas (HAYES et al., 2000; DINKOVA-KOSTOVA et al., 2001; KASPAR; NITURE; JAISWAL, 2009)

A indução desses genes é desencadeada por mecanismos de defesa em resposta ao estresse, regulado pelo elemento de resposta antioxidante/eletrófilo (ARE/EpRE) controlado pelo fator respiratório nuclear Nrf2, pertencente a uma família que contem o domínio do tipo *cap 'n' collar* (CNC) (JOHNSON; LAPADAT, 2002; KIM; HAN; CHAN, 2016). O Nrf2 é uma proteína *leucine zipper/CNC* presente no citoplasma na ausência de agentes estressores, associada ao seu inibidor denominado proteína associada à ECH tipo *Kelch1* (Keap1, *kelch-like ECH-associated protein 1*) ou analógo. Sob condições basais, o Keap1 associa-se ao Nrf2 no citoplasma, que visa o fator à degradação do proteassoma. O Nrf2 é um regulador chave do sistema de desintoxicação e antioxidante. Na etapa de indução, a dissociação Nrf2 da Keap1 é translocada para o núcleo e se heterodimeriza com os fatores transcricionais Maf ou Jun e liga-se a ARE/EpRE, induzindo a ativação dos genes de resposta antioxidante após a cascata de ativação mediada por proteína quinases tipo C (PKC, *protein kinase C*) (JOHNSON; LAPADAT, 2002; NITURE; KHATRI; JAISWAL, 2014).

A proteína p62/SQSTM1, também denominada sequestossomo, tem sua expressão supra-regulada de maneira dependente de Nrf2 sob condições de estresse químico/oxidativo (FIGURA 11B), e pode atuar no direcionamento de Keap1 para degradação proteolítica pela via autofágica, levando a ativação prolongada de Nrf2 e, consequentemente, a indução da resposta antioxidante (ISHII et al., 2000; WARABI et al., 2007; COPPLE et al., 2010; ICHIMURA et al., 2013).

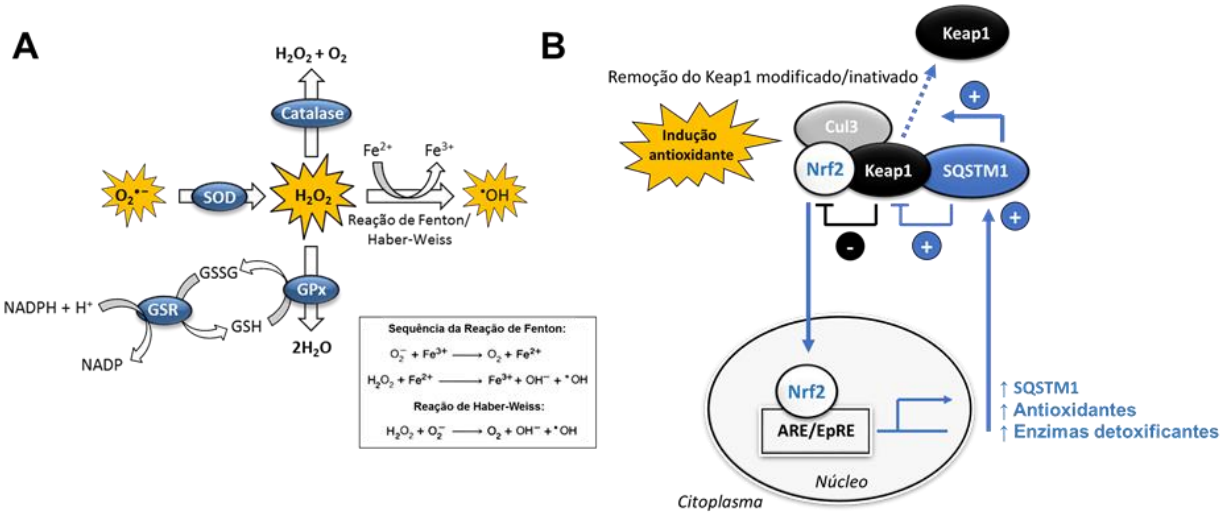

FIGURA 11. Resposta celular antioxidante. A) Balanço entre reações oxidativas e antioxidantes. Estão ilustrados os principais mecanismos enzimáticos de primeira linha responsáveis pela detoxificação de espécies reativas de oxigênio (EROs). A superóxido dismutase (SOD) atua na dismutação de ânios superóxido ($O_2^{\bullet-}$) em peróxido de hidrogênio (H_2O_2) e oxigênio (O_2). O H_2O_2 por sua vez, pode ser decomposto pela *(i)* catalase formando água (H_2O) e O_2; ou sofrer ação da glutationa peroxidase (GPx) formando $2H_2O$. A GPx requer glutationa reduzida (GSH) como substrato na catálise da reação. Os estoques de GSH são mantidos por ação da glutationa S-redutase (GSR) que reduz glutationa dissulfeto (GSSG) para formar glutationa sulfidril (GSH), um importane antioxidante celular. GSR pode ser regenerada por reações de transferência de hidrogênio mediada pelo NADPH + H^+ (fosfato de nicotinamida adenina dinucleotídeo). Essas reações contribuem mutuamente para manter o equíbrio oxidativo prevenindo dano tecidual. O H_2O_2 pode também, por exemplo, reagir com ferro ferroso (Fe^{2+}) proveniente da hemólise (reação de Fenton), ou reagir com $O_2^{\bullet-}$ (reação de Harber-Weiss) para formação de radical hidroxila ($OH^{\bullet}$) o mais reativo entre eles. O ferro férrico (Fe^{3+}) proveniente das reações de Fenton podem reagir com $O_2^{\bullet-}$ para formar Fe^{2+} em um processo cíclico [Adaptado de Chirico e Pialoux, 2012]. **B)** Mecanismo de indução e regulação da integridade da via de defesa antioxidante Keap1-Nrf2 pelo sequestossomo 1 (p62/SQSTM1). O Nrf2 é negativamente regulado pelo Keap1, uma proteína adaptadora de substrato para o complexo ubiquitina E3 ligase dependente de Culina (Cul3) (Cul3-Keap1-E3), que promove a ubiquitinação de Nrf2 e, subsequente, degradação pelo sistema de proteassoma ubiquitina (UPS, *ubiquitin proteasome system*). Em condições de estresse oxidativo ou indução de resposta antioxidante, o Nrf2 dissocia-se de Keap1 e sofre translocação para núcleo. Já o Keap1, sofre ubiquitinação e é direcionado para a degradação proteolítica. Após uma cascata de eventos mediado por proteínas quinases (PKs, *protein kinases*), o Nrf2 sofre ativação e é reconhecido pelo domínio Elemento de Resposta Antioxidante/Eletrófilo (ARE/EpRE) com indução da expressão de enzimas antioxidantes, detoxificantes e p62/SQSTM1. Deste modo, a expressão de p62/SQSTM1 é regulada inicialmente por Nrf2. Sob condições de ativação de Nrf2, a expressão elevada de p62/SQSTM1 *(i)* direciona Keap1 para via de degradação autofágica, potencializando a ativação de Nrf2; ou *(ii)* atua na reciclagem de Keap1 para restabelecer a homeostasia da via ARE. Nrf2 (ou NFE2L2): *Nuclear respiratory factor 2 ou* [*Nuclear factor (erythroid-derived 2)-like 2*]; ARE/EpRE: *antioxidant/electrophile response element*; Keap1: *kelch-like ECH-associated protein 1* [Adaptado de Copple et al., (2010)].

2.5 TRATAMENTO

A HU é uma droga mielossupressora utilizada no tratamento de doenças mieloproliferativas e também é referida como “padrão-ouro” aprovada pela FDA (*Food and Drug Administration*) para o tratamento da DF (WARE, 2010; NZOUAKOU et al., 2011; AGRAWAL et al., 2014; WONG et al., 2014). Os inúmeros benefícios constatados pela utilização da HU em várias pesquisas clínicas como melhoria

clínica, aumento da sobrevida e índices baixos de toxicidade, justificam a utilização desse fármaco no manejo terapêutico desses pacientes que, a partir de 2010, passou a compor o Protocolo Clínico e Diretrizes Terapêuticas para tratamento da DF (portaria N° 55, de 29 de janeiro de 2010), visando uma melhor qualidade de vida para esses pacientes (BRASIL, 2010). Segundo o protocolo atual (BRASIL, 2018), deve-se administrar dose inicial de 15 mg/kg/dia, por via oral, em dose única, e aumentar em 5 mg/kg/dia a cada 4 semanas até atingir a dose máxima de 35 mg/kg/dia (dose máxima tolerada), interrompendo a sua administração se houver toxicidade hematológica, ou outros efeitos adversos graves. A HU é utilizada em pacientes que apresentam manifestações clínicas mais graves, indicativas de mau prognóstico como: possuir Hb variante compatível com a DF; idade superior a 2 anos (ou após 9 meses em casos excepcionais); ocorrência de mais de três crises álgicas agudas (vasoclusão) com necessidade de hospitalização; ocorrência de dois episódios de síndrome torácica aguda com existência de infiltrado pulmonar e/ou insuficiência respiratória e necessidade de transfusão de hemoderivados; hipoxemia crônica; lesões crônicas de órgãos; concentração de Hb baixa persistente (< 6 g/dL após três medições fora do evento agudo) e HbF (< 8% após 2 anos de idade); leucocitose (> 20.000 /mm^3, após três medições fora do evento agudo); lactato desidrogenase elevada; e alterações no *eco-doppler* transcraniano (> 200 cm/s, com impossibilidade de regime transfusional crônico) (BRASIL, 2018).

Esta droga possui efeito dual (FIGURA 12), induzindo a biossíntese de HbF em um mecanismo dependente de NO, com inibição da ribonucleotídeo redutase, responsável pela biossíntese de deoxinucleotídeos, e redução da liberação de reticulócitos imaturos (reticulocitose) e leucócitos (leucocitose) no sangue periférico (YARBRO, 1992; COKIC et al., 2003; KING, 2003). Após a administração oral, a HU é rapidamente absorvida e, após sofrer peroxidação, libera NO. Este, por sua vez, ativa a guanilato ciclase solúvel (GCs) que, subsequentemente, induz a conversão de trifosfato de guanosina (GTP) em monofosfato cíclico de guanosina (GMPc) modulando a transcrição do gene da globina gama (*HBG*) para síntese de HbF (COKIC et al., 2008). Conforme comentado previamente, essa Hb possui como singularidade a alta afinidade pelo oxigênio. Essa condição proporciona estabilidade à $Hb\beta^S$ tornando-a solúvel, mesmo em seu estado desoxigenado (desoxi-HbS) e impedindo-a de iniciar a cascata de polimerização dentro do eritrócito.

Levando-se em consideração os efeitos primários e secundários da HU, Ware (2010) pontuou como principais benefícios: *(1)* a indução de HbF através da ativação da GCs; *(2)* diminuição na contagem de neutrófilos e reticulócitos pela inibição da ribonucleotídeo redutase e toxicicidade da medula; *(3)* diminuição da adesividade e melhora da reologia de neutrófilos circulantes e reticulócitos; *(4)* redução da hemólise e melhoria da hidratação eritrocitária, macrocitose e redução intracelular da falcização; e *(5)* liberação de NO como potencial vasodilatador local e melhoria da resposta vascular.

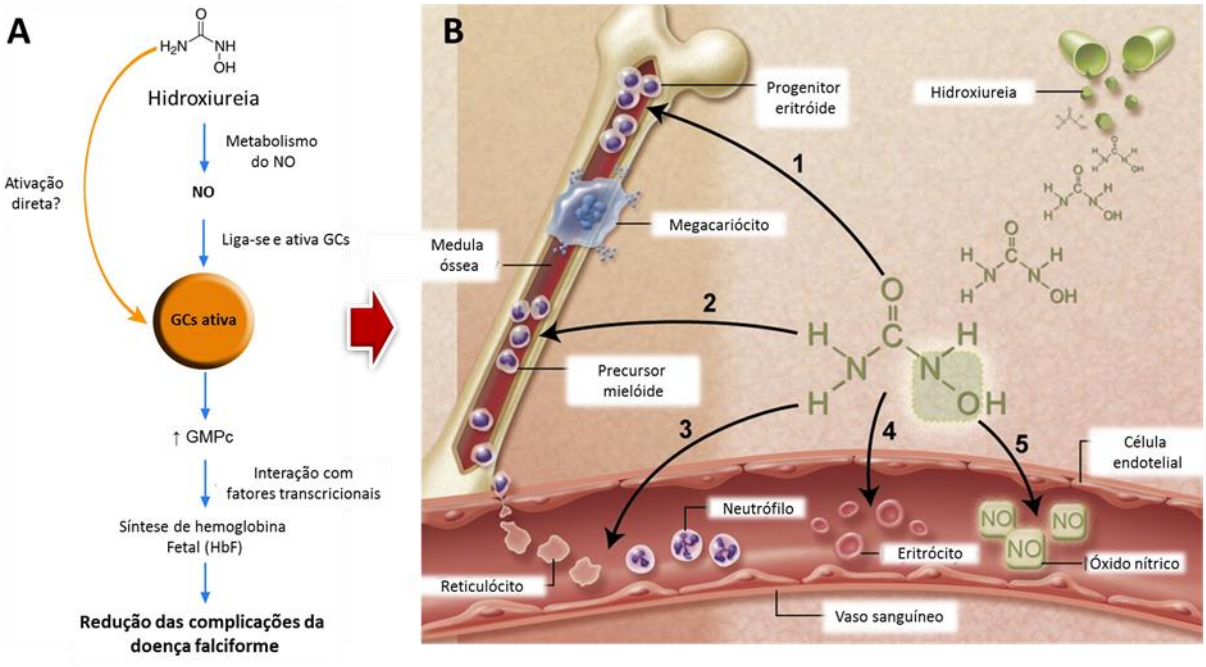

FIGURA 12. Benefícios da hidroxiureia no manejo terapêutico da doença falciforme. A) Mecanismo de ação da hidroxiureia. Após ingestão, a HU sofre biotransformação liberando NO, que se liga a guanilato ciclase solúvel ativando-a. A sua ativação converte trifosfato de guanosina (GTP) em monofosfato cíclico de guanosina (GMPc) que interage com fatores transcricionais modulando a translocação destes para o núcleo e ativando o gene da globina gama (*HGB*). Como resposta, ocorre produção de HbF que diminui a falcização eritrocitária e, consequentemente, os eventos vasoclusivos e hemolíticos [Adaptado de King (2003)]. **B)** Efeitos primários e secundários da HU. Inicialmente, a indução de HbF se dá pelo mecanismo por ativação da GCs descrito em "A"; (2) os efeitos da sua ação inibidora da ribonucleotídeo redutase refletem na diminuição da formação e liberação de leucócitos (p. ex., neutrófilos) e reticulócitos na circulação sanguínea; (3) diminuição da expressão de moléculas de adesão endotelial e leucocitária, como melhoria na reologia dos neutrófilos e reticulócitos circulantes; (4) redução da hemólise devido ao aumento da síntese de HbF e diminuição das reações de polimerização que promovem a falcização; (5) aumento da liberação de NO melhorando a vasodilatação e diminuindo a ativação de células endoteliais [Adaptado de Ware (2010)].

3. JUSTIFICATIVA E HIPÓTESES

3.1. JUSTIFICATIVA

A AF é uma doença molecular grave e complexa sendo referida como um problema de saúde pública pela OMS, devido a sua grande morbimortalidade. A condição fisiopatológica complexa da AF é orquestrada por eventos sucessivos de falcização, vasoclusão, lesão por isquemia-reperfusão e estresse oxidativo, que juntos contribuem para o estabelecimento da inflamação crônica observada nesses pacientes (STEINBERG, 2008; CHIRICO e PIALOUX, 2012).

O heme é considerado como molécula central no desencadeamento do processo inflamatório na hemólise, gerando danos sistêmicos e inflamação por várias vias (AFT e MUELLER, 1983; VINCENT, 1989; WAGENER et al., 2001; CARLSEN; MØLLER; SKIBSTED, 2005; DUTRA et al., 2014; DUTRA e BOZZA, 2014). Esta molécula pode promover oxidação de lipoproteínas formando produtos lipotóxicos altamente reativos e inflamatórios, como oxLDL e p-HDL gerando dano vascular (BALLA et al., 1991; JENEY et al., 2002; NAGY et al., 2010; OZTURK et al., 2013; ATAGA et al., 2015; SOUPENE et al., 2016). Além disso, foi demonstrado que a inflamação persistente na DF pode interferir na esterificação do colesterol e na função do HDL-C, refletindo na diminuição dos seus níveis circulantes (SHALEV et al., 2007). Em contraste, a literatura carece de estudos que avaliem a associação do heme com marcadores clínico-laboratorias rotineiramente empregados no manejo clínico de pacientes com AF.

A HU é considerada como droga “padrão-ouro” para o tratamento da AF sendo a principal opção terapêutica oficial recomendada para esta hemoglobinopatias, com uso em pacientes que apresentem prognóstico clínico mais grave. Embora seja também uma droga mielossupressora, a HU confere amplos benefícios com melhoria do bem-estar e sobrevida dos pacientes com AF (STEINBERG et al., 2010; SILVA-PINTO et al., 2013; AGRAWAL et al., 2014; DE ARAUJO et al., 2015).

No contexto terapêutico da AF, a melhoria clínica referida ao tratamento com HU tem sido atribuída ao seu mecanismo dual, *(i)* modulando a biossíntese de HbF em um mecanismo dependente de NO (KING, 2003); *(ii)* e por sua ação mielossupressora inibindo a ribonucleotídeo redutase, responsável pela biossíntese

de deoxinucleotídeos (envolvidos na biossíntese de DNA) reduzindo a liberação descompensada de leucócitos e reticulócitos no sangue periférico (YARBRO, 1992). Esses efeitos, quando avaliados de forma sistêmica, melhoram a oxigenação tecidual, diminuem as CVOs e hemolíticas, reduzem a liberação de Hb/heme livre e seus efeitos pró-oxidantes, diminuem a liberação de precursores eritróides/granulocíticos e, consequentemente, a inflamação (WARE, 2010). Os benefícios terapêuticos da HU foram comprovados em vários estudos in vitro e coortes (STEINBERG et al., 2010; SILVA-PINTO et al., 2013; DE ARAUJO et al., 2015) e superam os possíveis efeitos adversos atribuídos ao seu potencial clastogênico (OPPENHEIM e FISHBEIN, 1965), mutagênico (ZIEGLER-SKYLAKAKIS; SCHWARZ; ANDRAE, 1985), carcinogênico (SAKANO et al., 2001) e teratogênico (MURPHY e CHAUBE, 1964), descritos na literatura médica.

O estresse oxidativo e a inflamação merecem atenção especial na AF e podem ser tratados como alvos substanciais para a melhoria da condição clínica desses pacientes. Alguns trabalhos têm descrito a melhoria no perfil antioxidante em indivíduos tratados com HU, com o aumento da capacidade antioxidante, diminuição da peroxidação lipídica, elevação nos níveis de glutationa, diminuição de EROs, e capacidade antioxidante total no plasma (CAO e PRIOR, 1998; CHO et al., 2010; BEERS e KATO, 2012; BELINI JUNIOR et al., 2012; TORRES et al., 2012). Ensaios in vitro demonstram que a HU induz a expressão de HbF via sinalização de resposta antioxidante dependente de Nrf2 em células K562 (MACARI e LOWREY, 2011; PULLARKAT et al., 2014). O curioso aqui é o envolvimento do elemento de resposta antioxidante mediado pelo Nrf2, que é responsável pela indução de uma série de enzimas antioxidantes na indução de HbF.

Por outro lado, os efeitos da HU em mecanismos relacionados à inflamação não são claros. Estudos recentes têm demonstrado o envolvimento do inflamassoma NLRP3 como possível propagador da inflamação em indivíduos com AF (MENDONÇA et al., 2016; PITANGA, et al., 2016) ou DF (VOGEL et al., 2017), sendo referidos como alvos terapêuticos em potencial. No entanto, a avaliação de possíveis efeitos modulatórios da HU na formação e a ativação do inflamassoma não são descritos.

O aumento da condição redox e inflamatória proveniente dos distúrbios hemolíticos, com liberação contínua do heme, e a ampla utilização da HU como modalidade terapêutica da AF, justificam o desenvolvimento de novos estudos

visando ampliar a compreensão acerca dos mecanismos e efeitos da HU em diferentes vias, estimulando o desenvolvimento de estratégias terapêuticas mais eficientes.

3.2. HIPÓTESES

Hipótese 1 – O aumento da concentração plasmática de heme na AF está associado com alteração em biomarcadores clínicos e laboratoriais nesses pacientes.

Hipótese 2 – A HU regula negativamente a inflamação mediada por inflamassoma NRLP3 em leucócitos de pacientes com AF.

Hipótese 3 – A HU diminui o estresse oxidativo condicionado pelo heme e induz a resposta enzimática antioxidante in vitro.

4. OBJETIVOS

4.1. GERAL

Investigar as associações do heme com marcadores clínicos e laboratoriais em pacientes com AF; o papel de hemácias falciformes íntegras e lisadas na indução da expressão de componentes do inflamassoma NLRP3 e liberação de IL-1β e LTB_4, e se o tratamento de pacientes com HU interfere nestas vias; e por fim, investigar propriedades antioxidantes da HU.

4.2 ESPECÍFICOS

4.2.1 Manuscrito 1 – *Association of free heme plasma levels with hemolytic, hepatic, lipid markers and clinical outcome of stroke in sickle cell anemia.*

i. Avaliar os marcadores laboratoriais (parâmetros hematológicos, bioquímicos e inflamatórios) em indivíduos com AF;

ii. Determinar a concentração plasmática de heme livre em indivíduos com AF;

iii. Investigar as associações do heme com marcadores clínicos e laboratoriais;

iv. Investigar a associação dos haplótipos relacionados ao grupo de genes ligados a globina beta com a liberação de heme livre.

4.2.2. Manuscrito 2 – *Effect of hydroxyurea and sickle red cells on NLRP3 inflammasome and leukotriene B_4.*

i. Avaliar os marcadores bioquímicos, hematológicos e haplótipos ligados ao grupo de genes da globina β em indivíduos com AF;

ii. Avaliar se eritrócitos íntegros ou lisados de indivíduos com AF induzem diferentemente a expressão de componentes do inflamassoma NLRP3 e secreção de IL-1β e LTB_4;

iii. Avaliar se indivíduos com AF tratados com HU, apresentam diminuição na expressão dos componentes do inflamassoma *NLRP3* e diminuição na secreção de IL-1β e LTB_4.

4.2.3 Manuscrito 3 – *Hydroxyurea induces Nrf2-Antioxidant Response Element/electrophile Signaling Pathway regulated by p62/SQSTM1.*

i. Determinar a atividade antioxidante da HU;

ii. Avaliar a toxicidade da HU e hemina em células endoteliais de veia de cordão umbilical (HUVEC) e monocucleares de sangue periférico (PBMC);

iii. Avaliar os efeitos do tratamento com HU, hemina ou combinação de ambos no acúmulo de superóxido em sobrenadantes de PBMC;

iv. Determinar a produção de nitrito em HUVEC e PBMC desafiadas com HU e/ou hemina;

v. Avaliar o potencial indutor dos tratamentos com HU e/ou hemina na expressão de genes de resposta celular antioxidante em HUVEC e PBMC;

vi. Identificar vias relacionadas à indução de resposta celular antioxidante moduladas pela HU.

5. RESULTADOS

O presente estudo foi subdividido em três capítulos, apresentados na forma de manuscritos, descritos a seguir:

CAPÍTULO 1. *Association of free heme plasma levels with hemolytic, hepatic, lipid markers and clinical outcome of stroke in sickle cell anemia.*

CAPÍTULO 2. *Effect of hydroxyurea and sickle red cells on NLRP3 inflammasome and leukotriene B_4.*

CAPÍTULO 3. *Hydroxyurea induces Nrf2-Antioxidant Response Element/electrophile Signaling Pathway regulated by p62/SQSTM1.*

5.1. CAPÍTULO 1 – *Association of free heme plasma levels with hemolytic, hepatic, lipid markers and clinical outcome of stroke in sickle cell anemia*

Objetivo:

Investigar os efeitos do heme em marcadores laboratoriais rotineiramente empregados no monitoramento clínico dos pacientes com AF, e a sua possível contribuição para o estabelecimento da gravidade clínica desses indivíduos.

Principais resultados:

Neste trabalho, foi demonstrado que a liberação excessiva de heme em pacientes com AF pode contribuir para a gravidade da doença, estando associada com o aumento da contagem de monócitos, dos níveis de marcadores hepáticos (bilirrubina total e frações, e AST), triglicérides e VLDL-C; e associada com a diminuição dos níveis de HbF e HDL-C. Não foram encontradas associações entre o heme livre e os haplótipos relacionados ao grupo de genes da globina beta. Entretanto, níveis elevados de heme (98,2 ± 30,1 μM) apresentaram associação com a história clínica de AVC.

Situação: a ser submetido.

Association of free heme plasma levels with hemolytic, hepatic, lipid markers and clinical outcome of stroke in sickle cell anemia

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Conflict of Interest
All authors have no conflict of interest to declare.

ABSTRACT

The continuous and excessive release of heme occurring in hemolytic events in sickle cell anemia (SCA) and contributes substantially to the systemic damage and the disease severity, acting as a potent oxidizing agent in biomolecules such as lipids, proteins and DNA present in all tissues contributing for inflammation by different pathways. This study aimed to investigate the association of free heme with laboratory markers commonly used in clinical monitoring of SCA, as well as the different clinical outcomes of this hemoglobinopathy. SCA patients presented plasma heme concentration of 69.6 ± 30.3 μM, with a median of 66.1 μM. The free heme had a positive correlation with hemoglobin S (HbS) concentration (r = 0.4277; p = 0.0001) and negative correlation with fetal hemoglobin (HbF) (r = -0.3786; p = 0.0008). Patients with high free heme levels (≥ 66.1 μM) had increased count of monocytes and levels of hepatic markers related to hemolysis, such as total bilirubin, indirect bilirrubin, and aspartate transaminase (AST). In addition, the comparative analysis of 25th (44.3 μM) and 75th (87.3 μM) percentiles related to free heme revealed a association with direct bilirubin. Interestingly, heme also showed association with lipid markers, where its increase was associated with a decrease in high-density lipoprotein-cholesterol (HDL-C) levels and increase of triglycerides and very low-density lipoprotein-cholesterol (VLDL-C) levels. Correlation analysis reinforced our findings, where heme showed a positive correlation for monocyte count (r = 0.3025; p = 0.0064), total bilirubin (r = 0.3505; p = 0.0014) and fractions [direct bilirrubin (r = 0.2660; p = 0.0178) and indirect bilirubin (r = 0.2989; p = 0.0071)], AST (r = 0.3008; p = 0.0071), triglycerides (r = 0.5954; p < 0.0001) and VLDL-C (r = 0.6068; p < 0.0001); and negative correlation for HDL-C (r = -0.3596; p = 0.0011). We also evaluated the contribution of beta-globin gene cluster haplotypes on the production of HbF and whether they interfere in the release of free heme. Patients with BEN/BEN genotype presented higher HbF levels, and CAR/CAR had lower levels of HbF, but we found no association of these haplotypes with free heme release. However, elevated levels of heme (98.2 ± 30.1 μM) were associated with patients with previous history of stroke. Our data suggest that the excessive release of heme from recurrent hemolytic events in SCA may contribute substantially to the severity of the disease: *(i)* increasing the auto-oxidation of HbS; *(ii)* systemic inflammation and tissue damage in different organs; *(iii)* interfering with lipid metabolism; and *(iv)* increasing the risk for developing stroke (or recurrence).

Keywords: sickle cell anemia, heme, hemolysis, dyslipidemia, inflammation, stroke

LIST OF ABBREVIATIONS

A1AT	Alpha-1 antitrypsin
ALT	Alanine transaminase
ASO	Antistreptolysin O
AST	Aspartate transaminase
BEN	Benin haplotype linked to beta-globin gene cluster
CAR	Central African Republic haplotype linked to beta-globin gene cluster
C-RP	C-reactive protein
Hb	Hemoglobin
HBB	Beta-globin gene cluster
HbF	Fetal hemoglobin
HbS	Hemoglobin S
HDL-C	High-density lipoprotein-cholesterol
HPLC	High-performance liquid chromatography
HU	Hydroxyurea
LDH	Lactate dehydrogenase
LDL-C	Low-density lipoprotein-cholesterol
NO	Nitric oxide
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
SAUD	Saudi Arabia or India haplotype linked to beta-globin gene cluster SCA
SCA	Sickle cell anemia
SCD	Sickle cell disease
SEN	Senegal haplotype linked to beta-globin gene cluster SCA
VLDL-C	Very low-density lipoprotein-cholesterol

INTRODUCTION

Sickle cell anemia (SCA) has an autosomal recessive inheritance, which is associated with the presence of the variant hemoglobin (Hb) S (HbS), due to the GAG>GTG point mutation located in the sixth position of the gene that encodes the globin beta chain (*HBB*). This mutation promotes the replacement of glutamic acid with valine (glu6val). SCA is considered the most severe clinical manifestation of the sickle cell disease (SCD), in which individuals present mutation in both β-coding genes (HbSS) (ZAGO and PINTO, 2007; STEINBERG, 2008).

The pathophysiology of SCA is complex, due to the clinical heterogeneity observed among individuals, varying degrees of intermittent vasoconstrictive and hemolytic crises, followed by ischemia-reperfusion injury and increased redox status in the vascular microenvironment, in a cyclical process that promotes a chronic inflammatory state, causing systemic damage of multiple organs (STUART and NAGEL, 2004; BELCHER et al., 2010; CHIRICO and PIALOUX, 2012; NOURAIE et al., 2013). The existence of vaso-occlusive, hemolytic and dyslipidemic sub-phenotypes (KATO; GLADWIN; STEINBERG, 2007; SEIXAS et al., 2010; ALELUIA et al., 2017) has been described, and although they are overlapping in many clinical features, they may be useful in the more appropriate clinical management of the disease.

The clinical severity of the disease is directly related to variations of haplotypes linked to the beta-globin gene cluster (*HBB*), the concentration of HbS in the intra-erythrocyte microenvironment, its interaction with other variant types of hemoglobin, and the concentration of fetal hemoglobin (HbF) (NAGEL et al., 1987; STEINBERG and RODGERS, 2001; AKINSHEYE et al., 2011; STEINBERG et al., 2014; PIEL; STEINBERG; REES, 2017). These haplotypes have been described as genetic modifiers of risk of DF severity and are named according to their place of origin: Benin (BEN), Central African Republic (CAR) or Bantu, Senegal (SEN), Cameroon (CAMER) and Saudi Arabia and India (SAUD) (NAGEL, 1984; NAGEL; RANNEY, 1990).

Chronic intravascular hemolysis promotes the release of free plasma hemoglobin, bilirubin, lactate dehydrogenase and heme (IYAMU et al., 2005; HEBBEL, 2011; STANKOVIC and LIONNET, 2016). The saturation of proteins involved in the capture of free hemoglobin, heme and free iron, together with the

release of arginase, is the favorable environment for the SCA severity due to the decrease in the bioavailability of nitric oxide (NO) leading to endothelial dysfunction (ROTHER et al., 2005; STEINBERG, 2008). In this context, the continuous and excessive release of heme occurring in hemolytic events in SCA appears to play a crucial role in acting as a potent oxidizing agent generating damage in biomolecules, such as lipids, proteins and DNA present in all tissues, contributing to inflammation by different pathways (AFT and MUELLER, 1983; VINCENT, 1989; WAGENER et al., 2001; CARLSEN; MØLLER; SKIBSTED, 2005; DUTRA and BOZZA, 2014).

Considering the great contribution of heme to the establishment and maintenance of the inflammatory state and the lack of clinical studies to understand its pathophysiological relevance in SCA, this study aims to investigate its relevance in the alteration of biomarkers routinely employed in the clinical follow-up of patients.

METHODS

Subjects

We performed a cross-sectional study on 80 children and adolescents with SCA in steady state. Patients presented mean age (±S.D.) of 8.38 (±3.74) years, with a median (percentile 25^{th} – 75^{th}) of 8 years (6.0 –11.5), being 45 males (55.5%) and 36 females (44.45%). The recruitment of patients occurred from March 2010 to November 2012 at the outpatient pediatric hematology unit of the Bahia Hematology and Hemotherapy Foundation (HEMOBA). The eligibility criteria used to determine the steady state consisted of HbSS genotype, absence of crisis, transfusion regimen, hospitalizations or infections in the last 4 months. In addition, all patients reported regular use of folic acid and were not receiving hydroxyurea (HU) treatment and corticosteroids (10 days prior to blood sampling). This study was conducted in accordance with the Helsinki Declaration of 1975, and its revisions, and received approval from the Institutional Review Board of the Oswaldo Cruz Foundation-FIOCRUZ, Bahia-Brazil (CAAE: 0016.0.225.000-09). All study subjects or their legal guardians were, properly, advised about the research, agreed with the biological sample collection and signed the informed consent.

Blood sampling and clinical history of patients

Blood samples were taken during a regular clinical visit, and each patient's medical history was obtained from patient records. Blood samples were collected by venipuncture to perform different analyses, and were used for hematological analyses, hemoglobin profile characterization, DNA extraction for molecular analyses, free heme quantification, and biochemical and inflammatory markers analyses. Hematological and biochemical analyses were performed within a few hours after blood collection. Plasma aliquots were prepared and stored at -80 °C for further determination of free heme.

Determination of haplotypes to the β^s globin-gene cluster haplotypes

Genotypic determinations related to the β^s globin-gene cluster haplotypes were performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using restriction endonucleases for polymorphic sites: *Xmn*I (G gamma gene), *Hind*III (in the IVSII of G gamma and A gamma gene), *Hinc*II (3' and inside the gamma gene), *Hinf*I (5' of the beta gene) and, *Hpa*I (3' of the beta gene) for analysis of sites in beta-globin gene cluster haplotypes as described by Sutton and collegues (1989).

Hematological, biochemical, and immunological marker analysis

Hematological analyses were performed using CELL-DYN Ruby Hematology Analyzer (Abbott Diagnostics, Lake Forest, Illinois, USA), and hemoglobin profiling and quantification was performed by high-performance liquid chromatography (HPLC) using the VARIANT™ II Hemoglobin Testing System (Bio-Rad, Hercules, California, USA). Quantification of biochemical markers including lipid profile (triglycerides, total cholesterol and fractions), protein (total and fractions), bilirubin (total and fractions), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) were performed using A25 random access automatic analyzer (Biosystems SA, Barcelona, Spain). Ferritin was measured using Access® Immunoassay System X2 (Beckman Coulter, Fullerton, CA, USA). Alpha 1-antitrypsin (AAT) and C-reactive protein (C-RP) and antistreptolysin O (ASO) were determined using IMMAGE® 800 Immunochemistry System (Beckman Coulter, Fullerton, CA, USA). Genomic DNA was obtained from peripheral blood using QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Westphalia, Germany), according to the manufacturer's recommendations. Hematological and biochemical analyses were performed at the Laboratory of Clinical and Toxicological Analysis of the Faculty of Pharmacy (LACT-FAR) of the Federal University of Bahia (UFBA). Molecular analyses and heme levels were developed in the Laboratory of Investigation in Genetics and Translational Hematology (LIGHT) of the Gonçalo Moniz Institute (IGM – FIOCRUZ – BA).

Determination of free heme

Free heme was measured on plasma samples using QuantiChrom™ Heme Assay Kit (BioAssay Systems, Hayward, California, USA) following the manufacturer's protocol.

Statistical analysis

Statistical analysis and assembly of the graphs were performed using GraphPad Prism version v.6.0 (Graphpad software, San Diego, CA, USA). The variables selected were expressed as means (±SD), medians and percentiles. To evaluate the association of heme with laboratory markers, we stratified the patients into groups based on the percentiles: $< 50^{th}$ vs $\geq 50^{th}$; and $\leq 25^{th}$ vs $\geq 75^{th}$. The distribution of the quantitative variables was analyzed by the Shapiro-Wilk test. The significance of the difference between two groups was analyzed using *t*-test (for normal distributions) or Mann-Whitney U-test (for non-parametric distributions). Correlation analyses were performed using Pearson coefficient or Spearman (*r*) for normal or non-parametric distributions respectively, with 95% confidence interval. Values of $p < 0.05$ were considered significant. Non-significant analyses are presented as supplementary material (SUPPLEMENTARY FIGURES 1-4).

RESULTS

Laboratory data from patients enrolled in the study

Table 1 summarizes the results of important laboratory biomarkers used for clinical follow-up of SCA patients. Heme was included although it is not normally investigated in the clinical management of these patients. The mean plasma concentration (± SD) of heme among patients was 69.6 ± 30.3 μM, with a median (25^{th} – 75^{th}) of 66.1 μM (44.3 – 87.3). Thus, we used the median (50^{th}) and the 25^{th} and 75^{th} percentiles values to investigate the association of heme as a biomarker. For the purpose of analysis, the heme percentiles will be referred as high (≥ 50^{th} percentile) and very high (≥ 75^{th} percentile) concentrations of heme.

Heme is associated with a hemoglobin profile and monocyte count

We observed a association of heme with hemoglobin S (HbS) and fetal hemoglobin (HbF). High concentrations of heme showed increased levels of HbS (p = 0.0003) and decreased levels of HbF (p = 0.0020) (TABLE 1). These associations were maintained at very high concentrations of heme. Similarly, heme had positive correlation with HbS (%) (r = 0.4277; p = 0.0001) and negative correlation with HbF (%) (r = -0.3786; p = 0.0008) (FIGURE 1A-B). We also found the association (≥ 50^{th} percentile: p = 0.0005) and correlation (r = 0.3025; p = 0.0064) of heme with increased monocyte count to high concentrations of heme (FIGURE 1C).

Association of heme with hepatic and hemolytic markers

High or very high levels of heme are significantly associated with increased levels of AST (≥ 50^{th} percentile: p = 0.0077; ≥ 75^{th} percentile: p = 0.0055), total bilirubin (≥ 50^{th} percentile: p = 0.0021; ≥ 75^{th} percentile: p = 0.0031) and indirect bilirubin (≥ 50^{th} percentile: p = 0.0041; ≥ 75^{th} percentile: p = 0.0104) (TABLE 1). In addition, very high levels of heme are also associated with increase of direct bilirubin (≥ 75^{th} percentile: p = 0.0178) (TABLE 1). Similarly, we found positive correlations for AST (r = 0.3008; p = 0.0071), total bilirubin (r = 0.3505; p = 0.0014), direct bilirubin (r = 0.2660; p = 0.0178) and indirect bilirubin (r = 0.2989; p = 0.0071) (FIGURE 1 D-G).

Association of heme with lipid markers

Percentiles with high or very high heme concentrations are strongly associated with increased plasma triglycerides levels ($p < 0.0001$) and VLDL-C ($p < 0.0001$), and inversely associated with HDL-C (≥ 50th percentile: $p = 0.0094$; ≥ 75th percentile: $p = 0.0045$) (TABLE 1). Correlations analysis also demonstrated a strong positive correlation of heme with triglycerides ($r = 0.5954$; $p < 0.0001$) and VLDL-C ($r = 0.6068$; $p < 0.0001$) and a negative correlation with or HDL-C ($r = -0.3596$; $p = 0.0011$) (FIGURE 1 H-J).

Association of haplotypes related to the β^S globin-gene on fetal hemoglobin and free heme

The haplotypes analysis (in descending order) showed CAR/BEN (36.4%), BEN/BEN (27.3%), CAR/CAR (25.8%), CAR/atypical (4.5%), BEN/CAMER (3.0%), CAR/CAMER (1.5%), and BEN/atypical (1.5%) (TABLE 2). The most frequent alleles were BEN (47.7%), CAR (47.0%), atypical (4.0%), and CAMER (3.0%). To investigate the association of haplotypes with HbF expression and free heme, we selected the haplotypes with the highest genotypic frequency (CAR/CAR, BEN/BEN and CAR/BEN) (TABLE 2). The BEN/BEN (11.2 ± 5.9%) and CAR/BEN (10.9 ± 6.3%) haplotypes had significantly higher ($p < 0.05$) HbF levels than CAR/CAR (7.3 ± 5.9%). In contrast, CAR/CAR had higher levels of free heme (79.5 ± 29.7 μM) compared to BEN/BEN (65.9 ± 30.2 μM) and CAR/BEN (64.7 ± 30.1 μM), although these differences were not statistically significant.

High heme concentrations are associated with clinical history of stroke in SCA patients

We investigated a possible heme association with several clinical outcomes of sickle cell anemia based on previous clinical history such as cholelithiasis, transfusion therapy, infections, pneumonia, splenomegaly, splenectomy, cardiac abnormalities, cholelithiasis, cholestecystectomy, and stroke. Patients with a previous history of stroke had an association with high heme levels (FIGURE 2), presenting a mean concentration of 98.2 ± 30.1 μM ($p = 0.0227$), compared to the group of

patients who did not develop previous stroke (67.3 ± 29.3 μM). We did not find associations between heme levels and other clinical outcomes evaluated.

DISCUSSION

In this study, we investigated the association of heme with laboratory markers and clinical outcomes commonly used in the clinical monitoring of patients with SCA, in order to identify a possible contribution in different clinical contexts.

Initially, we demonstrated that increased levels of free heme are associated with increased HbS, and decreased HbF levels. Uzunova and collegues (2010) demonstrated in vitro that the heme concentration greater than 66 μM increases by two orders of magnitude the HbS metastable agglomerates, suggesting that the peaks of free heme concentration in the erythrocytes of patients with SCA may contribute to the complexity of the clinical manifestations of disease. On the other hand, the HbF presents stability and high affinity for oxygen, preventing the initiation of the polymerization of HbS and, consequently, the vaso-occlusive and hemolytic events (AKINSHEYE et al., 2011; STEINBERG et al., 2014). In agreement with these findings, the inverse relationship between heme and HbF suggests that higher levels of circulating heme are due to increased hemolytic crisis and decreased levels of HbF. Although heme release is directly related to hemolysis in SCA, the inverse and significant correlation found between plasma levels of free heme and HbF in the present study was not previously described in other studies, reinforcing the importance of HbF levels in the release of heme and that this molecule may be an indicator of the severity of SCA.

Next, we demonstrate that heme concentrations are associated with increased monocyte counts. The erythroid function is closely related to the biology of macrophages that are derived from the maturation of monocytes and may play important roles in the collection of free heme, iron recycling, erythropoiesis and inflammation (ALAM; DEVALARAJA; HALDAR, 2017). Degradation products from hemolysis may stimulate the production monocytes subsets that recognize the hemoglobin/heme-haptoglobin and hemopexin complexes, via CD163 and CD91 respectively, high affinity scavenger receptors present on the surface of monocytes/macrophages (KRISTIANSEN et al., 2001; MOESTRUP and MOLLER, 2004; HVIDBERG et al., 2005; ROTHER et al., 2005). High levels of heme also stimulate the differentiation of monocytes in iron recycling macrophages in the spleen via Spi-C transcription factor (HALDAR et al., 2014) and acts as an alarmin, stimulating a pro-inflammatory response via TLR4 activating the inflammasome and

secretion of proinflammatory cytokines (FIGUEIREDO et al., 2007; DUTRA et al., 2014; LI et al., 2014).

As expected, heme concentration is strongly associated with markers of hemolysis and hepatic function, with increased production of total bilirubin and fractions (direct and indirect), and AST. Heme is catabolized by heme oxygenase, generating biliverdin, carbon monoxide and iron ferreous (Fe^{2+}). After this step, biliverdin undergoes bilirubin reductase action to form bilirubin (TENHUNEN; MARVER; SCHMID, 1968). Both biliverdin and bilirubin have antioxidant properties, protecting tissues against oxidative insults (ZIBERNA et al., 2016). The increase of indirect bilirubin is related to increased hemoglobin degradation from excessive hemolysis, bile accumulation, and increased risk of cholelithiasis (STEPHENS and SCOTT, 1980; COATS; GARDNER; THEIN, 2014; MARTINS et al., 2017). On the other hand, direct bilirubin may be related to hepatic injury or biliary obstruction. Allied to this, increased AST is usually related to chronic liver damage, although it is not an exclusively hepatic marker and can be found in other tissues such as heart, kidneys, brain and muscles (BOTROS and SIKARIS, 2013). A case control study from our group demonstrated that patients with SCA have increased levels of heme and inflammatory mediators (CARVALHO et al., 2017). Taken together, these findings suggest that heme may contribute systemically to hepatic dysfunction, generating oxidative stress, tissue damage and inflammation, promoting damage in different organs.

We also found a association of heme with lipid markers, suggesting that its pro-oxidant properties may interfere in lipid metabolism in SCA. Heme had a very positive correlation with triglycerides and negative correlation with HDL-C levels. Several studies have demonstrated disturbances in lipid metabolism in patients with SCA or SCD (DJOUMESSI et al., 1994; SHORES et al., 2003; SEIXAS et al., 2010; ZORCA et al., 2010; OZTURK et al., 2013; SOUPENE et al., 2016; ALELUIA et al., 2017), and our research group has suggested the existence of the dyslipidemic subphenotype (SEIXAS et al., 2010; ALELUIA et al., 2017), although the mechanism of this dysfunction is poorly understood. Shalev et al. (2007) have suggested that the hypocholesterolemia observed in patients with chronic anemia is due to increased erythropoietic activity in the marrow, which mobilizes the pool of plasma cholesterol to produce new erythroid cells. HDL-C exerts antioxidant and anti-inflammatory properties by removing excess cholesterol from peripheral tissues and deposited in

the arteries, by reverse transport to the liver to be eliminated (NOFER et al., 2002; SEIXAS et al., 2010), conferring to patients less risk of hemolysis, endothelial dysfunction and inflammation, decreasing the risk of cardiovascular complications (DJOUMESSI et al., 1994; NAVAB et al., 2004) and pulmonary hypertension (ZORCA et al., 2010). In contrast, an antagonistic approach has shown that in oxidizing or inflammatory environments, HDL-C can undergo functional changes and proinflammatory properties (p-HDL) exacerbating oxidative stress (NAVAB et al., 2004; MCMAHON et al., 2006; WATANABE et al., 2007; NAVAB et al., 2009; OZTURK et al., 2013; ATAGA et al., 2015). Increased levels of hemoglobin/free heme from hemolysis and other pro-inflammatory factors in SCD, act as an oxidant challenge to HDL-C and contribute substantially to the formation of its inflammatory form (WATANABE et al., 2007; JI et al., 2016).

Increased levels of triglycerides and VLDL-C are strongly associated with many of these clinical complications (ZORCA et al., 2010). Triglycerides and membrane phospholipids are the primary targets for the hydroxyl ion-mediated attack generated by Fenton reactions mediated by Fe^{2+} and H_2O_2 (GUTTERIDGE, 1984; GARDNER, 1989) with formation of lipid radicals generating lipotoxicity (BIELSKI; ARUDI; SUTHERLAND, 1983; GUTTERIDGE, 1984), and have been correlated with biomarkers of hemolysis, endothelial activation, inflammation, and cardiovascular changes (ZORCA et al., 2010). In addition, chain oxidative reactions can promote the shortening of acyl chains by generating reactive molecules such as polyunsaturated α and β lipid aldehydes, with alterations in the architecture and functional properties of these lipids including ion transport, membrane-bound enzyme activity and and the function of cellular receptors (SPECTOR and YOREK, 1985; HAUCK and BERNLOHR, 2016). Soupene and collegues (2016) have demonstrated that the highly oxidative inflammatory environment of SCD interferes with cholesterol esterification and HDL-C function, which may explain the decrease in their circulating levels. In this context, it can be suggested that the oxidizing microenvironment generated by hemolysis products, may contribute to structural changes in these lipids and to some key point of lipid metabolism, including esterification, transport and/or recognition reactions, modifying the normal function of these molecules in metabolic homeostasis, resulting in the accumulation of triglycerides, decrease of HDL-C, or generating p-HDL.

The next step was to evaluate the contribution of beta-globin gene cluster haplotypes on the production of HbF, and whether they interfere in the release of free heme, since we previously found a negative association between heme and HbF. Patients with BEN/BEN genotype presented higher HbF levels, and CAR/CAR had lower levels. Our results are consistent with other reports (NAGEL et al., 1987; GREEN et al., 1993; GONÇALVES et al., 2003). Our results suggest that the presence of at least one BEN allele has a beneficial effect, even in the presence of the CAR allele, presenting equivalent levels of HbF compared to homozygous for BEN. In assessing the influence of these haplotypes on heme release, we did not observe statistically significant differences among the haplotypes studied, although we observed that individuals with BEN/BEN genotype had higher absolute levels of free heme compared to CAR/CAR, and CAR/BEN that presented intermediate levels.

We also demonstrated that concentrations of heme were significantly elevated in patients with a previous clinical history of stroke. Heme contributes systemically to inflammation generating oxidative stress and inflammation through various pathways favoring the state of hypercoagulability and endothelial dysfunction, which constitutes a fully favorable microenvironment for the development of stroke (MULLER-EBERHARD et al., 1968; WAGENER et al., 1997; REITER et al., 2002; SWITZER et al., 2006; FORTES et al., 2012; SPARKENBAUGH and PAWLINSKI, 2013; DUTRA et al., 2014; DUTRA and BOZZA, 2014; KATO; STEINBERG; GLADWIN, 2017). Further studies are needed to understand whether heme levels are elevated prior to the development of stroke, or whether those levels rise after their occurrence. Thus, considering that the occurrence of stroke is a predisposing factor for the occurrence of new events, and the contribution of heme to systemic inflammation and endothelial dysfunction, we suggest that elevated heme levels constitute a risk factor for the development of stroke, and deserves attention as a possible marker of disease severity prediction.

The combined analysis of these data suggests that the excessive release of heme from recurrent hemolytic events in SCA may contribute substantially to the severity of the disease: *(i)* increasing the auto-oxidation of HbS; *(ii)* systemic inflammation and tissue damage in different organs; *(iii)* interfering with lipid metabolism; *(iv)* increasing the risk for developing stroke (or recurrence).

CONFLICT OF INTEREST DISCLOSURE

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

SSS, MOSC, TNP and MSG conceived and designed the study; MOSC collected the samples, performed the experiments at IGM/FIOCRUZ and at Laboratory of Clinical and Toxicological Analysis of the Faculty of Pharmacy (LACT-FAR) of the Federal University of Bahia (UFBA); SSS and MOSC performed statistical analyses and wrote the paper. TAS, BAVC, NFL and CGB helped with the sample collection and performed the enzyme immunoassays. LCR, IML and VML assist the patients enrolled in the study. TNP and CSAA provided technical support, discussed the results and co-wrote the paper. MBN, VMB and MSG were involved in the design and coordination of the study, providing academic support, co-wrote and critically revised the manuscript. The manuscript has been critically reviewed and approved by all authors.

ACKNOWLEDGMENT

The authors would like to thank SCA patients for trust us and for making possible the realization of this work, and the physicians and laboratory technicians who provided technical support and assistance with the sickle cell patients and with routine laboratory tests. This work was supported by grants from the Brazilian National Council of Research (CNPq) (311888/2013-5) (MSG); the Foundation of Research and Extension of Bahia (FAPESB) (3626/2013, 1431040053063, and 9073/2007) (MS); PPSUS/FAPESB (020/2013 EFP00007295) (MSG); and MCD/CNPq/MS-SCTIE-DECIT (409800/2006-6) (MSG).

TABLE 1. Association of laboratory data and free heme in sickle cell anemia patients.

Markers	SCA N = 80 Median (25^{th} – 75^{th})	Heme free percentiles vs Markers				*p*-value	
		< 50^{th} (66.1 µM) n = 40 Mean ± S.D	**≥ 50^{th} (66.1 µM)** n = 40 Mean ± S.D	**≤ 25^{th} (44.3 µM)** n = 20 Mean ± S.D	**≥ 75^{th} (87.3 µM)** n = 20 Mean ± S.D	***P₁***	***P₂***
Hemoglobin profile							
HbS (%)	88.3 (81.4 – 91.6)	84.1 ± 6.3	89.1 ± 5.3	83.15 ± 5.8	89.3 ± 4.6	**0.0003**	**0.0006**
HbF (%)	7.7 (4.8 – 15.4)	12.0 ± 6.3	7.7 ± 5.1	13.0 ± 5.9	7.6 ± 4.4	**0.0020**	**0.0025**
Hematological plus hemolysis							
RBC (x 10^6 /mL)	2.6 (2.3 – 3.1)	2.9 ± 0.8	2.7 ± 0.6	2.9 ± 0.8	2.7 ± 0.6	0.2351	0.6539
Hemoglobin (g/dL)	7.7 (7.1 – 8.6)	8.2 ± 1.5	7.8 ± 1.3	8.2 ± 1.6	7.8 ± 1.1	0.3243	0.3640‡
Hematocrit (%)	24.1 (21.9 – 26.7)	25.4 ± 4.7	24.1 ± 4.1	25.3 ± 4.9	23.8 ± 3.5	0.1409	0.2986‡
MCH (pg)	29.9 (27.6 – 31.9)	29.1 ± 3.8	29.7 ± 3.3	29.2 ± 3.7	29.3 ± 3.5	0.4391	0.8996‡
MCHC (%)	32.4 (31.9 – 32.9)	32.3 ± 1.1	32.6 ± 0.8	32.4 ± 1.0	32.6 ± 0.8	0.1759	0.3861‡
MCV (fL)	92.5 (85.4 – 97.8)	90.0 ± 10.9	91.1 ± 9.2	90.0 ± 11.3	89.7 ± 9.4	0.7286	0.9111‡
Reticulocyte (%)	8.5 (6.0 – 12.7)	9.6 ± 4.5	8.7 ± 4.9	8.8 ± 4.7	7.8 ± 4.8	0.4115‡	0.5067‡
Erythroblast (%)	1.5 (1.0 – 3.0)	2.5 ± 3.1	1.9 ± 2.2	1.9 ± 2.5	1.7 ± 1.7	0.4640	0.9478
Heme (µM)	66.1 (44.3 – 87.3)	45.4 ± 11.6	93.8 ± 22.9	35.4 ± 6.0	111.5 ± 18.9	----	----
Platelets							
Platelet (x10^3 /mL)	426.0 (343.3 – 539.8)	427.8 ± 132.5	463.5 ± 137.5	404.5 ± 149.2	475.8 ± 135.0	0.4168	0.1213‡
Leukocytes							
WBC (x10^6 /mL)	13,550.0 (11,600.0 – 16,900.0)	14,153.0 ± 5,622.0	14,773.0 ± 3,988.0	14,455.0 ± 6,395.0	13,635.0 ± 2,527.0	0.3414	0.8357
Neutrophil (x10^6 /mL)	5,527.0 (4,059.0 – 7,940.0)	6,252.0 ± 3,762.0	6,660.0 ± 3,192.0	6,701.0 ± 4,336.0	5,940.0 ± 1,866.0	0.3793	0.7734
Eosinophil (x10^6 /mL)	675.0 (410.3 – 1,128.0)	955.9 ± 872.5	929.8 ± 884.4	1,115.0 ± 1,129.0	764.8 ± 401.0	0.7613	>0.9999
Monocyte (x10^6 /mL)	856.0 (682.5 – 1,128.0)	808.2 ± 386.6	1,041.0 ± 418.1	812.0 ± 428.7	951.2 ± 301.3	**0.0005**	0.0532
Lymphocyte (x10^6 /mL)	5,826.0 (4,651.0 – 7,335.0)	6,094.0 ± 2,304.0	6,089.0 ± 1,859.0	5,774.0 ± 2,129.0	5,949.0 ± 1,807.0	0.7503	0.7799‡
Hepatic plus Hemolysis							
AST (U/L)	50.5 (40.0 – 72.7)	47.7 ± 17.5	65.6 ± 32.7	47.4 ± 20.2	74.85 ± 37.7	**0.0077**	**0.0055**
Total Bilirubin (mg/dL)	3.0 (2.1 – 4.2)	2.8 ± 1.3	3.9 ± 1.9	2.2 ± 0.9	4.0 ± 2.3	**0.0021**	**0.0031**
Direct bilirubin (mg/dL)	0.8 (0.5 – 1.0)	0.8 ± 0.6	0.8 ± 0.3	0.6 ± 0.3	0.9 ± 0.3	0.3031	**0.0178**‡
Indirect bilirubin (mg/dL)	2.1 (1.5 – 3.4)	1.9 ± 0.9	3.1 ± 1.9	1.6 ± 0.8	3.2 ± 2.3	**0.0041**	**0.0104**
Iron serum (µg/dL)	107.5 (88.0 – 148.0)	129.4 ± 111.9	132.6 ± 102.6	135.8 ± 156.6	108.6 ± 58.1	0.8013	0.8104
LDH (U/L)	1,077.0 (672.8 – 1,456.0)	985.9 ± 460.0	1,151.0 ± 536.9	919.9 ± 470.2	1,240.0 ± 553.9	0.1444‡	0.0559 ‡
Lipid metabolism							
Triglycerides (mg/dL)	98.0 (77.0 – 127.0)	87.6 ± 21.2	138.8 ± 59.6	79.3 ± 15.6	158.4 ± 64.8	**< 0.0001**	**< 0.0001**‡
Total cholesterol (mg/dL)	121.0 (105.5 – 135.8)	118.4 ± 21.3	125.5 ± 31.9	117.9 ± 19.9	132.5 ± 39.5	0.2388	0.1484‡
HDL-C (mg/dL)	31.0 (24.2 – 38.0)	35.3 ± 10.7	29.3 ± 8.4	37.8 ± 12.3	28.0 ± 7.5	**0.0094**	**0.0045**‡
LDL-C (mg/dL)	63.0 (52.2 – 81.0)	65.15 ± 17.1	71.2 ± 26.6	63.7 ± 17.7	77.8 ± 29.6	0.5901	0.1290
VLDL-C (mg/dL)	20.0 (15.2 – 25.7)	17.5 ± 4.2	27.8 ± 11.8	15.7 ± 3.0	31.5 ± 12.6	**< 0.0001**	**< 0.0001**‡

TABLE 1. Association of laboratory data and free heme in sickle cell anemia patients (continued).

Hepatic							
ALT (U/L)	26.0 (18.0 – 35.5)	26.8 ± 14.6	37.7 ± 33.3	29.9 ± 18.7	40.8 ± 39.3	0.2667	0.5419
Total Protein (g/dL)	7.3 (6.8 – 8.0)	7.1 ± 1.1	7.5 ± 0.8	7.2 ± 0.7	7.6 ± 0.8	0.2919	0.0934‡
Albumin (g/dL)	4.1 (3.5 – 4.4)	3.9 ± 0.9	4,1 ± 0.7	3.9 ± 0.7	4.2 ± 0.7	0.3554	0.2075
Globulin (g/dL)	3.3 (2.7 – 4.0)	3.3 ± 0.93	3.4 ± 0.8	3.3 ± 0.9	3.4 ± 0.6	0.4517‡	0.7029‡
Renal							
Urea (mg/dL)	16.0 (12.2 – 20.7)	17.3 ± 7.6	17.2 ± 6.0	17.3 ± 9.7	17.1 ± 6.6	0.9065	0.6627
Creatinine (mg/dL)	0.4 (0.3 – 0.5)	0.47 ± 0.2	0.4 ± 0.2	0.5 ± 0.3	0.4 ± 0.2	0.4047	0.9947
Inflammation/infection							
C-RP (mg/dL)	3.0 (1.2 – 5.9)	7.0 ± 9.2	6.4 ± 11.0	7.5 ± 9.8	4.2 ± 3.7	0.1457	0.7975
Ferritin (ng/dL)	181.8 (103.0 – 405.8)	299.2 ± 265.1	433.4 ± 455.8	304.1 ± 304.2	360.5 ± 430.1	0.4504	0.6566
A1AT (mg/dL)	162.5 (145.5 – 188.8)	162.9 ± 44.3	168.7 ± 33.8	171.0 ± 42.9	167.2 ± 34.1	0.3714	0.7582‡
ASO (U/mL)	68.0 (25.0 – 125.0)	139.3 ± 160.0	290.1 ± 396.9	150.7 ± 195.6	215.1 ± 236.2	0.9475	0.2250

HbS: hemoglobin S; HbF: fetal hemoglobin; RBC: red blood cells; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; HDL-C: high-density lipoproteins-cholesterol; LDL-C: low-density lipoproteins-cholesterol; VLDL-C: very low-density lipoprotein-cholesterol; AST: aspartate aminotransferase; LDH: Lactate dehydrogenase; ALT: Alanine aminotransferase; C-RP: C-reactive protein; A1AT: alpha-1 antitrypsin; ASO: Antistreptolysin O. Mann-Whitney U-test or ‡*t*-test were used to calculate the difference of significance between the independent groups; *p*-values < 0.05 (in bold) were considered significant. *P1*: *p*-values of < heme 50th percentile vs ≤ 50th; or *P2*: *p*-values of ≤ heme 25th percentile vs ≤ 75th.

TABLE 2. Allelic and genotypic distribution related to the beta-globin gene cluster (*HBB*) haplotypes in sickle cell anaemia and HbF and haem levels based on haplotypes

Allele	Allele Frequency	Haplotype	Genotypic Frequence	% HbF based on haplotype	Haem (µM) based on haplotype	*p*-value‡	
	N = 132 (%)		N = 66 (%)	Mean ± S.D.	Mean ± S.D.	HbF	Heme
CAR	62.0 (47.0)	CAR/CAR	17.0 (25.8)	7.3 ± 5.9	79.5 ± 29.7	----	----
BEN	63.0 (47.7)	BEN/BEN	18.0 (27.3)	11.2 ± 5.9	65.9 ± 30.2	**0.0327**[a]	0.0976[b]
CAMER	3.0 (2.3)	CAR/BEN	24.0 (36.4)	10.9 ± 6.3	64.7 ± 30.1	**0.0307**[a]	0.0892[b]
Atypical	4.0 (3.0)	CAR/CAMER	1.0 (1.5%)	6.0	92.8	i.s.	i.s.
		BEN/CAMER	2.0 (3.0)	3.5	85.1 ± 2.5	i.s.	i.s.
		CAR/Atypical	3.0 (4.5)	0.2 ± 4.6	72.1 ± 33.2	0.3579[a]	0.8298[b]
		BEN/Atypical	1.0 (1.5)	2.8	136.7	i.s.	i.s.
		SCA (all)*	----	9.6 ± 6.1	71.3 ± 30.5	----	----

* Corresponds to the grouping of all haplotypes. ‡Mann-Whitney U-test was used to calculated the diferemce of significance between the independent groups; *p*-values < 0,05 (in bold) were considered significant. i.s.: insufficient samples to perform statistical analysis. HbF: Fetal haemoglobin. CAR: Central African Republic; BEN: Benin; CAMER: Cameron. [a] Comparison of fetal haemoglobin levels with CAR/CAR haplotype. [b] Comparison of free haem levels with CAR/CAR haplotype.

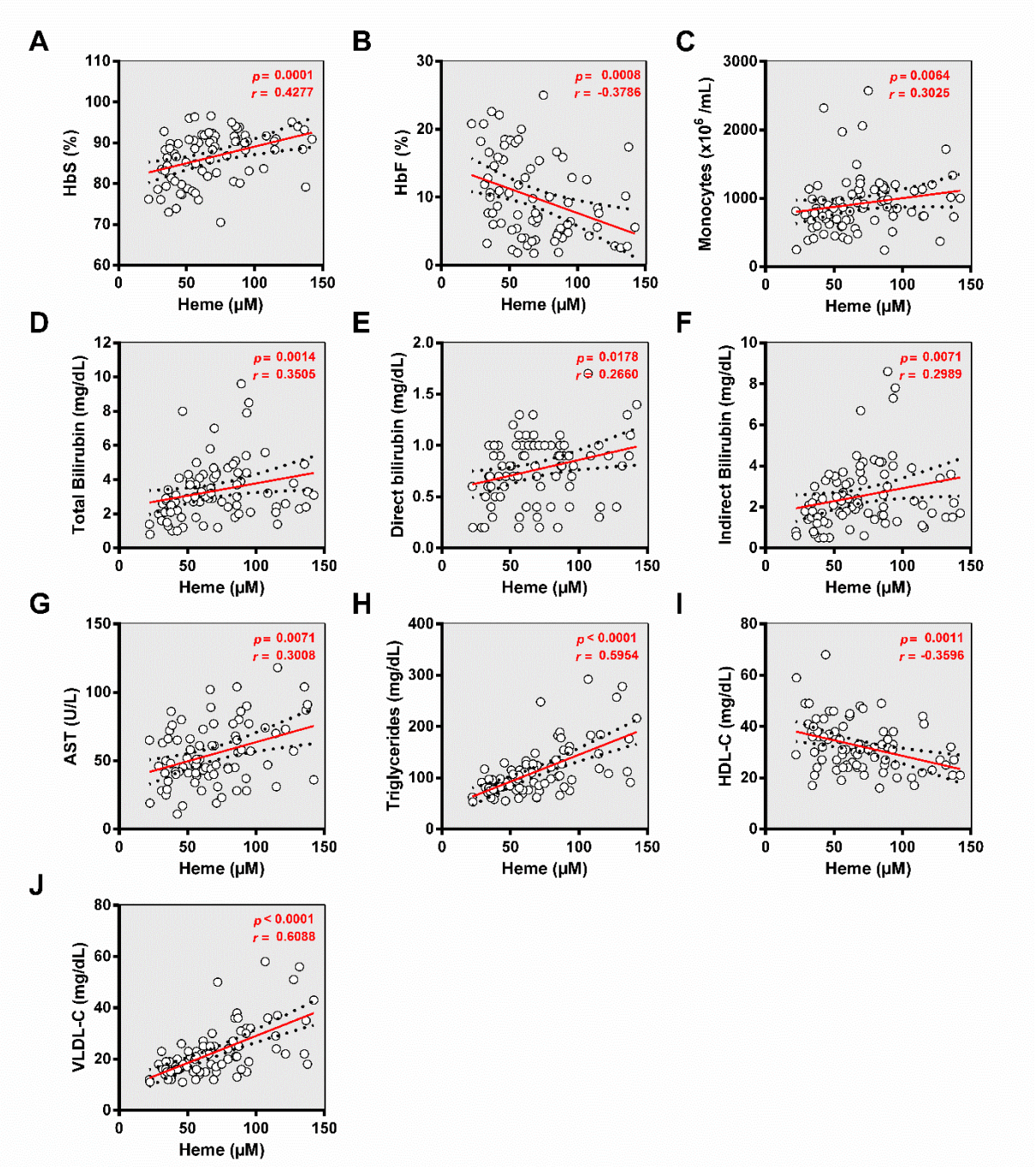


FIGURE 1. Heme correlation analyses with hemoglobin profiles, lipid and hepatic markers in patients with sickle cell anemia. (A) Hemoglobin S (HbS); **(B)** fetal hemoglobin (HbF); **(C)** Monocytes; **(D)** total bilirubin; **(E)** direct bilirubin; **(F)** indirect bilirubin; **(G)** aminotransferase (AST); **(H)** triglycerides; **(I)** high density lipoproteins-cholesterol (HDL-C); **(J)** very low density lipoprotein-cholesterol (VLDL-C). Spearman's correlation coefficient (*r*) was used to determine positive or negative correlations; *p*-value < 0.05 represent the significance of the correlation between the evaluated variables.

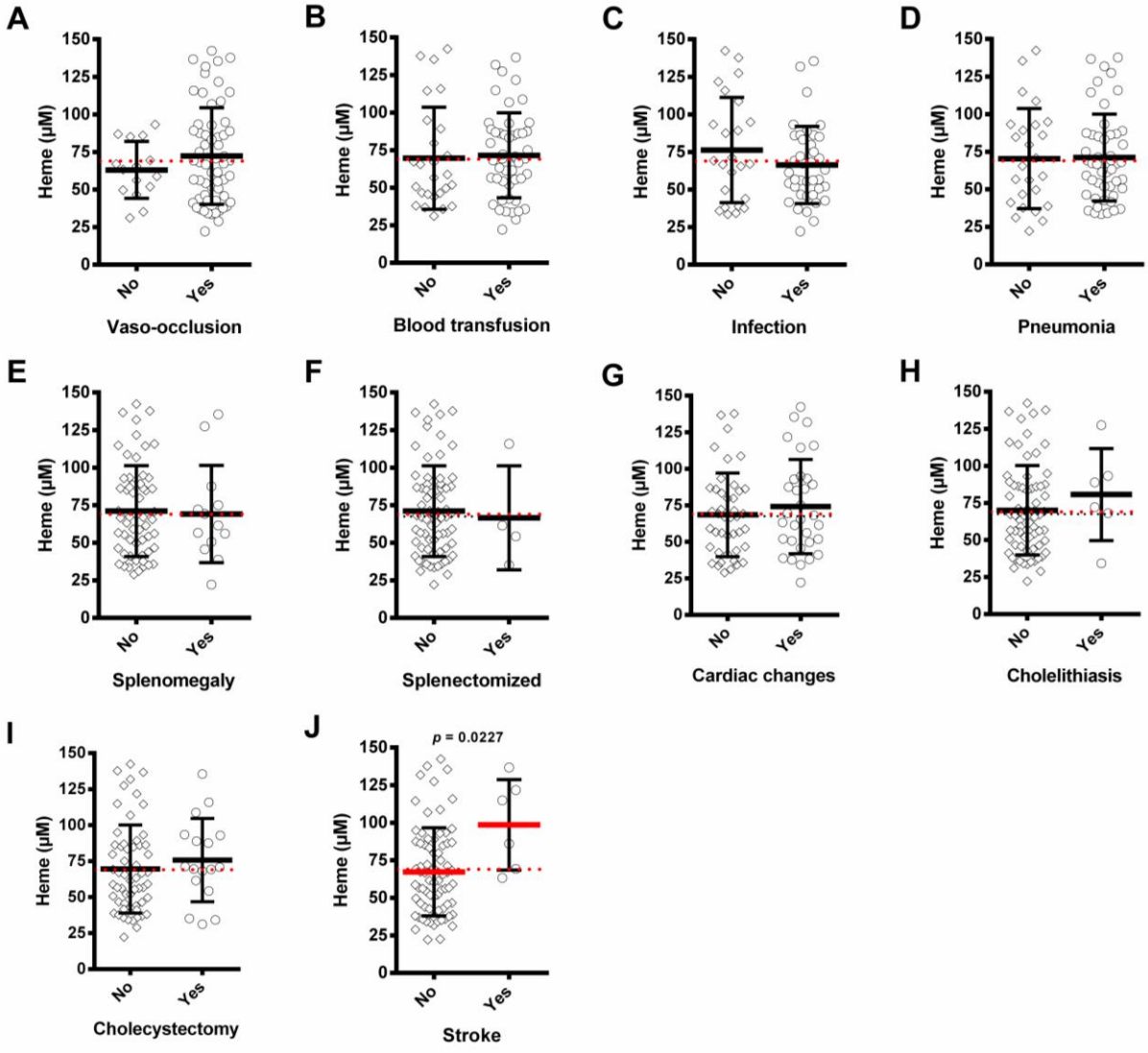
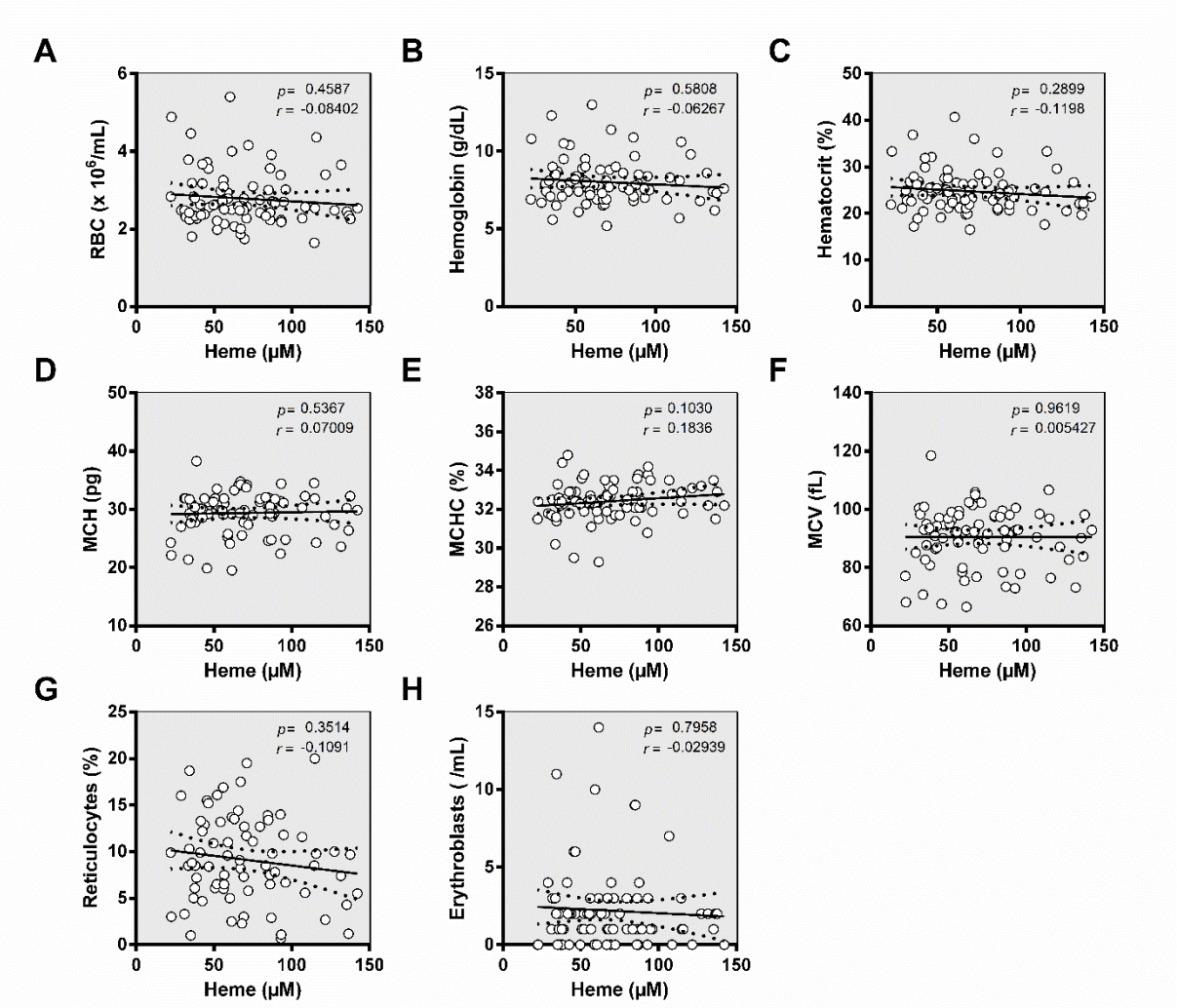
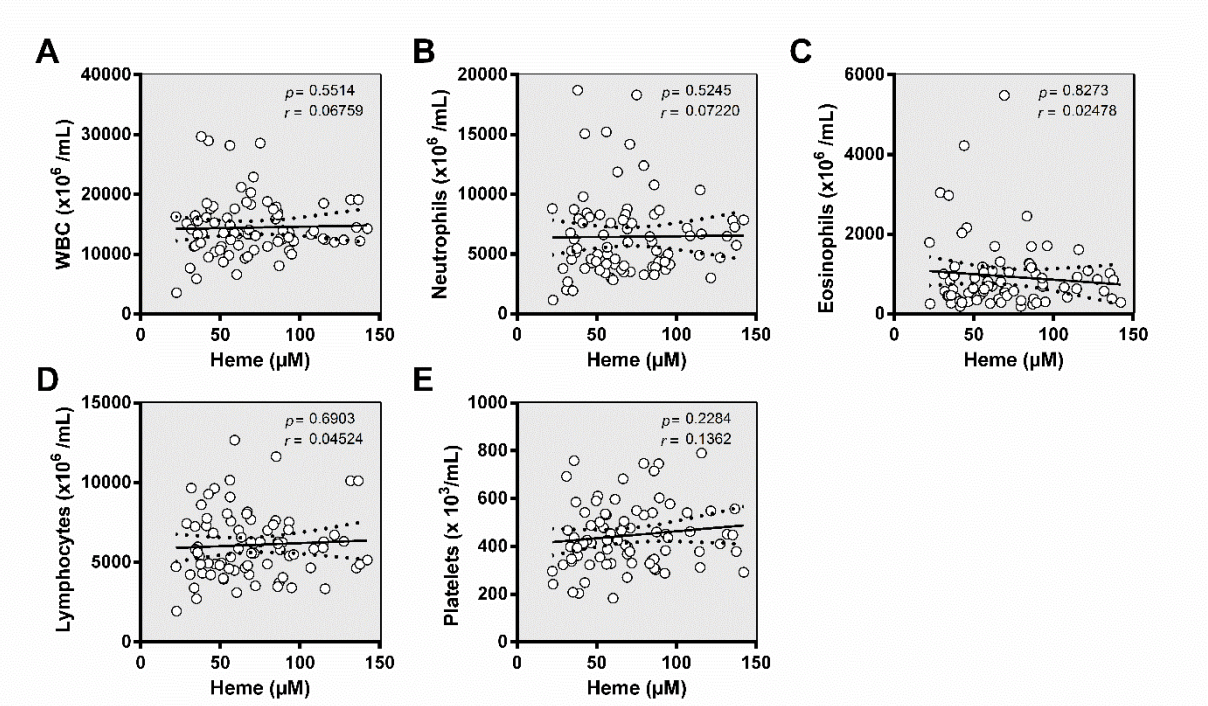


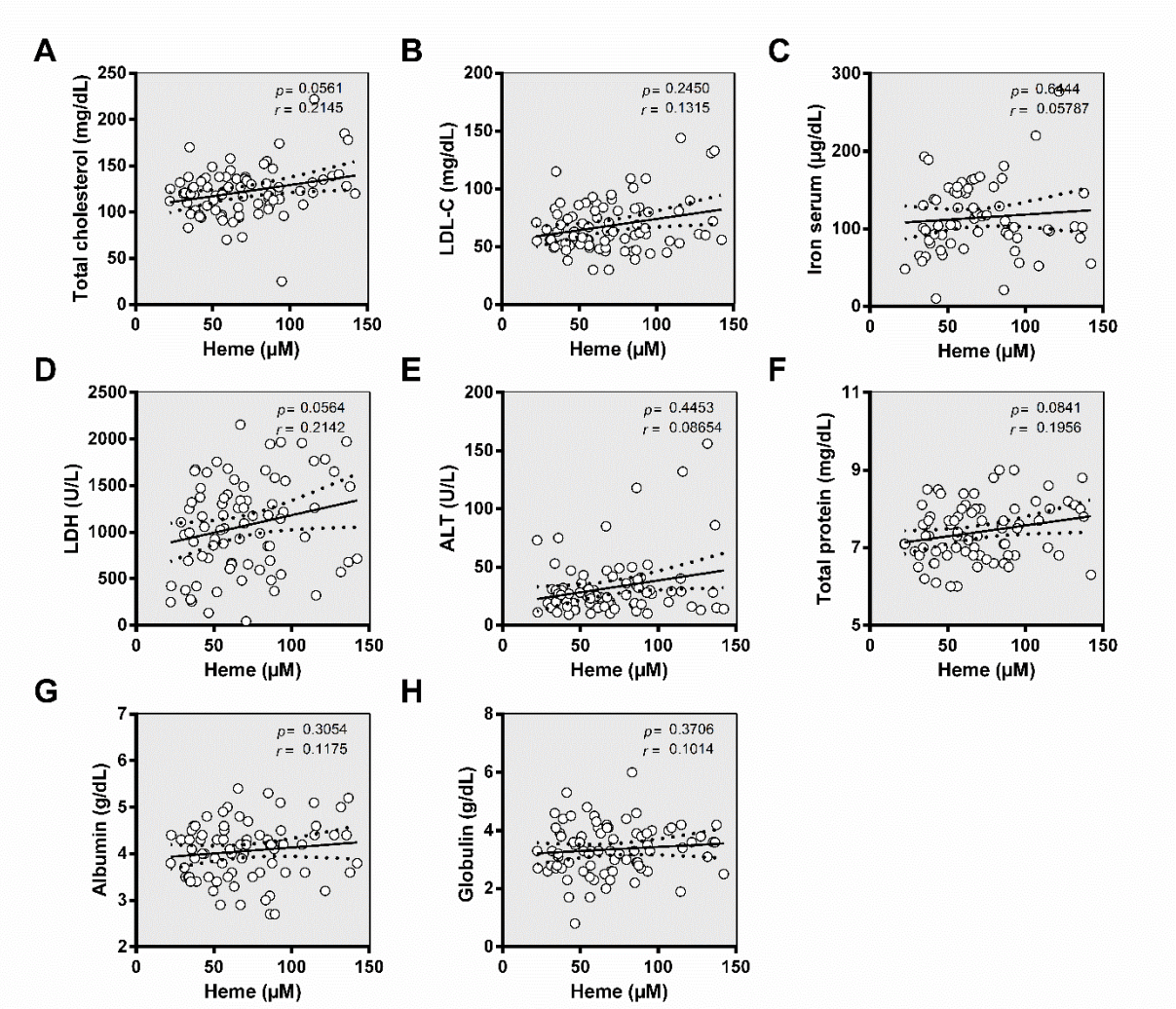
FIGURE 2. Association of free heme concentration with some clinical outcomes of sickle cell anemia. The clinical history of SCA patients (n = 80) was analyzed in relation to the previous occurrence of **(A)** vaso-occlusion, **(B)** blood transfusion, **(C)** infection, **(D)** pneumonia, **(E)** splenomegaly, **(F)** splenectomized, **(G)** cardiac change, **(H)** cholelithiasis, **(I)** cholecystectomy, and **(J)** stroke. Yes: patients with positive outcome; No: patients with negative outcome. Mann-Whitney U-test was used to calculate the difference of significance between the independent groups; *p*-values < 0.05 were considered significant.



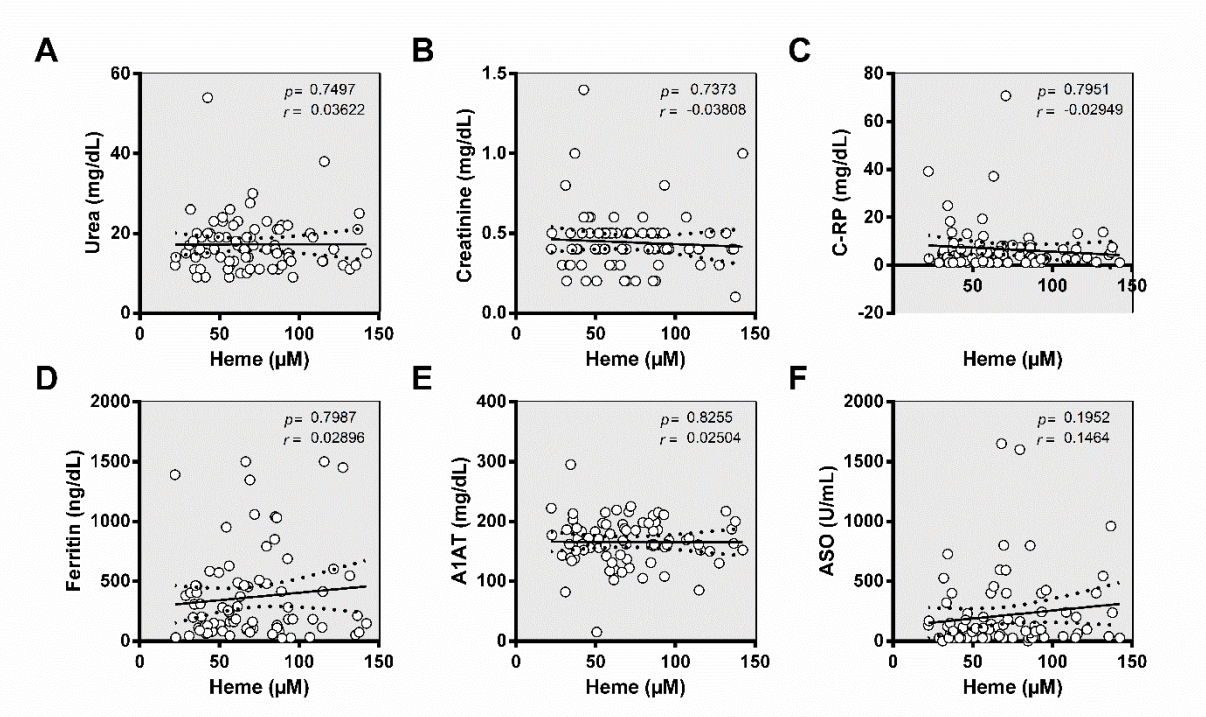
SUPPLEMENTARY FIGURE 1. Free heme correlation analyses with hematological markers. (A) Red blood cells count (RBC); **(B)** hemogobin; **(C)** hematocrit; **(D)** mean corpuscular hemoglobin (MCH); **(E)** mean corpuscular hemoglobin concentration (MCHC); **(F)** mean corpuscular volume (MCV); **(G)** reticulocytes; **(H)** erythroblasts. Spearman's correlation coefficient (*r*) was used to determine positive or negative correlations; *p*-value < 0.05 represent the significance of the correlation between the evaluated variables.



SUPPLEMENTARY FIGURE 2. Heme correlation analyses with leukocytes and platelets. (A) White blood cells (WBC); **(B)** neutrophils; **(C)** eosinophils; **(D)** lymphocytes; **(E)** platelets. Spearman's correlation coefficient (*r*) was used to determine positive or negative correlations; *p*-Value < 0.05 represent the significance of the correlation between the evaluated variables.



SUPPLEMENTARY FIGURE 3. Heme correlation analyses with lipid, hepatic and hemolytic markers. (A) Total cholesterol; **(B)** hemogobin; **(C)** hematocrit; **(D)** low density lipoproteins-cholesterol (LDL-C); **(E)** alanine aminotransferase (ALT); **(F)** total protein; **(G)** albumin; **(H)** globulin. Spearman's correlation coefficient (*r*) was used to determine positive or negative correlations; *p*-value < 0.05 represent the significance of the correlation between the evaluated variables.



SUPPLEMENTARY FIGURE 4. Heme correlation analyses with renal and inflammatory markers. **(A)** Urea; **(B)** creatinine; **(C)** C-reactive protein; **(D)** ferritin (LDL-C); **(E)** alpha-1-antitrypsin (AAT1); **(F)** **a**nti-streptolysin O (ASO). Spearman's correlation coefficient (*r*) was used to determine positive or negative correlations; *p*-value < 0.05 represent the significance of the correlation between the evaluated variables.

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5.2. CAPÍTULO 2 – *Effect of hydroxyurea and sickle red cells on NLRP3 inflammasome and leukotriene B_4*

Objetivo:

Investigar o papel de hemácias falciformes íntegras e lisadas na indução da expressão de componentes e ativação do inflamassoma NLRP3, assim como a produção de LTB_4, e se o tratamento de pacientes com HU interfere nessas vias inflamatórias.

Principais resultados:

Hemácias íntegras e lisadas atuam como DAMPs, induzindo diferentemente a expressão de componentes do inflamassoma e a produção de IL-1β e LTB_4, que contribuem para o estabelecimento da inflamação. Além disso, a ativação do inflamassoma é mais pronunciada após estimulo com hemácias falciformes em relação a hemácias de voluntários sadios. O tratamento com HU não alterou a produção de citocinas pró-inflamatórias como a IL-1β, embora tenha sido comprovada a diminuição na indução do NLRP3 do inflamassoma e secreção de LTB_4.

Situação: a ser submetido.

Effect of hydroxyurea and sickle red cells on NLRP3 inflammasome and leukotriene B_4

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Conflict of Interest
All authors have no conflict of interest to declare.

ABSTRACT

Sickle cell anemia (SCA) is characterized by systemic inflammation with frequent vaso-occlusive phenomena, painful episodes and ongoing haemolysis, with periods of exacerbation of haemolysis. Since erythroid contents can act as danger signals or damage associated molecular pattern molecules (DAMPs) activating NLR family pyrin domain containing 3 (NLRP3)-inflammasome platform pathways. Thus, this study tested the hypothesis that sickle red blood cell (SS-RBC) can induce inflammasome NLRP3 components such as *NLRP3*, *CASP1*, *IL1B* and *IL18* gene expression in peripheral blood mononuclear cells (PBMCs) as well as interleukin-1β (IL-1β) and leukotriene B_4 (LTB_4) production. Additionally, we investigated the effect of hydroxyurea (HU) treatment in these inflammatory markers. PBMCs from healthy donors (AA-PBMC) were challenged with intact and lysed RBCs from SCA patients (SS-RBC) and from healthy volunteers (AA-RBC). *NLRP3*, *IL1B*, *IL18* and Caspase-1 gene expression levels were assessed by quantitative PCR (qPCR). IL-1β and LTB_4 protein levels were measured by ELISA. We observed that lysed SS-RBC induced the expression of inflammasome NLRP3 components, but this increase was more prominent for CASP1 and IL18 expression levels. Moreover, we observed that intact SS-RBC induced higher production of IL-1β and LTB_4 than lysed SS-RBC. Although SCA patients treated with HU have reduction in NLRP3 gene expression and LTB_4 production, this treatment did not modulate the expression of other inflammasome components or IL-1β production. Thus, our data suggest that caspase-1, IL-1β and IL-18 may contribute to the inflammatory status observed in SCA and that HU treatment may not interfere in this inflammatory pathway.

Keywords: Sickle cell anemia, NLRP3-inflammasome, hemolysis, hydroxyurea, heme.

LIST OF ABBREVIATIONS

AA	Arachnoic acid
AA-PBMC	Peripheral blood mononuclear cells from healthy donors
AA-RBC	Red blood cell from healthy individuals
ASC	Apoptosis-associated speck-like protein containing card
β^S	Beta(S)-globin
BEN	Benin haplotype linked to beta-globin gene cluster
CAR	Central African Republic haplotype linked to beta-globin gene cluster
CASP-1	Caspase-1
DAMPs	Damage-associated molecular pattern molecules (DAMPs)
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
H_2O_2	Hydrogen peroxide
HbF	Fetal hemoglobina
HMGB1	High-mobility group box protein B1
HU	Hydroxyurea
HV	Healthy volunteers
IL	Interleukin
IL1B	IL-1β gene
IL-1β	Interleukin 1β
LTB_4	Leukotriene B_4
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptors
NLRP3	NOD-like receptor family, pyrin domain containing 3
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
RBC	Red blood cell
SCA	Sickle cell anemia
SCD	Sickle cell disease
SS-RBC	Red blood cells from sickle cell anemia patients
SS-RBC	Red blood cells from sickle cell anemia patients
TNF	Tumor necrosis fator

INTRODUCTION

Sickle cell anemia (SCA) is a molecular disorder characterized by systemic inflammation with frequent vaso-occlusive phenomena and ongoing hemolytic anemia. Patients present a wide range of clinical manifestations related to the pathophysiological mechanism, such as vaso-occlusive and painful episodes, increased susceptibility to infections and intravascular hemolysis which contribute to the chronic inflammatory status (STEINBERG, 2001; TAYLOR et al., 2008).

Free hemoglobin and heme released during intravascular hemolysis are able to activate several cells, including endothelial cells and leukocytes, leading to systematic inflammation, painful crises and vascular injury (BELCHER et al., 2003; WOOD; HEBBEL; GRANGER, 2004). Cellular residues, endogenous molecules, such as leukotriene B4 (LTB_4) (SETTY e STUART, 2002; PITANGA et al., 2016) and inflammatory cytokines (VICARI et al., 2015), released during intravascular hemolysis, are able to act as signals for tissue injury, and are recognized by NOD-like receptors (NLRs) (DUTRA et al., 2014). High-Mobility Group Protein B1 (HMGB1) and heme are danger-associated molecular patterns (DAMPs) considered to be NLRs agonists (DUTRA et al., 2014; GLADWIN and OFORI-ACQUAH, 2014; GUPTA, 2014; XU et al., 2014).

The LTB_4 synthesis from arachidonic acid is catalyzed by 5-lipoxygenase and Leukotriene-A4hydrolase (LTA4H) that is mainly produced by leukocytes during inflammatory response. LTB_4 is able to recruit and activate neutrophils, monocytes and eosinophils (SETTY and STUART, 2002).

The NLR family pyrin domain containing 3 (NLRP3) inflammasome is expressed in a variety of cell types, including neutrophils, dendritic cells, epithelial cells, monocytes and T lymphocytes. Upon activation, NLRP3 recruits the apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) (ASC) adaptor protein and pro-caspase-1 in a process that requires participation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), as a first-step of NLRP3 activation, and NLRP3 oligomerization itself as a second-step of activation, in order to convert pro-caspase-1 into its active form. Then, active caspase-1 converts the inflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) into active forms, which consist of cytokines associated with the innate immune response in both infection and aseptic inflammation,

contributing to the pathology severity (MARTINON; BURNS; TSCHOPP, 2002; AGOSTINI et al., 2004; MARIATHASAN e MONACK, 2007; BAUERNFEIND et al., 2009; BAUERNFEIND et al., 2011; ZHOU et al., 2011; GHONIME et al., 2014).

Levels of IL-1β and lL-18 or polymorphisms of *IL1B* and *IL18* have been associated to clinical inflammatory conditions, such as multiple sclerosis, cancer, Alzheimer's disease, arthritis and SCA (GUMA et al., 2010; CERQUEIRA et al., 2011; KITAZAWA et al., 2011; PITANGA et al., 2013; RAMIREZ-RAMIREZ et al., 2013; KETELUT-CARNEIRO et al., 2015; TAS et al., 2015; VICARI et al., 2015; PITANGA et al., 2016), contributing to the disease severity, and to the therapeutic failure (ASARE et al., 2010; CERQUEIRA et al., 2011; PITANGA et al., 2013; VICARI et al., 2015).

The therapy with hydroxyurea (HU) seems to be the best treatment for SCA patients, since it increases fetal hemoglobin (HbF) levels and reduces leukocyte count. HU exhibits its pharmacological effect by inhibiting ribonucleotide reductase enzyme. The treatment with HU leads to fewer symptoms, less severe hemolytic anemia and lower mortality (DE MONTALEMBERT et al., 2006; PLATT, 2008; LEBENSBURGER et al., 2010). In a previous study the HU therapy was associated with high tumor necrosis factor (TNF) levels and increased plasma levels of the anti-inflammatory IL-10 (LANARO et al., 2009).

A previous study from our group has shown that intact sickle red blood cells (SS-RBC) induce the production of inflammatory mediators and gene expression of NLRP3 inflammasome components in peripheral blood mononuclear cells (PBMC) (PITANGA et al., 2016). This study is a sequence of a previous article from our group in this subject, from which the main difference is the addition of lysed RBCs, as an attempt to compare the effects of the release of internal RBC content with the effects of intact RBCs on NLRP3 inflammasome. Other difference is that now we work with whole blood from SCA patients treated and untreated with HU, to evaluate whether inflammasome components gene expression are modulated by this pharmacotherapy.

Based on these findings, our group decided to evaluate the gene expression of *NLRP3*, *CASP1*, *IL1B* and *IL18* and production of IL-1β and LTB_4 in AA-PBMC challenged with intact or lysed RBC isolated from both SCA patients and healthy individuals. In addition, we investigated whether SCA patients treated with HU have a

different profile of gene expression and inflammatory mediators' production in comparison to untreated patients.

METHODS

Patients and healthy volunteers

Patients were recruited from the Bahia Hematology and Hemotherapy Foundation (HEMOBA), while healthy volunteers (individuals without hematological disorders or inflammatory conditions) were enlisted from the Faculty of Pharmaceutical Sciences (FacFAR) of the Federal University of Bahia (UFBA). A cross-sectional study included 137 children with SCA in steady state and 30 healthy volunteers. None of the included patients required blood transfusion four months prior to blood draw nor presented any evidence of infection, vaso-occlusive events or hospitalizations. Although all patients were being treated with folic acid, none reported taking antibiotics, steroidal or nonsteroidal anti-inflammatory drugs. A total of 29 patients were on HU therapy with initial single dose of 17 mg/kg/day. The demographic, hematological and biochemical parameters of both groups are shown in Table I. This study was conducted in accordance with the Helsinki Declaration of 1975, and its revisions, and received approval from the Institutional Review Board of the Oswaldo Cruz Foundation–FIOCRUZ, Bahia-Brazil (CAAE: 04733612.7.0000.0040). All study subjects or their legal guardians were properly advised about the research, agreed with the biological sample collection and signed a term of informed consent.

Blood sampling, isolation of human peripheral mononuclear and red blood cells

Venous blood collected from SCA patients in steady state and from healthy volunteers (HV) were subjected to hemoglobin profiling by high-performance liquid chromatography, as well as to obtain RBC for experimentation and PBMCs for gene expression analysis.

PBMCs were obtained from the peripheral blood of a healthy donors by gradient centrifugation using Ficoll-Hypaque (GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA) as previously described (PITANGA et al., 2016). PBMC cultures were resuspended in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL

penicillin and 100 µg/mL of streptomycin (Gibco, Grand Island, NY, USA). PBMCs were then counted, distributed in a 24-well plate (Costar, Corning, NY, USA) at a concentration of 3 x 10^6 mononuclear cells/mL and challenged with 3% of lysed or intact RBCs for overnight incubation at 37°C with 5% CO_2.

Lysed RBC were obtained using a previously described technique (GAO et al., 2014; PITANGA et al., 2016) with minor modifications. Briefly, whole blood was collected and centrifuged at 500 g for 10 min at 4 °C. Plasma was removed by aspiration and discarded. The RBC pellet was washed 3 times in sterile phosphate buffered saline (PBS) (pH 7.3) for 5 min at 170 g. The RBC concentrate was evaluated for leukocyte and platelet contamination by staining with trypan blue and RBCs under 97% of purity were not considered. A phase contrast microscope (Olympus CK2, Center Valley, PA, USA) was used for examination and images were obtained via Image Pro Plus 6.1 software (Media Cybernetics, Rockville, MD, USA). RBCs were lysed by freezing concentrate in liquid nitrogen for 10 minutes, followed by three thawing cycles consisting of 5 minutes at 37 °C.

Heme and H_2O_2 solutions

The experimental heme solution was prepared by diluting bovine hemin (Sigma, St. Louis, MO, USA), according to a previously described technique (DA GUARDA et al., 2016). A previous study performed in our laboratory found an average heme plasma concentration of 28.5 µM in healthy subjects, and 68.6 µM in HbSS patients (DA GUARDA et al., 2006), which led us to use 70 µM of heme in the experiments herein. Hydrogen peroxide (H_2O_2) (Proquimios, RJ, Brazil) was used at a concentration of 20 µM, identical to what has been described in inflammatory conditions (PITANGA et al., 2014).

Leukotriene-B_4 and IL-1β production

Supernatants were collected from in vitro cultured PBMCs challenged with lysed or intact RBCs. EIA was used to detect LTB_4 using Leukotriene B_4 EIA kit in accordance with manufacturer instructions (Cayman Chemical Company, MI, USA). IL-1β production was assessed using IL-1β ELISA kits (R&D Systems, Minneapolis, USA) in accordance with manufacturer instructions. Serum and supernatant LTB_4

and IL-1β dosages were similarly conducted for both HU-treatead and untreated SCA patients.

Determination of β^S haplotypes

The beta(S)-globin gene haplotypes CAR (Central African Republic) and BEN (Benin) were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques in according to previously study (ADORNO et al., 2004).

Gene expression quantification

Total RNAs were obtained with the use of Trizol reagent (Invitrogen, Life Technologies, CA, USA), according to the manufacturer's recommendations, and quantified using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). RNAs were used in the reverse transcription reactions using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, CA) in accordance with the manufacturer's instructions. Primer sequences were: *NLRP3* (Fw: *TGC CCC GAC CCA AAC C*; Rev: *GAA GCC GTC CAT GAG GAA GA*), *IL1B* (Fw: *AGC TAC GAA TCT CCG ACC AC*; Rev: *CGT TAT CCC ATG TGT CGA AGA A*), *IL18* (Fw: *ATC GCT TCC TCT CGC AAC A*; Rev: *TCT ACT GGT TCA GCA GCC ATC TT*), *Caspase-1* (Fw: *AAA AAA TCT CAC TGC TTC GGA CAT*; Rev: *TCT GGG CGG TGT GCA AA*), *GAPDH* (Fw: *CAC ATG GCC TCC AAG GAG TAA*; Rev: *TGA GGG TCT CTC TCT TCC TCT TGT*) and *β-ACTIN* (Fw: *CCT GGC ACC CAG CAC AAT*; Rev: *GCC GAT CCA CAC GGA GTA CT*).

Gene expression was analyzed by quantitative PCR (qPCR) using an ABI 7500 FAST Real-Time PCR system (Applied Biosystems TM, Foster City, CA). Amplification reactions were performed using Power SYBR® Green (Applied Biosystems TM, Foster City, CA), also in accordance with the manufacturer instructions. Cycle threshold (Ct) values were obtained using the Operational Program 7500™ System (Applied Biosystems™, Foster City, CA, USA). Intra-assay precision and normalization of expression levels were performed according to previously study (PITANGA et al., 2016). Relative expression folds were calculated based on $2^{-\Delta\Delta Ct}$ method (PFAFFL, 2001; PITANGA et al., 2016).

Statistical analysis

Comparisons of quantitative variables between groups were made using the Mann-Whitney U-test for non-normally distributed data. The non-parametric Kruskal-Wallis test was used to compare among three or more groups. Values of $p < 0.05$ were considered significant. All data were analyzed using Prism 5.1 software (GraphPad, San Diego, USA).

RESULTS

Hematological and biochemical markers in SCA patients under hydroxyurea treatment

Data regarding the hematological and biochemical markers of SCA patients either in steady state, i.e., untreated (n = 108), or treated (n = 29) with HU, and healthy volunteers (HV, n = 30) are represented in Table 1. HU treatment was able to reduce levels of heme (p = 0.0247) and increase ferritin (p = 0.0103) and C-Reactive Protein (p = 0.0010) levels. There was no statistically significant difference in fetal hemoglobin between the groups treated and not treated with HU. However, patients with Central African Republic (CAR) haplotype and treated with HU presented HbF levels increased suggesting that this haplotype influences the improve of this marker production in response to HU.

Intact and Lysed SS-RBC induce the expression of NLRP3 inflammasome components

To compare the impact of intact and lysed RBC on the induction of NLRP3 inflammasome component gene expression, PBMCs were challenged with intact or lysed SS-RBC or with AA-RBCs. Our results show that the PBMCs stimulated with intact AA-RBC did not exhibit any significant expression of *NLRP3*, *CASP1*, *IL1B* or *IL18*, in comparison to the basal expression of these receptors (FIGURE 1). Lysed AA-RBCs induced significantly higher expression of *NLRP3* (4.57 ± 1.45-fold; p = 0.022) and *CASP1* (3.94 ± 0.67-fold; p = 0.0179), but lower *IL1B* (3.69 ± 1.91-fold; p = 0.0119) and *IL18* (0.26 ± 0.06-fold; p = 0.0238) gene expression when compared with unstimulated PBMC cultures (0.38 ± 0.06-fold).

To further investigate any discrepancies between hemolytic and nonhemolytic state *in vitro*, PBMCs were challenged with intact or lysed SS-RBC under the same conditions described above. As shown in Figure 1, stimulation with both intact and lysed SS-RBC induced significant expression of all the NLRP3 inflammasome components. Furthermore, we found substantially increased *CASP1* (FIGURE 1B) and *IL18* (FIGURE 1D) gene expression in the PBMCs challenged with lysed SS-RBC populations, as compared to intact SS-RBC.

In general, the challenge of PBMCs with heme and H_2O_2 induced the gene expression *NLRP3* inflammasome components. However, no significant changes in *CASP1* gene expression were observed upon stimulation with H_2O_2 (0.85 ± 0.43-fold; $p = 0.1000$), or in *IL18* expression under heme (0.57 ± 0.56-fold; $p = 0.6286$).

Lysed SS-RBC induce the production of IL-1β and LTB_4

Supernatants from cultured PBMCs stimulated with both intact (265.20 ± 95.17 pg/mL; $p = 0.0179$) and lysed (92.97 ± 22.37 pg/mL; $p = 0.0286$) SS-RBCs showed increased production of IL-1β (FIGURE 2A) when compared with unstimulated PBMC cultures (11.62 ± 2.98 pg/mL). No statistically significant differences were seen in the levels of IL-1β in response to intact (21.54 ± 5.93 pg/mL; $p = 0.1000$) or lysed (71.65 ± 10.46 pg/mL; $p = 0.1000$) AA-RBCs when compared to unstimulated cell cultures.

Both intact and lysed SS-RBC and AA-RBC were found to induce LTB_4 production in PBMCs (FIGURE 2B). Significant statistical differences were observed when comparing LTB_4 production upon stimulation with intact (3.19 ± 0.27 pg/mL; $p = 0.0275$) and lysed (4.02 ± 0.63 pg/mL; $p = 0.0238$) AA-RBC, as well as between intact (6.08 ± 2.29 pg/mL; $p = 0.0286$) and lysed (2.33 ± 0.34 pg/mL; $p = 0.0290$) SS-RBC. Moreover, enhanced LTB_4 production was detected in PBMCs stimulated with intact SS-RBC in comparison to those stimulated with lysed SS-RBC ($p = 0.0286$).

In addition, stimulation of cultured PBMCs with heme and H_2O_2 induced the production of IL-1β and LTB_4 by these cells.

HU treatment affects NLRP3 gene expression and LTB_4 production

A comparison of gene expression between untreated and treated SCA patients (FIGURE 3) revealed that *CASP1*, *IL1B* and *IL18* were unaffected by HU treatment. However, the patients treated with HU exhibited decreased *NLRP3* gene expression (6.25 ± 1.36-fold; 3.30 ± 1.49-fold, $p = 0.0245$).

The Figure 4 illustrates that SCA patients treated with HU showed no changes in IL-1β (6.43 ± 1.53; 7.50 ± 1.51, $p = 0.2632$); however, HU treatment did induce a decrease in LTB_4 (921.80 ± 454.20; 540.00 ± 129.80, $p = 0.0245$), reaching levels similar to those seen in healthy volunteers.

DISCUSSION

The NLRP3 inflammasome is the most common and fully characterized inflammasome and has been shown to be activated by several endogenous dangers signals, such as heme and hemolysis products (SCHRODER and TSCHOPP, 2010; DUTRA et al., 2014). In the activation of the inflammasome, and if the treatment of the HU patients interfere in its activation in the context of SCA. Our results showed that SS-RBCs can act as DAMPs, as indicated by the induction of the expression of NLRP3 inflammasome components by the stimulated PBMCs. When comparing the effects of lysed and intact SS-RBC, we observed a higher induction of *CASP1* and *IL18* gene expressions with lysed SS-RBC than with intact SS-RBC. On the other hand, the comparison between intact and lysed AA-RBC showed that only lysed AA-RBC were able to induce the gene expression of these NLRP3 inflammasome components. Although further studies are needed, our results suggest that erythrocyte composition differs between AA-RBCs and SS-RBCs regarding inflammasome pathway activation.

In compare to healthy donors, sickle RBCs present loss of lipid symmetry with increased expression of phosphatidylserine and adhesion molecules such as ICAM-4, CD44 and CD47, loss of the red cell membrane fluidity, and increased susceptibility to oxidative damage (DE OLIVEIRA and SALDANHA, 2010). Ren and collegues (2006) previously demonstrated that RBCs from SCA patients show an increase in arachidonic acid (AA), and a decrease in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in compare to RBCs from healthy volunteers suggesting that RBC alterations on membrane could justify the contrast observed between healthy individuals and SCA patients. These previous findings may reinforce the differences between AA-RBCs and SS-RBC, and intact and lysed RBCs.

A different behavior was observed for *IL1B,* since PBMCs challenged with intact SS-RBC showed higher *IL1B* gene expression levels than those challenged with lysed SS-RBC. Despite the observed effects of lysed AA-RBCs on *IL1B*, *CASP1* and *NLRP3* expression, it is important to consider that significant intravascular hemolysis does not occur physiologically in healthy volunteers, whereas a large proportion of SCA patients exhibit this condition (TAYLOR et al., 2008; VILAS-BOAS et al., 2010). Hence, the inflammation observed in SCA patients could be a consequence of constant exposure to DAMPs originated from intact or lysed RBCs (DUTRA et al.,

2014) during intravascular hemolysis, which leads to the increased expression of NOD-like receptors and can promote leukocyte recruitment in a mechanism dependent of inflammasome components.

In addition to assess gene expression, we also evaluated the protein levels of IL-1β and LTB_4 in the supernatant from PBMC challenged with SS-RBC. We observed that both intact and lysed SS-RBC were able to induce IL-1β production, while neither lysed AA-RBC nor intact showed this effect. This finding suggests that IL-1β induction may be related to DAMPs found only in SS-RBCs.

LTB_4 is an inflammatory mediator that has previously shown to be increased in SCA patients (SETTY and STUART, 2002; PITANGA et al., 2016). LTB_4 acts as a chemoattractant that enhances leukocyte migration and adhesion to vascular endothelium, promoting the inflammatory scenario in these patients. Our results show that both AA-RBC and SS-RBC were able to induce LTB_4 production. However, intact SS-RBC were able to induce a higher LTB_4 production than lysed SS-RBC did, reflecting differences between these conditions. Despite initial concerns over hemolysis products, such heme, increase LTB_4 levels (MONTEIRO et al., 2011), our results suggesting that intact SS-RBC membrane components may show an increased ability to induce LTB_4 production.

Since hematological and biochemical markers are known to differ substantially between SCA individuals and healthy volunteers, we evaluated the effect of HU treatment with respect to these parameters. In SCA, the main therapeutic target consists of attempting to shift hemoglobin production from sickle hemoglobin to fetal hemoglobin. HU yields therapeutic benefits by increasing HbF levels, improving red cell rheology and reducing red cell adhesion (KUMKHAEK et al., 2008; PLATT, 2008). It is known that the main effect of HU is attributable to increases in HbF, which aids the inhibition of HbS polymerization. However, in the present study, there was no significant difference in HbF between the groups treated and not treated. It is known that $ß^S$ gene haplotypes have an important role in the fetal Hb synthesis. Some studies have shown that this HbF has the highest concentration in patients with Benin (BEN) haplotype in compared to Central African Republic (CAR) haplotype (ADORNO et al., 2004; REZENDE et al., 2016). This could justify the greater responsiveness to treatment with HU of patients with only CAR haplotype (CAR/CAR, p = 0.042) in compare to CAR/BEN (p = 0.9150) or BEN/BEN (p =

0.9990). Taken together, these results demonstrate the ability of HU treatment to improve HbF production in SCA patients with CAR haplotype.

As expected, levels of heme and C-reactive protein were higher in SCA patients compared to healthy volunteers (REITER et al., 2002; MOHAMMED et al., 2010; REES and GIBSON, 2012). Moreover, we showed that HU treatment was able to reduce levels of heme and increase C-reactive protein levels. It is well known that free heme has pro-oxidant and pro-inflammatory properties (RYTER et al., 2002), and that the levels of C-RP is associated with endothelial injury and the chronic inflammatory state observed in SCA patients (REES and GIBSON, 2012). Thus, this finding may support the hypothesis that SCA patients may have persistent inflammation even under treatment with HU, since this treatment does not interfere with all inflammatory mechanisms.

Ferritin is a biomarker found to be elevated in inflammation and autoimmune diseases, such as systemic lupus erythematosus (VANARSA et al., 2012) and in SCA vaso-occlusive crisis and infections (AL-SAQLADI; BIN-GADEEM; BRABIN, 2012). We observed that the variation of ferritin levels in patients treated with HU and untreated was very high. Subjects submitted to HU therapy usually have a more severe clinical history, which usually improves significantly after therapy. However, iron released in the previous hemolytic crisis associated with the transfusional regime at the time of life, promote iron accumulation with an increase in plasma levels of ferritin (ADAMKIEWICZ et al., 2009; BOURBON FILHO et al., 2011; BARCELLINI and FATTIZZO, 2015), which may explain the great variation between the two groups, and the increase observed in treated patients with HU. In addition, this large variation observed among patients using or not using the drug can be explained by clinical heterogeneity of SCA.

Regarding the NLRP3 inflammasome analysis performed with whole blood-derived mRNA from SCA patients untreated in comparison with those treated with HU, we observed that HU treatment did not interfere in the expression of *CASP1*, *IL1B* and *IL18*, suggesting that HU may not affect inflammasome-dependent inflammatory conditions observed in SCA patients, as a previous work from our group has shown, that *in vitro* PBMCs challenged with SS-RBCs and HU did not stimulate *NLRP3* inflammasome gene expression (PITANGA et al., 2016). However, herein, our results suggested that SCA patients treated with HU decrease *NLRP3* gene expression. HU treatment did not change IL-1β levels in SCA patients, yet it

decreased LTB_4 serum levels. LTB_4 is an important chemoattractant for neutrophils (MONTEIRO et al., 2011). Thus, reduced *NLRP3* gene expression and levels of LTB_4 may suggest a better prognostic, demonstrating that HU therapy can decrease inflammation by these pathways.

Our previous studies have demonstrated that PBMCs from SCA patients exhibited high expression of *NLRP3* and *IL1B,* and that cultured AA-PBMC challenged with intact SS-RBC exhibited increased gene expression of components of the NLRP3 inflammasome (PITANGA et al., 2016). The present study observed that the NLRP3 inflammasome is modulated by lysed SS-RBC, as well as by HU treatment. Herein we show that AA-PBMC challenged with lysed SS-RBC presented increased *caspase-1* and *IL18* gene expression, as well as increased IL-1β and LTB_4 levels; moreover, patients treated with HU showed reduced NLRP3 gene expression in whole blood-derived mRNA, in addition to lower levels of LTB_4. The present study serves to complement a previous report by our group in that PBMCs were presently challenged with lysed SS-RBC, versus intact SS-RBC previously, and the results of each investigation are compared herein.

This study showed novel evidences for NLRP3-inflammasome platform as an important inflammatory pathway in SCA, that may contribute to the clinical inflammatory status observed in SCA patients. Interestingly, we observed that HU therapy did not change the production of potent pro-inflammatory cytokines, such as IL-1β. Thus, further studies are necessary to evaluate the biological association of products of hemolysis and the immune-mediated inflammatory process that is prominent on SCA patients. This knowledge could lead to the development of new targets for therapeutic intervention, preferentially, sickle cell DAMPs or NLRP3-inflammasome-associated molecules.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no competing interests.

AUTHORSHIP

TNP, SSS, RRO and MSG: conceived the study design. TNP and SSS: performed all the experiments. DLZ, CCG and RPS: assisted in all experiments. VMLN and IML: responsibles for patients' follow-up. VVM, MMA, JRDF, JBL and GQC: discussion and accomplishment of some experiments. MOSC: assisted in the collection of samples from patients. VMB, RRO and MMA: discussion of study design, experiments and all results. TNP, SSS, RRO and MSG: analyzed the data and wrote the paper. The manuscript has been critically reviewed and approved by all authors.

ACKNOWLEDGEMENTS

The authors would like to thank SCA patients and all healthy volunteers for trust us and for making possible the realization of this work. We thank the laboratory technicians for help us in the collection of the samples. We would also like to thank Wendell Vilas Boas and Bruno Cerqueira for excellent discussions. The authors would like to thank Andris K. Walter for his critical analysis and English revision of the final manuscript. TNP received a scholarship from CAPES. V.M.B. and MSG have productivity scholarship from CNPq. This work was supported by grants from the Brazilian National Council of Research (CNPq) (311888/2013-5) (MSG); the Foundation of Research and Extension of Bahia (FAPESB) (3626/2013, 1431040053063, and 9073/2007) (MSG); PPSUS/FAPESB (020/2013 EFP00007295) (MSG); and MCD/CNPq/MS-SCTIE-DECIT (409800/2006-6) (MSG).

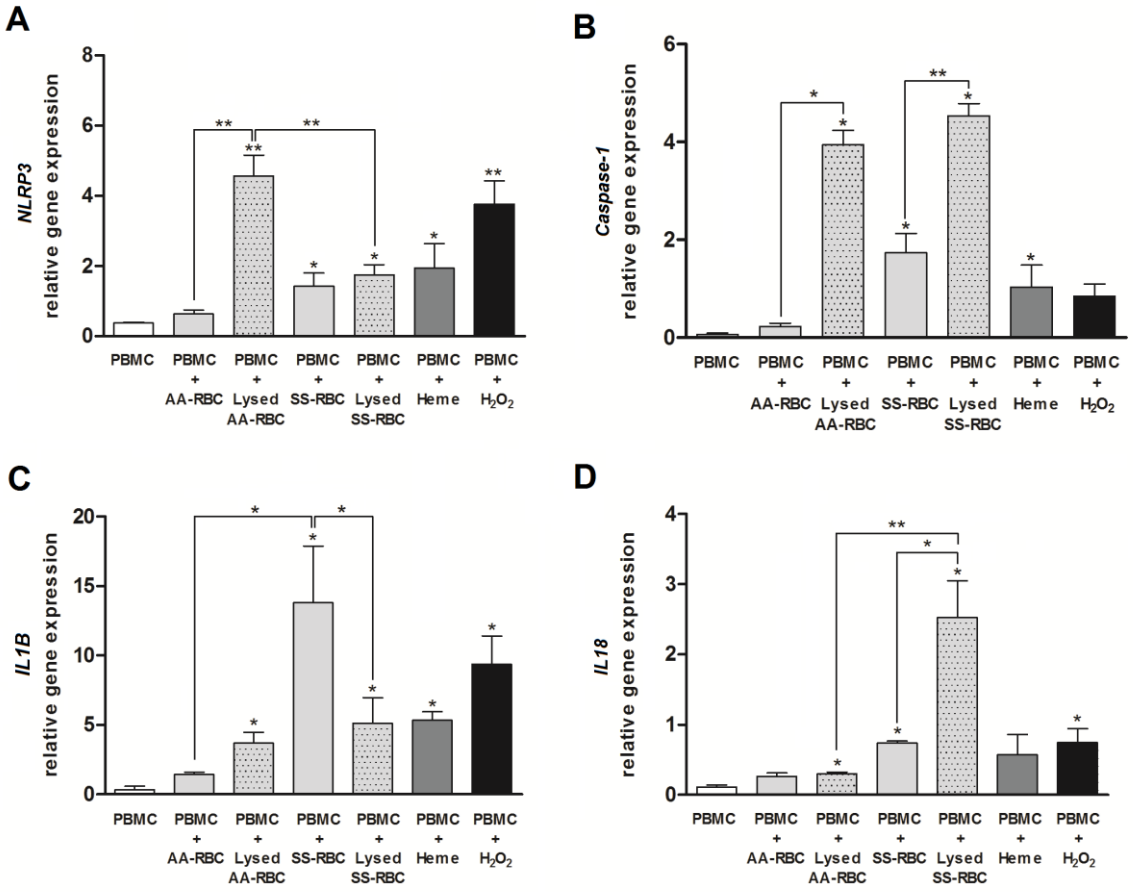

FIGURE 1. Intact and lysed sickle red cells induce NLRP3 inflammasome components expression in PBMC culture in vitro. *NLRP3* **(A)**, *Caspase-1* **(B),** *IL1B* **(C)**, *IL18* **(D)** mRNA expression were evaluated in PBMC from healthy donors (AA-PBMC, n = 4), challenged in triplicates for each stimulus, with RBC from healthy volunteers (AA-RBC, n = 10) or from SCA patients (SS-RBC, n = 8), for 24 h at 37 °C and 5% CO_2. For control of cell activation, 70 µM heme and 20 µM H_2O_2 were used. Asterisk marker above each bar represents statistic significant difference between this and the negative control bar (untreated PBMC). Asterisk marker above each bar represents statistic significant difference between this and the negative control bar (PBMC). Asterisk marker above horizontal lines represents statistic significant difference between evaluated groups (*$p < 0.05$; **$p < 0.01$), Mann Whitney test. Relative expression folds were calculated based on $2^{-\Delta\Delta Ct}$ method using non-stimulated PBMCs for calibration. NLRP3, NOD-like receptor family, pyrin domain containing 3; IL, interleukin; PBMC, peripheral blood mononuclear cells; RBC, red blood cells; SCA, sickle cell anemia; H_2O_2, hydrogen peroxide.

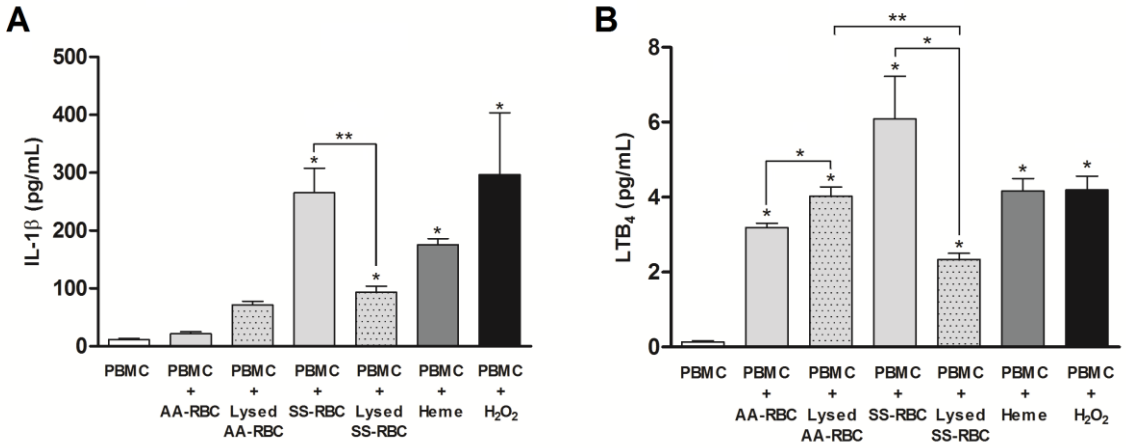

FIGURE 2. Intact and lysed sickle red blood cells induce IL-1β and LTB_4 production in PBMC culture in vitro. After PBMC from healthy donors (AA-PBMC, n = 4) have been challenged with AA- or SS-RBC, levels of IL-1β **(A)** and LTB_4 **(B)** were measured in cells supernatant cultured in triplicates with RBC from healthy volunteers (AA-RBC, n = 10) or from SCA patients (SS-RBC, n = 8), for 24 h at 37 °C and 5% CO_2. For control of cell activation, 70 µM heme and 20 µM H_2O_2 were used. Asterisk marker above each bar represents significant statistic difference between this and the negative control bar (untreated PBMC). Asterisk marker above horizontal lines represents significant statistic difference between designated groups (*$p < 0.05$; **$p < 0.01$), Mann-Whitney test. LTB_4, leukotriene B4; IL, interleukin; PBMC, peripheral blood mononuclear cells; SCA, sickle cell anemia; RBC, red blood cells.

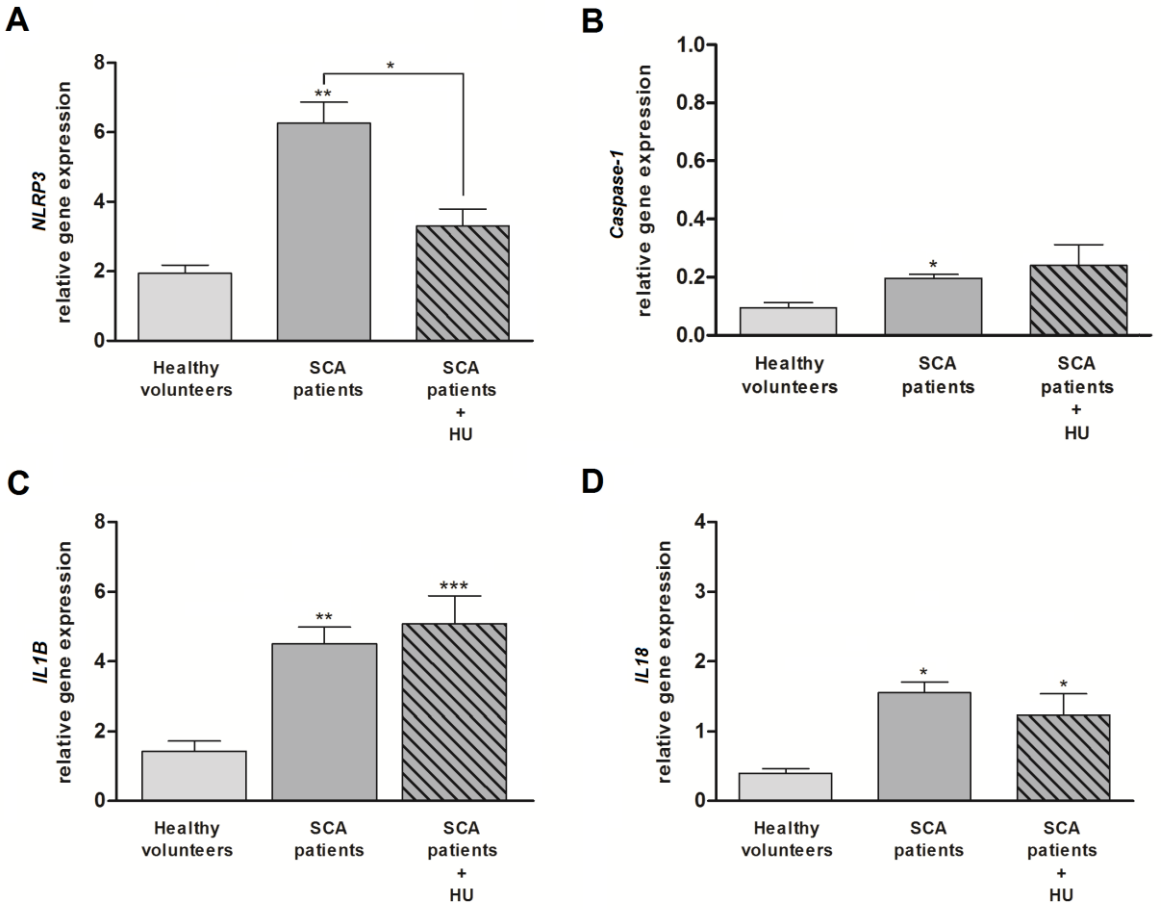

FIGURE 3. Whole blood derived-mRNA presented decreased NLRP3 gene expression in SCA patients treated with HU. NLRP3 (A), *Caspase-1* **(B)**, *IL1B* (C), *IL18* **(D)** mRNA expression were evaluated in whole blood from SCA patients treated with HU (n = 13) comparing to untreated patients (n = 15) and healthy volunteers (n = 20). Asterisk marker above each bar represents significant statistic difference between this and the control group (healthy volunteers). Asterisk marker above horizontal lines represents significant statistic difference between evaluated groups (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$), Mann Whitney U-test. Relative expression folds were calculated based on $2^{-\Delta\Delta Ct}$ method using non-stimulated PBMCs for calibration. NLRP3, NOD-like receptor family, pyrin domain containing 3; IL, interleukin; SCA, sickle cell anemia; HU, hydroxyurea.

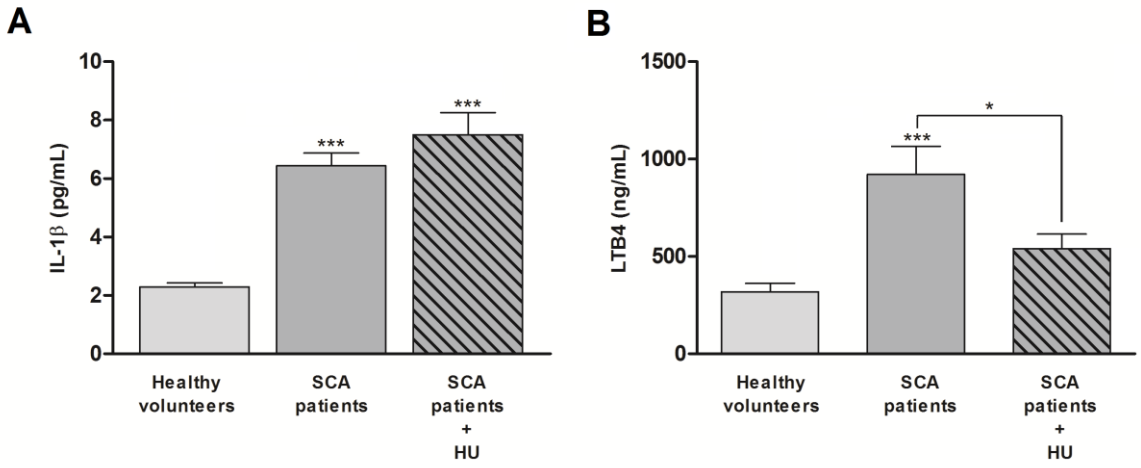

FIGURE 4. HU treatment decreases LTB_4 production and did not change IL-1β production in SCA patients. Levels of IL-1β **(A)** and LTB_4 **(B)** were measured in serum from SCA patients untreated (n = 15) or treated (n = 13) with HU, and healthy volunteers (n = 20). Asterisk marker above each bar represents significant statistic difference between this and the control group (healthy volunteers). Asterisk marker above horizontal lines represents significant statistic difference between evaluated groups (*$p < 0.05$; ***$p < 0.001$), Mann Whitney U-test. LTB_4, leukotriene B_4; IL, interleukin; SCA, sickle cell anemia; HU, hydroxyurea.

TABLE 1. Hematological and biochemical markers of steady SCA patients, either treated with HU or untreated, and healthy volunteers

	SCA		HV	*P*-value§	*P*-value†	*P*-value‡
	Untreated (Mean ± SD) (n=108)	**Treated with HU** (Mean ± SD) (n=29)	(Mean ± SD) (n=30)			
Age (years)	10.80 ± 3.11	10.24 ± 2.91	9.62 ± 3.12	0.3860	0.0576	0.3374
Hemolysis markers						
Red blood cell (x10^6/mL)	2.67 ± 0.49	2.64 ± 0.38	4.67 ± 0.42	0.7045	**< 0.0001**	**< 0.0001**
Hemoglobin (g/dL)	8.07 ± 1.06	8.13 ± 0.67	12.84 ± 1.27	0.3788	**< 0.0001**	**< 0.0001**
Hematocrit (%)	23.11 ± 3.09	23.72 ± 2.92	38.40 ± 3.39	0.2154	**< 0.0001**	**< 0.0001**
Mean orpuscular volume (fL)	87.94 ± 7.81	90.20 ± 7.24	82.18 ± 4.38	0.2253	**< 0.0001**	**< 0.0001**
Mean corpuscular hemoglobin (pg)	30.75 ± 3.33	31.73 ± 3.10	27.48 ± 1.65	0.2304	**< 0.0001**	**< 0.0001**
Reticulocytes (%)	6.94 ± 2.08	6.95 ± 2.18	0.77 ± 0.18	0.9649	**< 0.0001**	**< 0.0001**
Lactate dehydrogenase (U/L)	1,153.00 ± 468.90	1,007.00 ± 435.70	417.90 ± 94.61	0.1084	**< 0.0001**	**< 0.0001**
Heme (µg/mL)	4.95 ± 1.01	3.06 ± 0.58	1.64 ± 0.65	**0.0247**	**0.0018**	**0.0112**
Platelets						
Platelets (x10^9/mL)	459.50 ± 129.90	432.70 ± 160.20	301.30 ± 61.06	0.4476	**< 0.0001**	**0.0030**
Leukocytes						
Leukocytes (x10^9/mL)	13,326.00 ± 3,238.00	12,941.00 ± 4,901.00	6,988.00 ± 1,864.00	0.3126	**< 0.0001**	**< 0.0001**
Monocytes (x10^9/mL)	936.10 ± 390.60	909.60 ± 443.10	438.40 ± 186.90	0.7462	**< 0.0001**	**< 0.0001**
Neutrophils (x10^9/mL)	6,337.00 ± 2,738.00	5,935.00 ± 2,988.00	3,263.00 ± 1,444.00	0.2852	**< 0.0001**	**< 0.0001**
Lymphocytes (x10^9/mL)	4,881.00 ± 1,648.00	5,358.00 ± 2,517.00	4,881.00 ± 1,648.00	0.5964	**< 0.0001**	**< 0.0001**
Iron metabolism						
Ferritin (ng/mL)	262.40 ± 246.10	317.50 ± 162.60	32.13 ± 16.67	**0.0103**	**< 0.0001**	**< 0.0001**
Iron	92.34 ± 43.81	89.93 ± 43.09	72.52 ± 34.55	0.6861	**0.0310**	0.1374
Inflammation						
C-reactive protein (mg/L)	5.07 ± 4.05	8.52 ± 6.56	1.29 ± 0.57	**0.0010**	**< 0.0001**	**< 0.0001**
Hemoglobin pattern						
Fetal Hemoglobin (%)	9.07 ± 5.75	9.42 ± 5.29	0.30 ± 0.12	0.5901	**< 0.0001**	**< 0.0001**
Fetal Hemoglobin levels based on haplotype (%)						
CAR/CAR*	4.47 ± 2.80	10.50 ± 4.95	-	**0.0200**	**-**	-
CAR/BEN**	9.05 ± 6.00	8.24 ± 4.89	-	0.9640	**-**	-
BEN/BEN***	7.68 ± 5.98	10.65 ± 7.22	-	0.3930	-	-

SCA, sickle cell anemia; HU, hydroxyurea; HV, healthy volunteers; BEN, Benin haplotype; CAR, Central African Republic haplotype; SD, standard deviation.. §*P*-values < 0.05 were considered significant among untreated SCA patients and those treated with HU (Mann-Whitney test). †*P*-values < 0.05 were considered significant among untreated SCA patients and HV individuals (Mann-Whitney U-test). ‡*P*-values < 0.05 were considered significant among treated SCA patients and HV individuals (Mann-Whitney U-test). Genotype frequency in SCA patients: *CAR/CAR: 16.2%; **CAR/BEN: 55.0%; ***BEN/BEN: 20.0%. Significant *P*-values are shown in bold.

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5.3. CAPÍTULO 3 – *Hydroxyurea induces Nrf2-Antioxidant Response Element/electrophile Signaling Pathway regulated by p62/SQSTM1*

Objetivo:

Investigar propriedades antioxidantes da hidroxiureia in vitro, a fim de compreender os seus efeitos farmacológicos na diminuição do estresse oxidativo e estimulação do sistema antioxidante de defesa em células endoteliais e leucócitos.

Principais resultados:

Neste trabalho, foi demonstrado que a hidroxiureia pode contribuir com a diminuição do estresse oxidativo por inibição direta de radicais livres ou por indução do sistema antioxidade celular, através da estimulação da via de sinalização Nrf2 dependente do sequestossomo.

Situação: a ser submetido.

Hydroxyurea induces Nrf2-Antioxidant Response Element/electrophile Signaling Pathway regulated by p62/SQSTM1

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Conflict of Interest
All authors have no conflict of interest to declare.

ABSTRACT

Few studies have given a distinct focus on hydroxyurea (HU) and its alternative mechanisms that broaden the field of knowledge about its action and its systemic effects, in hemolytic diseases, especially sickle cell anemia (SCA). In this work, we evaluated the action of HU on the inhibition/minimization of Reactive Oxygen and Nitrogen Species (ROS/RNS) and stimulating the defense antioxidant system in human peripheral blood mononuclear cells (PBMC) of healthy volunteers and human umbilical vein endothelial cells (HUVEC) in the presence or absence of hemin, an important pro-oxidant molecule released in hemolysis. In vitro inhibition assays, with free radical DPPH, have demonstrated that HU presents considerable scavenging activities at equimolar concentrations to the plasma of patients taking the drug. PBMC and HUVEC showed no decrease in viability after treatments with 70 μM hemin or HU, or the combination of both. PBMC treated with HU showed no significant decrease in the accumulation of superoxide anions. In addition, PBMC and HUVEC treated with HU in combination with a hemin significantly stimulated nitrate/nitrite production in culture supernatants. HU promoted significant induction of superoxide dismutase-1 (*SOD1*) in HUVEC and PBMC, and glutathione peroxidase (*GPx*) in PBMC and a significant increase in glutathione S-reductase (*GSR*) expression in HUVEC and PBMC treated with HU and hemin. Microarray analyzes in HUVEC showed that HU increased the expression rate of antioxidant response genes, kinases and p62/sequestosome (*p62/SQSTM1*) than under Nrf2 activation conditions, directed the cytoplasmic inhibitor Keap1 (iNrf2) to autophagic degradation promoting Nrf2-mediated antioxidant induction. In contrast, a decrease in BACH1 transcriptional factor expression was observed. Upstream analyses demonstrated prediction of activation for Jun, miR-155-5p and mir-141-3p. These results suggest that HU can induce the Nrf2-dependent antioxidant/electrophilic response (ARE/EpRE) pathway regulated by SQSTM1.

Keywords: sickle cell anemia, hydroxyurea, oxidative stress, Nrf2, p62/SQSTM1

LIST OF ABBREVIATIONS

ARE/EpRE	Antioxidant Response Element/Electrophile Response Element
BACH1	BTB (Broad-Complex, Tramtrack and Bric a brac) Domain And CNC Homolog 1, Basic Leucine Zipper Transcription Factor 1
CAT	Catalase
CO	Carbon monoxide
Cul3	Cullin 3
DPPH	2,2-Diphenyl-1-picrylhydrazyl
GCS	γ-glutamate-cysteine synthetase
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSR	Glutationa S-redutase
H_2O_2	Hydrogen peroxide
HbF	Fetal hemoglobin
HbS	Hemoglobin S
HMGB1	High-mobility group box 1
$^{\bullet}OH$	Hydroxyl radical
HO-1	Heme oxigenase-1
HMOX1	Heme oxigenase-1 gene
HU	Hydroxyurea
Keap1	Kelch-like ECH-associated protein1
MAPK	Mitogen-activated protein kinase
NO_3^-	Nitrate
NQO1	NAD(P)H:quinone oxidoreductase 1
NQO2	NRH:quinone oxidoreductase 2
Nrf2	Nuclear factor erythroid 2 (NF-E2) p45-related factor 2
$O_2^{\bullet-}$	Superoxide anion
NO	Nitric oxide
$ONOO^-$	Peroxynitrite
p62/SQSTM1	Sequestosome1
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
RNS	Reactive nitrogen species
ROS	Reactive oxygen stress
SCA	Sickle cell anemia
SOD-1	Superoxide dismutase-1

INTRODUCTION

Sickle cell anemia (SCA) is a hereditary autosomal recessive disease, characterized by the homozygous beta S (β^S) (HbSS) allele, whose alteration is associated with the GAG>GTG mutation located at the sixth position of the beta globin gene (*HBB*) encoding the beta globin chain (STEINBERG and RODGERS, 2001). The complex pathophysiology condition of the SCA, including sickling, vaso-occlusion and ischemia-reperfusion injury, has a cyclic nature with large production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), assuming an important maintenance mechanism of the inflammatory condition (STEINBERG, 2008; BELCHER et al., 2010; NUR et al., 2011; CHIRICO and PIALOUX, 2012). Oxidative stress is commonly used term to describe the organic imbalance between the production of ROS or RNS and the antioxidant defense mechanism, with a pro-oxidant status increase. Under these conditions, both ROS and RNS can cause lipid peroxidation, DNA damage and affect the function of cellular proteins, culminating in a chronic and marked inflammatory condition (GIROTTI, 1985; BEETSCH et al., 1998; KATO et al., 2009).

The imbalance caused by the increased oxidation-reduction status (redox) in the vascular microenvironment in SCA, is a critical point due to deleterious effects (NUR et al., 2011; SILVA et al., 2013). Thus, the individuals with SCA can present *(i)* intra and extravascular hemolysis with free heme release using $Fe^{2+/3+}$ in Fenton reaction catalysis; *(ii)* autoxidation of hemoglobin (HEBBEL et al., 1988; BELCHER et al., 2010); *(iii)* nitric oxide (NO) depletion and endothelial dysfunction (BONAVENTURA et al., 2002; REITER et al., 2002); *(iii)* ischemia-reperfusion events occasioned by vaso-occlusive crises (KAUL and HEBBEL, 2000); *(iv)* marked dysfunction of leukocytes, conferring a non-effector response against pathogens, and deregulation in the control system of inflammation with increased susceptibility to secondary infections (SCHIMMEL et al., 2013; CHEN et al., 2014; BALANDYA et al., 2016).

Considering the pro-oxidant condition resulting from hemolytic disorders due to destruction of SS-erythrocytes, the enzymatic and non-enzymatic defense mechanisms of antioxidant play a pivotal role in the maintenance of pro and antioxidant balance. Antioxidant enzyme systems are the first line of defense against free radicals preventing the accumulation of superoxide radical anion ($O_2^{\bullet -}$) and

hydrogen peroxide (H_2O_2), such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (SHIH; YEH; YEN, 2007; BIRBEN et al., 2012; CHIRICO and PIALOUX, 2012). Other enzymes act directly or indirectly on the biotransformation/detoxification of xenobiotics or electrophilic drugs, for example NAD(P)H:quinone oxidoreductase 1 (NQO1), NRH:quinone oxidoreductase 2 (NQO2), glutathione S-transferase (GST), and γ-glutamate-cysteine synthetase (GCS), heme oxygenase-1 (HO-1), as well as molecules such as reduced glutathione (GSH) and metallothioneins (HAYES et al., 2000; DINKOVA-KOSTOVA et al., 2001; KASPAR; NITURE; JAISWAL, 2009).

The induction of these genes is triggered by defense mechanisms in response to oxidative/electrophilic stress, regulated by the antioxidant response/electrophile response element (ARE/EpRE) controlled by cap 'n' collar (CNC) family of Nuclear Respiratory Factor (Nrf) (JOHNSON and LAPADAT, 2002; NITURE; KHATRI; JAISWAL, 2014; KIM; HAN; CHAN, 2016). The nuclear factor erythroid 2–related factor 2 (Nrf2) is a leucine zipper/CNC protein present in the cytoplasm in the absence of stressor agents that is found in the cytoplasm associated with its inhibitor Kelch-like ECH-associated protein1 (Keap1) or analog. Under basal conditions, Keap1 associates with Nrf2 in the cytoplasm, which targets the factor to the proteasome degradation. Nrf2 is a key regulator of detoxification and antioxidant system. In the induction step, Nrf2 dissociates from Keap1 is translocated to the nucleus and heterodimerizes with small Maf or Jun and binds to ARE/EpRE inducing the activation of the antioxidant response genes after a activation cascade mediated by protein kinases type C (PKC) (JOHNSON and LAPADAT, 2002; MARTIN et al., 2004; NITURE; KHATRI; JAISWAL, 2014). The p62/SQSTM1 protein also named sequestosome, has its expression up-regulated in a Nrf2-dependent manner under conditions of chemical/oxidative stress, and may enable the targeting of Keap1 for degradation via autophagy, leading to a prolonged activation of Nrf2 (ISHII et al., 2000; WARABI et al., 2007; COPPLE et al., 2010; ICHIMURA et al., 2013).

Hydroxyurea (HU) is classically a myelosuppressive drug that acts by inhibiting the ribonucleotide reductase, and is also considered a gold standard drug and the only official therapeutic option recommended for treatment of SCA (CHARACHE, 1997; AGRAWAL et al., 2014). Regarding the primary and secondary effects of HU therapy, Ware (2010) pointed out as main benefits *(i)* the induction of fetal hemoglobin (HbF) through the activation of guanylate cyclase; *(ii)* decrease in

neutrophil and reticulocyte counts by inhibition of ribonucleotide reductase and bone marrow toxicity; *(iii)* decreased adhesiveness and improved rheology of circulating neutrophils and reticulocytes; *(iv)* reduction of hemolysis and improvement of erythrocyte hydration, macrocytosis and intracellular reduction of sickling; and *(v)* release of NO as a potential local vasodilator and improvement of vascular response.

However, a few studies have given a distinct focus on the action of HU in alternative mechanisms that broaden the field of knowledge about its action and systemic effects. In this work, we evaluated the action of HU in human peripheral blood mononuclear cells (PBMC) and umbilical cord vein endothelial cells (HUVEC in the presence or absence of hemin, an important pro-oxidant molecule released in hemolysis (MARTIN et al., 2004; BELCHER et al., 2010; BEAN et al., 2012). In our hypothesis, we believe that HU can act directly on decreasing ROS/RNS, and stimulating defense antioxidant system in endothelial cells and leukocytes.

METHODS

Drugs

Hydroxyurea, L-ascorbate and Butylated hydroxytoluene were purchased from Sigma Aldrich Louis (St. Louis, MO, USA) and prepared in accordance with the manufacturer's instructions. After complete solubilization, drugs were sterilized by filtration using a 0.22 µm polyethersulfone membrane (PES) (Jet Biofil, Guangzhou, China) for use in culturing assays.

Preparation of hemin

Hemin (Sigma Aldrich, St. Louis, MO, USA) was prepared from a 5 mM stock solution solubilized in 0.1 M NaOH using non-pyrogenic water under dark conditions. The hemin solution was then diluted in RPMI 1640 medium (Gibco, New York, NY, USA) to obtain optimal concentrations. Finally, non-pyrogenic hemin solutions were obtained following 0.22 µm PES-membrane filtration (Jet Biofil, Guangzhou, China) for use in cell culture assays.

Scavenging activity assay of 2,2-diphenyl-1-picrylhydrazyl (DPPH)

DPPH free scavenging activity was assessed by a modified method previously described by Li et al (2002) for microplate assaying. Initially, 200 µM stock solution of 2,2-diphenyl-1-picrylhydrazyl (Sigma Aldrich, St. Louis, MO, USA) was prepared in methanol (Synth, Diadema, SP, Brazil) 10-15 min prior to performing the assay, stored in a sealed bottle and kept away from light. For this assay, stock drug solutions were prepared using methanol at concentrations ranging from 0.78 to 800 µM/well. HU, as well as the antioxidant external controls Butylated hydroxytoluene and L-ascorbate, were incubated at a volume of 0.1 mL in 96-well flat-bottom microtiter plates (Greiner Bio-one, Monroe, North Carolina, USA) at a ratio of 1:1 (v/v), with the addition of 200 µM DPPH stock solution (100 µM/well) for 30 and 60 min. All plates were covered to minimize evaporation interference and kept under dark conditions to avoid photosensitization of DPPH radicals. Finally, the solutions in each well were homogenized for 5 seconds and absorbance was measured on a

microplate reader (SpectraMax 190, Molecular Devices Corporation, Sunnyvale, CA) using Softmax software v. 5.0 (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 517 nm. DPPH radical scavenging activity was determined using the following equation: *Scavenging activity of DPPH (%) = [(*Abs_{dpph} *–* Abs_{drug}*) × 100] /* Abs_{dpph}.

Cell cultures

Immortalized human umbilical cord vein endothelial cells (HUVEC) were kindly donated by Dr. Ana Moretti, Ph.D. and Dr. Heraldo Possolo de Souza, M.D., from the São Paulo State University Medical School (FMUSP), São Paulo-Brazil. HUVEC were cultured in cell culture flasks with a surface area 25 cm^2 (Costar, Corning, NY, USA) containing 5 mL RPMI 1640 medium (Gibco, New York, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, New York, NY, USA), 20 mM glutamine (Sigma Aldrich, St. Louis, MO, USA), 10 mM HEPES, 5 mM NaOH and the following antibiotics: 100 U/mL penicillin and 10 mg/mL streptomycin (Sigma Aldrich, St. Louis, MO, USA). For all assays, HUVEC were used in passages 1 – 5 and phenotypically characterized by the evaluation of typical cobblestone morphology and tissue factor expression (CD142) (SUPPLEMENTARY FIGURE 1). Human peripheral venous blood samples were taken from healthy volunteers (hemoglobin AA genotype) to obtain peripheral blood mononuclear cells (PBMC). Written informed consent was obtained from all study participants, and the present protocol was conducted in accordance with the 1975 Helsinki Declaration and its amendments, and its revisions, and received approval from the Institutional Review Board of the Oswaldo Cruz Foundation–FIOCRUZ, Bahia-Brazil (CAAE: 04733612.7.0000.0040). PBMCs were obtained by Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation in accordance with manufacturer instructions. Both HUVEC and PBMCs were cultivated in a humidified atmosphere at 37 °C under 5% CO_2.

Cytotoxicity assays

The cytotoxic effects of the drugs and heme on HUVEC were evaluated using a resazurin sodium salt reduction colorimetric assay. To this end, 2 x 10^4 cells/well

(0.2 mL) were plated on 96-well plates (Costar, Corning, NY, USA) and cultivated under the culture conditions described above for 20-24 h to obtain a confluency of 70-80%. HU and/or hemin stimuli were then added to the cultures and reincubated for an additional 24 h. Next, the medium was aspirated and the wells were gently washed once with preheated (37 °C) 0.85% saline solution to avoid cell damage and detachment. Finally, 0.1 mL of 12.5 µM resazurin sodium salt solution (Sigma Aldrich, St. Louis, MO, USA) solubilized in RPMI 1640 and 10% FBS was added to each well, followed by incubation at 37 °C under 5% CO_2 in a humidified atmosphere for 3 h. Upon the conclusion of incubation, absorbance values were read at simultaneous wavelengths of 570 and 600 nm on a microplate reader. The percentage of viable cells was determined by measuring the percent reduction of sodium salt (deep blue fluorescent compound) that reduced to resorufin (pink fluorescent product). For the cytotoxicity assays involving PBMCs, 3 x 10^5 cells were challenged shortly after isolation with HU and/or hemin for 24 h. The cytotoxicity of PBMC induced by the stimuli was evaluated using propidium iodide (BD, Pharmigen) in accordance with the manufacturer's specifications. For each sample, 20,000 events were acquired using a BD LSRFortessa™ cytometer (Biosciences, San Jose, CA, USA).

Nitrite and superoxide anion accumulation in supernatants

Superoxide ion production was indirectly quantified via the accumulation of nitrite in cell supernatants by the hydroxylamine oxidation reaction (ELSTNER; HEUPEL, 1976; KHOURI et al., 2009) after a 24 h challenge with HU and/or hemin stimuli using the Griess method (BRYAN and GRISHAM, 2007) First, 1.2 x 10^6 PBMCs/well (0.3 mL) were seeded on 48-well plates with RPMI medium supplemented with 10% FBS in the presence of the stimuli, either with or without 0.5 mM hydroxylamine. After challenge, the supernatants were collected by centrifugation (1500 rpm, 5 min at 4 °C) and the Griess method was used to measure total nitrite. The accumulation of superoxide ions ($O_2^{\bullet-}$) was determined by the following equation: $O_2^{\bullet-}$ *(µM) = µM nitrite accumulation of stimuli with hydroxylamine – µM nitrite accumulation of stimuli without hydroxylamine*; where the µM accumulation of total nitrite (µM superoxide ions converted to nitrite plus µM real nitrite) corresponds to the wells containing hydroxylamine, and the real nitrite

accumulation corresponds to the non-hydroxylamine wells. As a positive control, Hydrogen peroxide (200 µM H_2O_2) and phorbol 12-myristate 13-acetate (200 nM PMA) (Sigma Aldrich, St. Louis, MO, USA) were used as inducers of $O_2^{\bullet-}$. For this reaction, 50 µL (1:1, v/v) of the supernatant was added to Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in acid solution – Sigma Aldrich, St. Louis, MO, USA) for 5 min. Absorbance was measured on a microplate reader at a wavelength of 550 nm. The conversion of absorbance into micromolar concentrations of NO was deduced from a standard curve using a known concentration of $NaNO_2$ diluted in RPMI medium. The standard curves used to determine molar concentrations assumed a coefficient of determination (R^2) ≥ 0.999.

Gene expression and RNA extraction assays

HUVEC and PBMCs were challenged with different concentrations of hydroxyurea in the presence and absence of 70 µM hemin for 4 h. Gene expression assays were performed by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from the HUVEC and PBMC samples using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. The concentration and purity of the extracted RNA were determined at the optical densities of 260 and 280 nm using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Rockford, IL, USA) with an absorbance ratio $A_{260/280}$ of 1.90–2.02. Reverse cDNA synthesis by reverse transcription of RNA (RT-PCR) was performed using 250 ng of the RNA transcript in a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Rockford, IL, USA) in accordance with the manufacturer's specifications. Real-time PCR was performed on an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following cycling conditions: 95 °C for 20 seconds, 95 °C for 1 second, 60 ºC for 20 seconds for 40 cycles. For the qRT-PCR reactions, mixtures containing SYBR® Green PCR Master Mix (SYBR® Green I dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, passive reference 1 - ROX) (Applied Biosystems, Foster City, CA, USA), the primers specific to the target genes and 2 µL of the cDNA sample product were added to the optical plates. The primers used for quantitative PCR were as follows: [heme oxygenase-1 (*HMOX1*): 5’-ATG GCC TCC CTG TAC CAC ATC-3’ (forward); 5’-TGT TGC GCT CAA TCT CCT CCT-3’ (reverse);

superoxide dismutase-1 (*SOD1*): 5'-TGG CCG ATG TGT CTA TTG AA-3' (forward); 5'-CAC CTT TGC CCA AGT CAT CT-3' (reverse); catalase, (*CAT*): 5'-GCC ATT GCC ACA GGA AAG TA-3' (forward); 5'-CCT TGG TGA GAT CGA ATG GA-3' (reverse); glutathione-disulfide reductase S-reductase (GSR): 5'-ACT TGC CCA TCG ACT TTT TG-3' (forward); 5'-GGT GGC TGA AGA CCA CAG TT-3' (reverse); glutathione peroxidase (*GPx*): 5'-CCA AGC TCA TCA CCT GGT CT-3' (forward); 5'-TCG ATG TCA ATG GTC TGG AA-3' (reverse); β-actin: forward, 5'-CCT GGC ACC CAG CAC AAT-3'; reverse, 5'-GCC GAT CCA CAC GGA GTA CT-3'; tubulin isotype a1C: forward, 5'-TCA ACA CCT TCT TCA GTG AAA GG-3'; reverse, 5'-AGT GCC AGT GCG AAC TTC ATC. After determination of the threshold cycle (CT), gene expression was measured by relative quantification using the following expression: fold change = $2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT_{target} - CT_{housekeeping}$ and $\Delta(\Delta CT) = \Delta CT_{treated} - \Delta CT_{control\ (medium)}$. Beta-actin and tubulin isotype a1C were used as housekeeping genes.

Microarray assays with HUVEC

Microarray gene expression analysis was performed using a HumanHT-12 v.4 Expression BeadChip Kit (Illumina Inc., San Diego, CA, USA) and a TargetAmp™ Nano Labeling Kit for Illumina® Expression BeadChip® (Epicenter Technologies, Madison, Wisconsin, USA) in accordance with the manufacturers' specifications. Fluorescence values were acquired on an Illumina HiScan using iScan Control software (Illumina Inc., San Diego, CA, USA). After quality control assessment, the generated data were exported for analysis using Genome Studio software (Illumina Inc., San Diego, CA, USA). We excluded results with a detected *p-value* > .05 and a differential score < .05. After validation, 18,792 transcripts were selected and analyzed in Ingenuity Pathway Analysis (IPA) software (QIAGEN). Experiments were performed in triplicate and results are expressed based on relative expression (log fold change > 1.5) after comparing the HUVEC treated with 200 μM HU to untreated cells.

Statistical analysis

Data correspond to mean values ± standard deviation (SD) of at least one representative experiment. All experiments were performed in triplicate. One-way ANOVA was used to test variance among multiple groups, while Tukey's post-hoc test was used to assess significance among the detected differences. Significance was considered when $p < .05$. GraphPad Prism software version v.6.0 was used for statistical analyses and graphing (GraphPad, San Diego, CA, USA).

RESULTS

Hydroxyurea has scavenging properties of free radicals

To investigate the possible antioxidant effects of HU, we performed assays to evaluate radical scavenging activity using DPPH, a stable free radical. Initially, we evaluated the scavenging activity of HU after 30 and 60 min of free radical DPPH incubation. Hydroxyurea demonstrated significantly superior scavenging activity at 60 min of challenge (100 μM DPPH) (SUPPLEMENTARY FIGURE 2). From these findings, the remaining scavenging assays were performed at 60 min following DPPH incubation. Hydroxyurea showed positive dose-dependent scavenging activity at all concentrations evaluated. Our global analysis found that HU (IC_{50} = 38.68 ± 0.47) demonstrated lower DPPH radical scavenging than the reference antioxidant compounds BHT (IC_{50} = 23.07 ± 2.64, $p < .05$) and L-ascorbate (IC_{50} = 18.22 ± 5.93, $p < .001$) (TABLE 1). However, we observed scavenging activity equivalent or superior to BHT at concentrations equal to or greater than 200 μM (FIGURE 1). Based on these findings, HU was used in the other assays at concentrations of 100 and 200 μM. In addition, these therapeutic plasma concentrations are commonly found in patients on hydroxyurea, as described in previous in vitro studies (ELIAS et al., 2014; LOPES et al., 2014; DA GUARDA et al., 2016).

Hydroxyurea and hemin presented no toxicity in HUVEC and PBMC samples

Prior to performing the assays using cell cultures to investigate the antioxidant properties of HU, we first evaluated whether different concentrations of this drug and 70 μM of hemin decreased cell viability in HUVEC and PBMC samples. To carry out toxicity testing in HUVEC, we initially standardized the time required to reduce resazurin salt, equivalent to the percentage of cell viability in unstimulated cells. We found that 180 min was the time required to reduce 12.5 μM of resazurin salt (SUPPLEMENTARY FIGURE 3A). Next, we investigated whether hemin induces cytotoxicity in HUVEC. No decreases in cell viability were seen at the concentrations evaluated, ranging from 6.25 to 100 μM of hemin (SUPLEMENTARY FIGURE 3B). The cytotoxicity of PBMCs to the stimuli was evaluated using propidium iodide. Incubation with HU and hemin did not cause toxicity in HUVEC and PBMC samples

at any of the concentrations assessed in comparison to controls (unstimulated cells) (FIGURE 2).

Hydroxyurea decreases the production of superoxide anion in PBMCs and increases nitrate/nitrite production in cells treated with HU and hemin

The potential of HU to induce the production of nitrate/nitrite and inhibit superoxide ions ($O_2^{\bullet-}$) in the presence or absence of hemin was evaluated indirectly in PBMCs by determining the micromolar accumulation of nitrite after the addition of hydroxylamine using the Griess reaction (FIGURE 3A). The H_2O_2 and PMA were used as positive controls. Hydroxyurea treatment, as well as hemin alone, did not significantly interfere with nitrite production, alhtough a discrete, similar and non-significant decrease in $O_2^{\bullet-}$ was observed under treatment with 100 μM HU (0.65 ± 0.17 μM) and 200 μM HU (0.66 ± 0.22 μM) compared to untreated PBMC (0.96 ± 0.54 μM). When we evaluated the combined treatment of HU with hemin versus untreated PBMCs, we observed a significant increase in nitrite production for 100 μM HU (1.1 ± 0.15 μM, $p < .05$) and 200 μM HU (1.14 ± 0.38 μM, $p < .01$) compared to non-stimulated PBMCs (0.96 ± 0.54 μM). A significant increase in nitrite production was also observed in the combined treatment with 200 μM HU and hemin when compared to PBMC treated with 70 uM hemin ($p < .05$). In addition, combined treatments of HU with hemin similarly decreased $O_2^{\bullet-}$ production (100 μM HU+hemin: 1.19 ± 0.35 μM; 200 μM HU+hemin: 1.22 ± 0.28 μM) when compared to treated PBMC with 70 μM hemin (1.55 ± 0.48 μM), although these differences were not statistically significant.

Hydroxyurea increases nitrate/nitrite production in the presence of hemin in HUVEC

Similarly to what was observed in PBMCs, HU treatment alone did not alter nitrite production in HUVEC at the concentrations evaluated (100 μM HU: 12.43 ± 0.23 μM and 200 μM HU: 12.38 ± 0.40 μM), when compared to untreated cells (12.13 ± 0.23 μM) (FIGURE 3B). However, higher absolute values of nitrite accumulation were observed in HUVEC compared to PBMCs. Treatment with hemin significantly stimulated nitrite production (22.37 ± 0.47 μM, $p < .0001$) compared to unstimulated

HUVEC. An increase in nitrite production was seen under the combined treatments of 100 μM HU (24.99 ± 0.83 μM, $p < .01$) and 200 μM HU (25.04 ± 1.03 μM, $p < .01$) plus hemin in comparison to HUVEC treated with 70 μM hemin alone (22.37 ± 0.47 μM).

Treatments with hydroxyurea alone or combined with hemin induce heterogeneous expression of important antioxidant enzymes in HUVEC and PBMC

Using HUVEC and PBMCs, we investigated the role of hydroxyurea in the induction of antioxidant enzyme genes that play an important role in the inhibition or control of free radicals and non-radical reactive species within the cellular microenvironment. To this end, superoxide dismutase-1 *(SOD1)*, glutathione peroxidase *(GPx)*, glutathione S-reductase *(GSR)* and heme oxygenase-1 *(HMOX1)* were evaluated (FIGURE 4).

In the absence of hemin, HU was shown to promote the increased expression of SOD-1 in a dose-dependent manner in PBMCs and HUVEC (FIGURE 4A). PBMCs stimulated with 100 and 200 μM HU showed, respectively, an increase of SOD-1 at 2.13 ± 0.86-fold and 2.57 ± 0.86-fold ($p < .05$) compared to the control (medium). HUVEC stimulated under the same conditions (100 μM HU: 1.37 ± 0.30-fold; 200 μM HU: 1.84 ± 0.36-fold, $p < .01$) exhibited similar dose-dependent responses in *SOD1* induction, although to a lesser extent. Although stimulation with hemin induced elevated levels of *SOD1* expression in PBMCs (1.51 ± 0.61-fold) and HUVEC (1.30 ± 0.09-fold), these differences were not statistically significant. On the other hand, 100 and 200 μM of HU in combination with hemin promoted a statistically significant increase of 3.37 ± 0.42-fold ($p < .01$) and 3.39 ± 0.37-fold ($p < .01$), respectively, in *SOD1* expression in PBMCs compared to 1.72 ± 0.15-fold ($p < .05$) and 1.53 ± 0.07-fold, respectively, in HUVEC. When compared to hemin, PBMCs challenged with 100 and 200 μM of HU combined with hemin promoted an increase of 1.86 ± 0.42-fold ($p < .05$) and 1.88 ± 0.37-fold ($p < .05$) and 0.43 ± 0.15-fold, and 0.24 ± 0.07-fold, respectively, in HUVEC.

Considerable *GPx* expression was observed in PBMCs challenged with 100 μM (2.27 ± 0.14-fold, $p < .0001$) and 200 μM HU (2.40 ± 0.12-fold; $p < .0001$) (FIGURE 4B). Similar expression values were observed in hemin-challenged PBMCs

at both HU concentrations (2.25 ± 0.05-fold, $p < .001$ and 2.11 ± 0.11-fold, respectively). In contrast, *GPx* expression levels did not vary in HUVEC in response to the treatments.

Treatment with HU alone did not promote increases in *GSR* expression at any of the concentrations evaluated in either PBMCs or HUVEC (FIGURE 4C). However, we observed an increase in *GSR* expression in PBMCs and HUVEC submitted to combined HU+hemin treatment. PBMCs treated with 100 μM or 200 μM of HU and 70 μM of hemin presented increases in *GSR* expression of 1.79 ± 0.22-fold ($p < .05$) and 1.42 ± 0.43-fold, respectively, while HUVEC presented 1.30 ± 0.07 ($p < .05$) and 1.48 ± 0.15-fold ($p < .001$) elevations in *GSR* expression in response to the same treatments in comparison to the control.

Significant levels of *HMOX1* were observed in hemin-treated PBMCs and HUVEC, regardless of HU treatment protocol (FIGURE 4D). In PBMCs, the increase in *HMOX1* expression was 45.5 ± 6.2-fold ($p < .0001$) versus controls. HUVEC exhibited modest expression values (4.6 ± 0.32-fold, $p < .0001$) of *HMOX1* compared to PBMCs, despite a high degree of statistical significance. HU+hemin combined treatments did not interfere with *HMOX1* expression, despite the slight decrease in expression observed in PBMCs treated with 100 μM (41.5 ± 4.6-fold) and 200 μM HU (40.31 ± 10.2-fold) when compared to the control.

Microarray analysis in HUVEC suggests that HU induces Nrf2-Antioxidant Response Element/electrophile Signaling Pathway regulated by p62/SQSTM1

A preliminary analysis identified 39 genes related to Nrf2-mediated oxidative stress response in HUVEC (TABLE 2). Increased expression levels were observed for superoxide dismutase 2 (*SOD2*; 1.852 Expr Log Ratio), glutathione disulfide reductase (*GSR*; 2.882 Expr Log Ratio), glutathione S-transferase mu 2 (*GSTM2*; 2.210 Expr Log Ratio), microsomal glutathione S-transferase 1 (*MGST1*; 1.733 Expr Log Ratio) and carbonyl reductase 1 (*CR1*; 1.727 Expr Log Ratio). We also observed increases in the expression of phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta (*PIK3C2B*; 1.892 Expr Log Ratio) and phosphoinositide-3-kinase regulatory subunit 3 (*PIK3R*; 1.597 Expr Log Ratio), protein kinases C beta (*PRKCB*; 4.026 Expr Log Ratio) and zeta (*PRKCZ*; 1.902 Expr Log Ratio), and glycogen synthase kinase 3 beta (*GSK3B*; 1.607 Expr Log Ratio). We also observed increased

expression of p62/sequestosome (*p62/SQSTM1;* 1.639 Expr Log Ratio) with decreased BTB domain and CNC homolog 1 (*BACH1;* -1.721 Expr Log Ratio), and ubiquitin conjugating enzyme E2 K (*UBE2K*; -1.830 Expr Log Ratio). Upstream analyses have indicated the predicted activation of mature microRNAs, miR-155-5p (*activation z-score* = 2.840) and miR-141-3p (*activation z-score* = 2.801), and transcription regulator, Jun (*activation z-score* = 2.560).

DISCUSSION

The present study proposes the approach of antioxidant properties of HU, the gold standard drug used in the treatment of SCA. We evaluated the benefits of HU treatment by focusing on its direct antioxidant properties as well as effects on the modulation of the antioxidant cellular response. To demonstrate these benefits, we investigated the effects generated by administering HU in HUVEC and PBMCs at concentrations equivalent to those found in the plasma of patients taking the drug. In an attempt to mimic the pathological inflammatory microenvironment, we used hemin, which is classically known to exert various cytotoxic and pro-inflammatory effects, an inherent condition in hemolytic diseases (JENEY et al., 2002; CHEN et al., 2014; DUTRA et al., 2014; CARVALHO et al., 2017; GUARDA et al., 2016).

Initially, we performed assays to evaluate whether HU was able to act directly via free-radical scavenging activity after 1 h of incubation with the radical compound DPPH. Despite the higher IC_{50} in comparison to the antioxidant controls evaluated, concentrations of 100 and 200 μM HU demonstrated considerable scavenging activities of the drug (73.5 and 90%). These concentration variations are consistent with plasma levels found in patients treated with HU, and have been used extensively in in vitro studies (ELIAS et al., 2014; LOPES et al., 2014; ALMEIDA; SOUZA, 2015; DA GUARDA et al., 2016).

From these findings, we applied these same HU concentrations in our other assays. The potential scavenging effect of HU can be explained by its ability to act as an hydrogen atom electron donor in the neutralization of the radical compound DPPH (BLOIS, 1958). These findings indicate that HU can act directly by neutralizing free radicals in the extracellular microenvironment. This property is of great importance, since it can potentially confer an important protective effect against direct oxidative attacks on membrane phospholipids, as well as prevent/minimize the triggering of activation responses involved in initiation of the oxidative cascade and establishment of inflammation (HALLIWELL and GUTTERIDGE, 1984; GRIJALBA et al., 1998; NOOR et al., 2005; MITTAL et al., 2014).

We next assessed whether the present treatment protocols would promote toxicity in HUVEC and PBMCs. Our strategy consisted of investigating, separately, HU and hemin treatments in endothelial cells, which represent the interface between the tissues and the vascular microenvironment, as well as in peripheral blood

mononuclear cells (monocytes and lymphocytes) – important players in the modulation of the innate and adaptive immune responses due to interaction in a double-handed pathway with the vascular endothelium. The concentration of 70 μM of hemin was chosen based on findings by Carvalho et al (2017) in accordance with the mean free heme plasma concentrations found in patients with sickle cell disease in a state of crisis. Interestingly, we did not observe any significant decreases in viability in either PBMCs or HUVEC after 24 h, regardless of treatment protocol. This non-toxicity may be explained by the cellular resilience presented by both cell types to the pro-oxidant microenvironment promoted by hemin. Heme-induced heme oxygenase-1 has been shown to confer a cytoprotective effect by inhibiting apoptosis in monocytes, despite positively inducing caspase-3 (LANG et al., 2005). Free heme also promotes differentiation of monocytes into iron-recycling macrophages by induction of the Spi-C transcription factor (HALDAR et al., 2014). Other studies have shown that heme inhibits neutrophil cell death by apoptosis by a phosphoinositide 3-kinase (PIK3), MAPK and NF-κB dependent mechanism, with the generation of ROS by induction of protein kinase-C (PKC) and increased secretion of IL-8 (GRACA-SOUZA et al., 2002; ARRUDA et al., 2004).

To rule out the possibility of the possible inactivation of hemin used in our study, we analyzed gene expression of *HMOX1*, an essential enzyme involved in heme catabolism. Since hemin promoted the induction of high levels of *HMOX1* in both cell types, the confirmation of this activity may explain the protective effect seen in response to heme stress, thus preventing cell death.

Functional assays were carried out to demonstrate whether HU treatments were able to inhibit the production of superoxide anions in PBMCs in vitro. Our results demonstrated that HU was capable of decreasing the production of superoxide anions, despite a lack of statistical significance. In addition, HU+hemin treatments decreased the production of $O_2^{\bullet-}$ in PBMCs in comparison to hemin treatment alone. To explain this, we offer three hypotheses: *(i)* this inhibition may have occurred due to the scavenging properties of HU, thereby abolishing $O_2^{\bullet-}$ formation; *(ii)* due to the induction of SOD, which acts on the dismutation of $O_2^{\bullet-}$ in H_2O_2 and O_2; or *(iii)* as a result of the preformed NO-mediated inhibition of $O_2^{\bullet-}$. Combined treatments with HU and hemin were also shown to induce nitrate/nitrite production, differently from what was observed in PBMCs with hemin alone. Similarly, HUVEC treated with HU+hemin also showed a significant increase in nitrate/nitrite

production relative to the control, as well as HUVEC challenged with hemin. The increase in NO production may be explained by the fact that HU reacts with hemoglobin to produce iron nitrosyl hemoglobin (HbNO), nitrite, and nitrate (HUANG et al., 2002; HUANG; KIM-SHAPIRO; KING, 2004; KING, 2004; LOCKAMY et al., 2004; KING, 2005). This would seem to suggest that the combined treatment protocol induced the production of NO, one of the most striking and well-known properties of HU. NO is known to act on the regulation of vascular tone and is an important modulating substrate for the induction of fetal hemoglobin in erythroid precursor cells (COKIC et al., 2003; ALMEIDA and SOUZA, 2015).

We further evaluated the expression of antioxidant response genes to verify whether HU was able to act in the modulation of cytoprotective antioxidant responses. A heterogeneous expression profile was observed in the different cell types investigated after 4 h of treatment with HU and/or hemin. PBMCs demonstrated more expressive levels of expression than HUVEC, which unsurprisingly can be explained by the substantial capacity for recognition and effector responses of phagocytes/lymphocytes, especially monocytes. These cells are usually more sensitive to environmental disturbances (CHAPLIN, 2010; GLADWIN and OFORI-ACQUAH, 2014).

Treatments with HU, as well as those combined with hemin, promoted a significant induction of *SOD* in both cell types investigated. Normally, under stress conditions, the accumulation of $O_2^{\bullet-}$ is a trigger for the induction of an antioxidant response, resulting in the induction of *SOD1* expression via activation of the Nrf2-dependent antioxidant response pathway. Here, we endeavor to suggest that HU may be modulating the induction of *SOD1* due to the fact that HU treatment was shown to decrease the production of superoxide anions in HUVEC.

PBMCs treated with HU showed increased expression of *GPx*, regardless of combined treatment with hemin, while no differences in *GPx* expression were seen in HUVEC. Other studies have portrayed the benefits of HU in inducing the *GPx*-mediated NO-cGMP pathway in patients with SCA (CHO et al., 2010; EK MD et al., 2016). Two hypotheses are proposed to explain this induction: *(i)* HU can act by inducing the action of transcriptional factors by modulating the expression of *GPx*; or *(ii)* HU metabolites can promote the generation of H_2O_2, controlled by the expression of *GPx,* which uses reduced glutathione (GSH) synthesized by GSR (MALEC et al., 1984; IYAMU et al., 2001; SAKANO et al., 2001; NAGAI et al., 2003; HUANG; KIM-

SHAPIRO; KING, 2004). In addition, an increase in the statistically significant induction of *GSR* was observed in the HUVEC and PBMCs challenged with combined treatment protocols. GSR uses glutathione disulfide (GSSG) as the substrate at the expense of NADPH to form sulfhydryl GSH, which is an important cellular antioxidant. On the other hand, glutathione S-transferases (GST), phase II metabolic isoenzymes, act by catalyzing the reduction of GSH in xenobiotic substrates for the purpose of detoxification (HODGES; MINICH, 2015). Many studies have shown that GSTs functions physiologically as a hemin-binding and/or transport protein to perform various important cellular functions (HARVEY and BEUTLER, 1982; KIRSCHNER-ZILBER; LAUFER; SHAKLAI, 1989; BOYER and OLSEN, 1991; KHAN and QUIGLEY, 2011). Herein, it is suggested that the increase in *GSR* expression observed in PBMC and HUVEC treated with HU and hemin is due to a decrease in the bioavailability of GSH, resulting from GST-mediated transfer of GSH to hemin reduction. Thus, with the decrease of basal levels of GSH, induction if GSR is necessary to restore their basal levels. We have also shown that HU does not interfere in the expression of *HMOX1*, suggesting that the induction of the antioxidant response mediated by it involves a different activation mechanism.

Preliminary microarray analyzes were performed at HUVEC to investigate the effects of HU treatment on pathways related to the induction of antioxidant response. HUVEC treated with HU showed increased expression rate of detoxifying enzymes and antioxidants such as *SOD2*, *GSR*, *GSTM2*, *CR1* and *MGST1*. Increased expression of p62/SQSTM1 and decrease of *BACH1* and *UBE2K* were also observed. The expression of p62/SQSTM1 is positively regulated in the Nrf2 dependent form under certain chemical/oxidative stress conditions (ISHII et al., 2000; WARABI et al., 2007). Under conditions of Nrf2 activation induced by antioxidant or electrophilic response, high expression of p62/SQSTM1 can lead to the fragmentation of Keap1 in the autophagic degradation pathway, allowing the Nrf2 translocation to the ARE/EpRE domain that will initiate the induction of the systems antioxidants (COPPLE et al., 2010; FURFARO et al., 2016; TANIGUCHI et al., 2016). On the other hand, BACH1 and UBE2K exert antagonistic effects to the induction of the antioxidant response. BACH1 acts as a negative regulator of Nrf2 that binds to the ARE/EpRE domain, preventing the induction of the antioxidant response; and UBE2K (E2), is part of the polyubiquitination complex (E1, E2 and E3) and is responsible for catalyzing the conjugation of ubiquitin to the Cul3-Keap1-E3 complex,

which directs Nrf2 to degradation via ubiquitin proteasome system (UPS) (CULLINAN et al., 2004, HE et al., 2006, COPPLE et al., 2010). Thus, the decrease in *BACH1* and *UBE2K* expression is a fully favorable condition for the induction of antioxidant response via the Nrf2 signaling pathway.

Additionally, we observed increased expression of the phosphatidylinositol-4-phosphate 3-kinase subunits (*PIK3C2B* and *PIK3R3*) and protein kinases (*PRKCB* and *PRKCZ*) involved in the antioxidant response induction phase; and *GSK3B*, which acts in the post-induction phase of antioxidant response, reinforcing the evidence that it is an induction of the antioxidant response via Nrf2 signaling (NAKASO et al., 2003; NITURE; KHATRI; JAISWAL, 2014). In the post-induction phase, *GSK3B*, when activated, phosphorylates Src family members and these enter the nucleus to phosphorylate Nrf2Tyr568, which triggers its export from the nucleus followed by degradation of Nrf2 and inhibition of the antioxidant response (JAIN; JAISWAL, 2006; NITURE et al., 2017).

Upstream analysis demonstrated activation of miR-155-5p, a specific inhibitor of BACH1 (GU et al., 2017); miR-141-3p, which exerts a suppressive effect on Keap1 (CHENG et al., 2017); and Jun, a transcriptional regulator of AP-1 protein that is involved in the activation of the Nrf2-mediated antioxidant pathway (NITURE; KHATRI; JAISWAL, 2014; PULLARKAT et al., 2014).

These results suggest that HU may be inducing an Nrf2-dependent antioxidant response and activating p62 SQSTM1, which may act by regulating the integrity of the Keap1-Nrf2 complex (COPPLE et al., 2010).

Thus, it is suggested that HU or electrophilic products from their metabolic degradation are inducing antioxidant or electrophilic response via Nrf2, mediated by regulation of p62/SQSTM1. These mechanisms are involved in the detoxification of endogenous and exogenous electrophiles that may react with cellular components such as DNA (MALEC et al., 1984; HAYES et al., 2000; NAGAI et al., 2003; NITURE; KHATRI; JAISWAL, 2014; HODGES, MINICH, 2015, FURFARO et al., 2016).

Classical studies with HU showed that after its oral administration, it is absorbed and converted into a nitroxide radical in vivo, and transported to the active site of the M2 subunit of the ribonucleotide reductase protein, inactivating the enzyme and generating cytotoxic suppression, which suggests the induction of antioxidant response (YARBRO, 1992; ZHOU et al., 2001).

However, collecting these data allows us to suggest that HU can act as *(i)* extra/intracellular radical scavenger due to its reducing properties and being easily absorbed; *(ii)* source of NO in oxidant microenvironment (presence of hemin); *(iii)* stimulates antioxidant cellular response by electrophilic induction, due to the induction of the ARE/EpRE mediated by Nrf2 under regulation of p62/SQSTM1; *(iv)* induces heterogeneous expression of antioxidant genes in PBMC and HUVEC; *(v)* not interfering with *HMOX1* expression.

Finally, we believe that the elucidation of the regulatory mechanisms of Nrf2 via p62/SQSTM1 proposed in this work will allow the development of new studies aimed at the research for this therapeutic target, increasing the antioxidant responses of cells against marked oxidative insults in hemolytic diseases, among others.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no competing interests.

AUTHORSHIP

SSS, TNP and MSG conceived, designed the study, performed statistical analyses and wrote the paper. SSS performed all the experiments. JMS, DLZ, CSAA assisted some experiments and provided technical support, discussed the results and co-wrote the paper. MSG and TNP critically revised the manuscript. The manuscript has been critically reviewed and the final version has been approved by all authors.

ACKNOWLEDGEMENTS

The authors would like to thank Andris K. Walter for his critical analysis and English revision of the final manuscript. This work was supported by grants from the Brazilian National Council of Research (CNPq) (311888/2013-5) (MSG); the Foundation of Research and Extension of Bahia (FAPESB) (3626/2013, 1431040053063, and 9073/2007) (MSG); PPSUS/FAPESB (020/2013 EFP00007295), (MSG); and MCD/CNPq/MS-SCTIE-DECIT (409800/2006-6) (MSG).

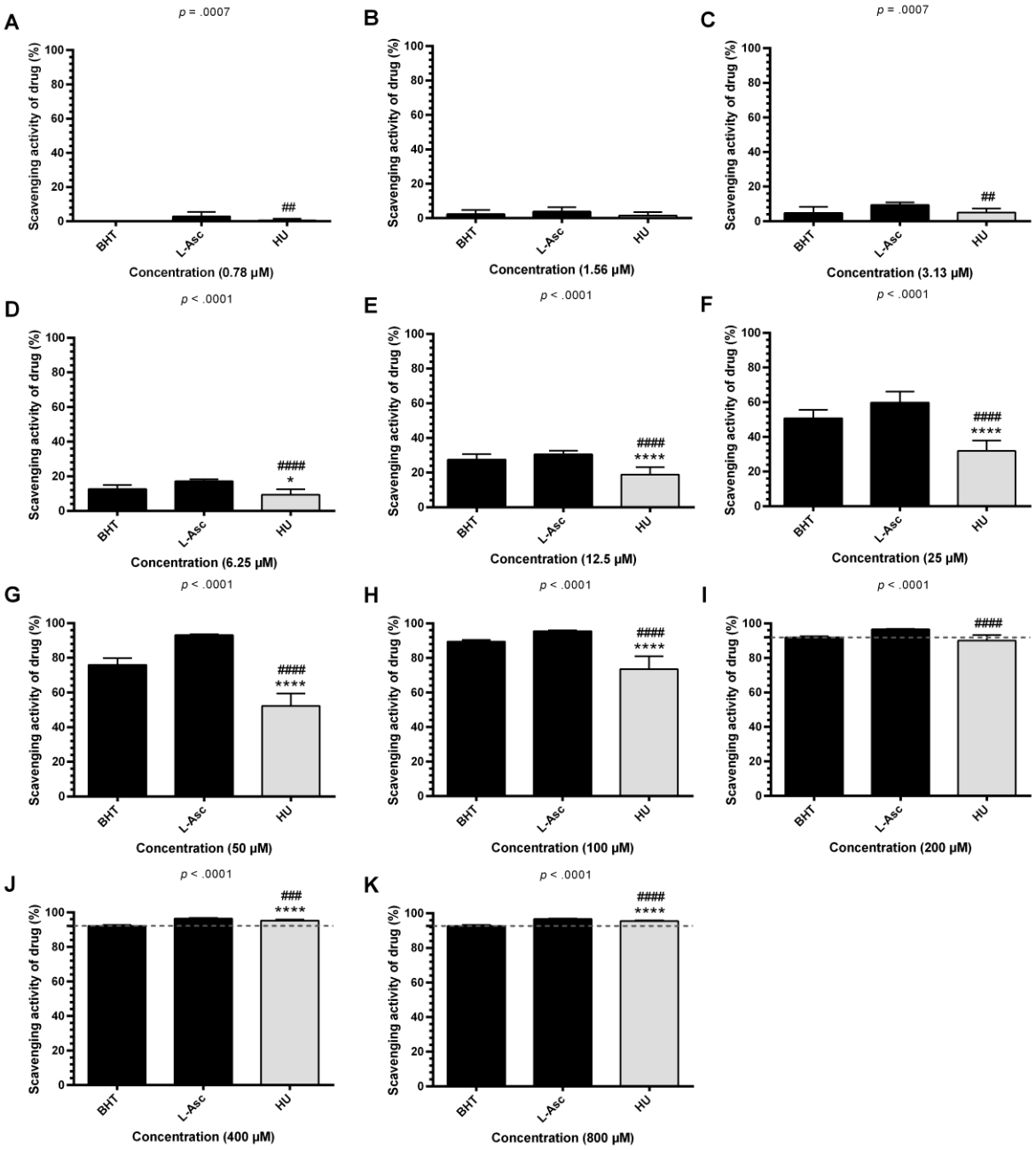


FIGURE 1. Scavenging activity of the free radical 100 µM DPPH with different concentrations of hydroxyurea. The antioxidant activity was measured by the DPPH free radical scavenging activity. Each value corresponds to the mean value ± mean standard deviation of three independent experiments (n = 4). BHT and L-acorbate were used as reference antioxidant compounds. HU: hydroxyurea; L-Asc: L-ascorbate; BHT: butylated hydroxytoluene. One-way ANOVA, p < .0001; Tukey's post-hoc: HU *vs* BHT, $^{*}p < .05$, $^{****}p < .0001$; HU *vs* L-Asc, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$, $^{\#\#\#\#}p < .0001$.

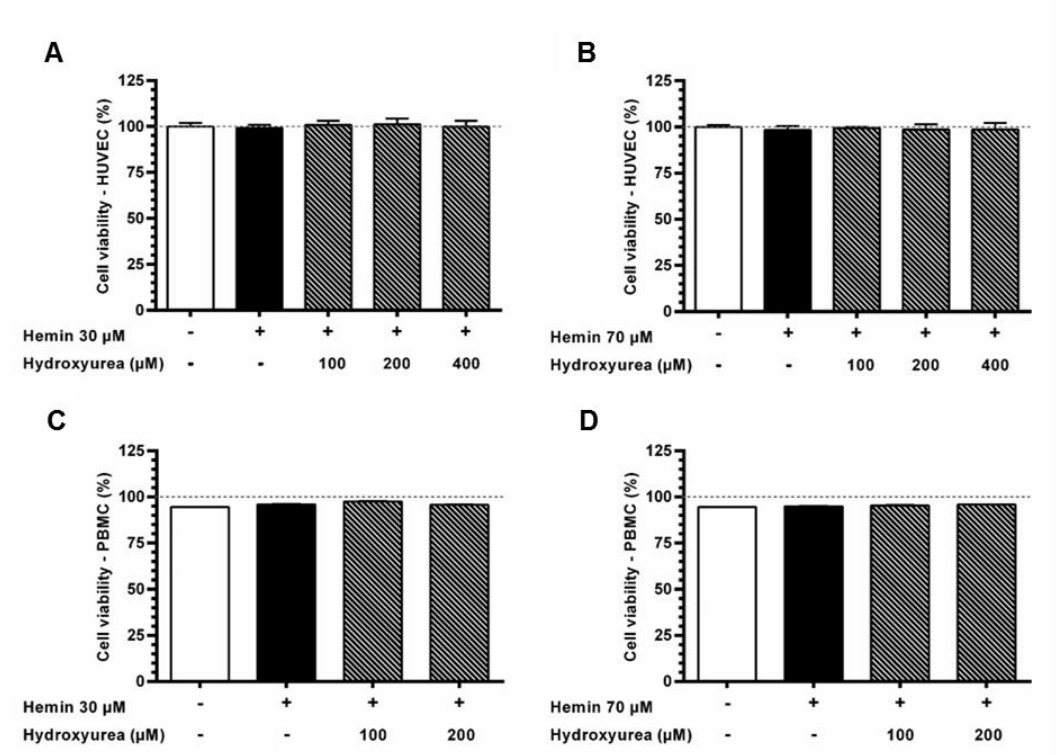


FIGURE 2. Evaluation of the cytotoxicity of hemin and hydroxyurea in HUVEC and PBMC. The cytotoxicity in HUVEC was assessed after challenge with hemin at concentrations of **(A)** 30 and **(B)** 70 µM and HU 100, 200 and 400 µM using resazurin salt (12.5 µM) reduction assay. Each value corresponds to the mean ± standard deviation (n = 4). Cytotoxicity in PBMC was assessed after challenge with hemin at concentrations of **(C)** 30 and **(D)** 70 µM and HU 100 and 200 µM using propidium iodide (PI) labeling by flow cytometry.

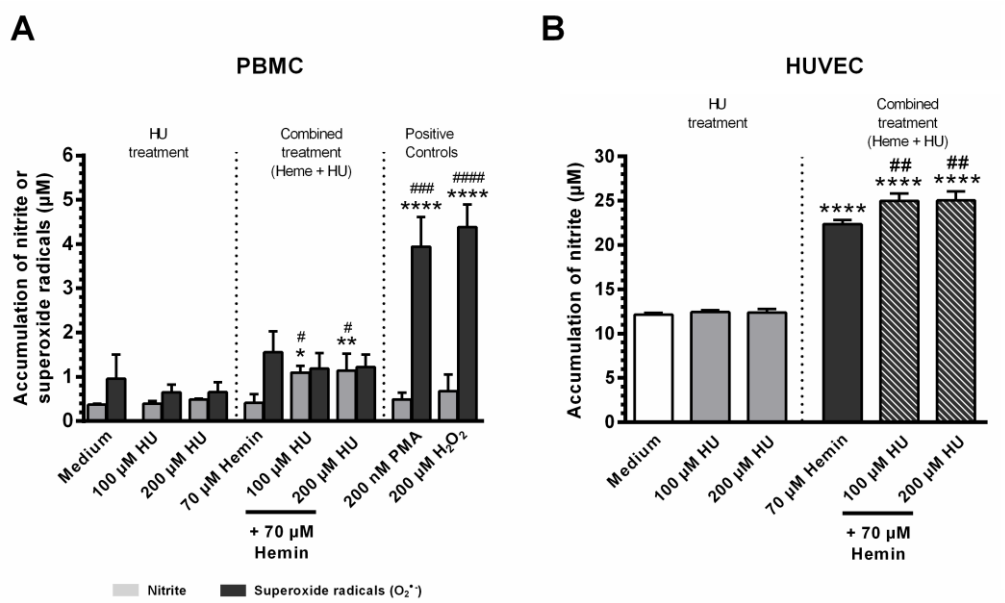


FIGURE 3. Production of superoxide radicals in PBMC, and nitrite metabolites in PBMC and HUVEC stimulated with hemin and/or hydroxyurea. A) Superoxide radicals ($O_2^{\bullet-}$) produced by PBMCs during the challenge were converted to nitrite by reaction with hydroxylamine and measured in supernatants by the Griess reaction after 24 h (as previously described in the materials and methods section). The nitrite production was measured directly in supernatants without hydroxylamine. Each value corresponds to the mean ± SD (n = 3). One-way ANOVA, $p < .0001$; Tukey's pos-hoc: HU alone or associated with hemin *vs* medium, or hemin *vs* medium, $^{*}p < .05$; $^{**}p < .01$; HU with hemin *vs* hemin, $^{\#}p < .05$. **B)** Production of nitrite in supernatants of HUVEC after challenge. Each value corresponds to the mean ± SD (n = 3). One-way ANOVA, $p < .0001$; Tukey's pos-hoc: HU alone or HU + hemin vs medium, or hemin *vs* medium, $^{****}p < .0001$; HU + hemin *vs* hemin, $^{\#\#}p < .01$.

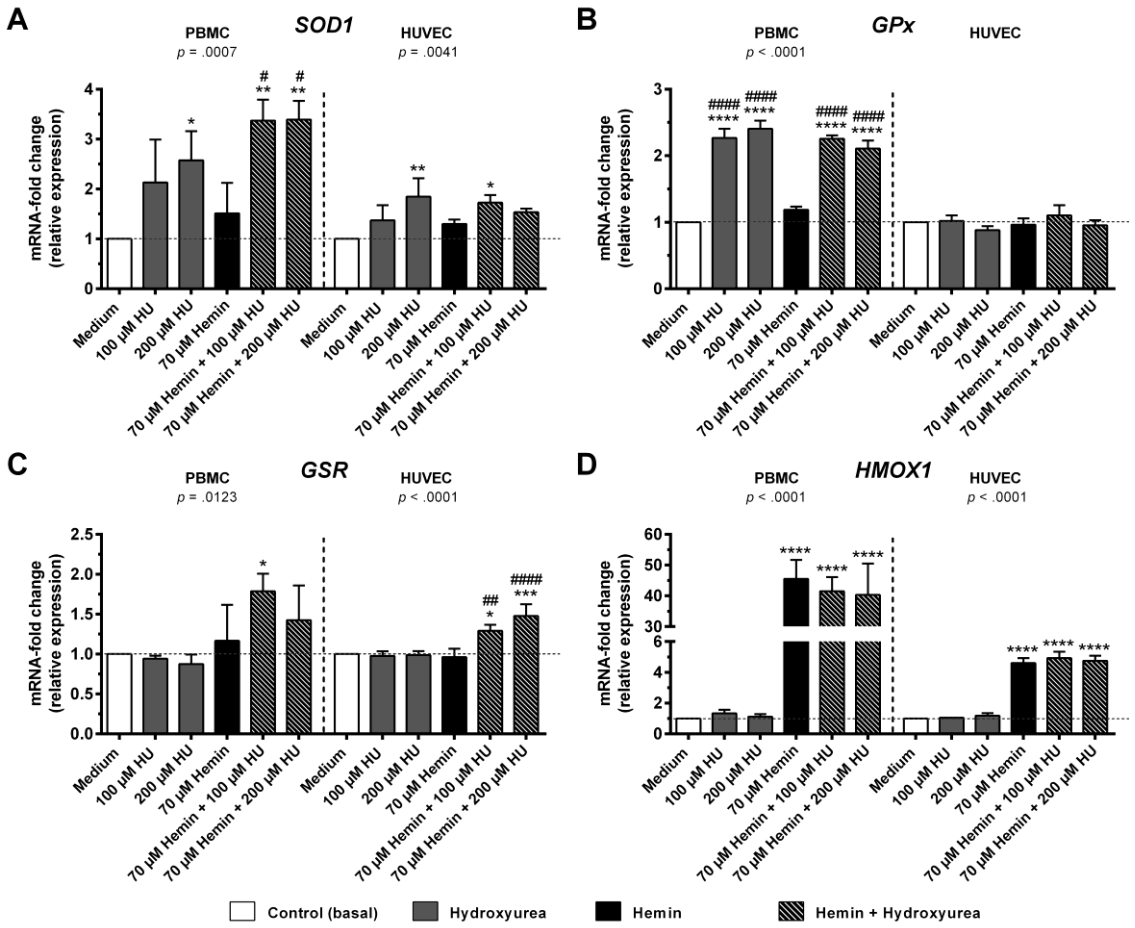


FIGURE 4. Effect of Hydroxyurea on induction of antioxidant response genes in PBMCs and HUVEC. Both PBMCs and HUVEC were treated with different concentrations of hydroxyurea (100 and 200 µM) in the presence or absence of 70 µM hemin for 4 h and important antioxidant genes were evaluated. **A)** Heme-oxygenase 1 (*HMOX1*); **B)** superoxide dismutase-1 (*SOD1*); **C)** Glutathione S-reductase (*GSR*); **D)** Glutathione peroxidase (*GPx*). Each value corresponds to the mean ± standard deviation (n = 3). Expression values was determined by relative quantification using the following expression: fold change = $2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT_{target} - CT_{housekeeping}$ and $\Delta(\Delta CT) = \Delta CT_{treated} - \Delta CT_{control}$ (medium). Data are normalized as fold expression over control for each gene. One-way ANOVA, $p < .05$; Tukey's post-hoc: HU alone or HU + hemin *vs* medium, or hemin *vs* medium, *$p < .05$; **$p < .01$, ***$p < .001$, ****$p < .0001$; hemin + HU *vs* hemin, $^{\#\#}p < .01$; $^{\#\#\#\#}p < .0001$.

TABLE 1. Scavenging activity of 50% of 100 µM DPPH free radical of hydroxyurea, L-ascorbate and butylated hydroxytoluene

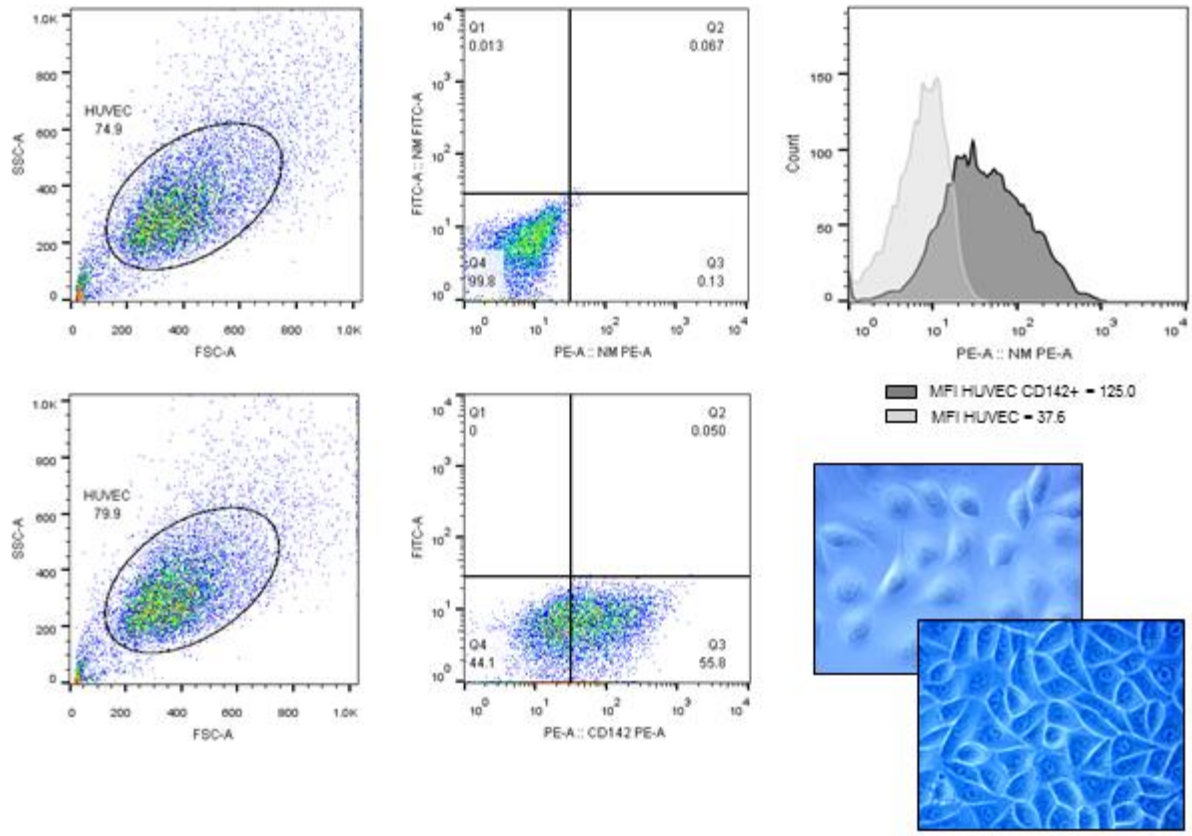
Drugs	IC_{50}* (µM) ± SD**	One-way ANOVA	Tukey's post-hoc		
			HU vs BHT	HU vs L-Asc	L-Asc vs BHT
Hydroxyurea	38.68 ± 0.47				
Butylated hydroxytoluene	23.07 ± 2.64	$p < .0001$	$p < .0001$	$p < .0001$	$p < .05$
L-Ascorbate	18.22 ± 5.93				

* Each value corresponds to the mean of inhibitory concentration (n = 3) of three independent experiments.
** Standard deviation. HU: Hydroxyurea; BHT: Butylated hydroxytoluene; L-Asc: L-Ascorbate.

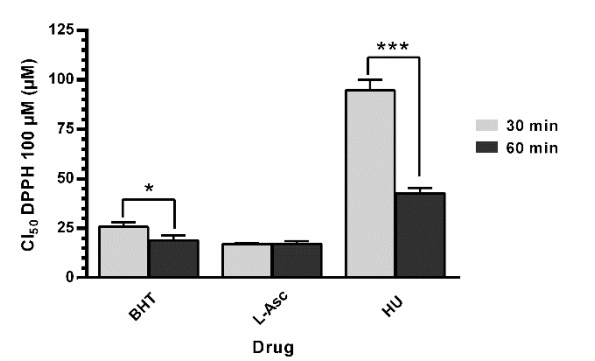
TABLE 2. Differential expression of genes obtained by Microarray analysis of HUVEC challenged with hydroxyurea related to Nrf2-antioxidant/electrophile response element signaling pathway

Symbol	Entrez Gene Name	Expr Log Ratio ‡	Location	Type(s)
Enzymes				
GSR	Glutathione-disulfide reductase	2.882	Cytoplasm	Enzyme
GSTM2	Glutathione S-transferase mu 2	2.210	Cytoplasm	Enzyme
KLB	Klotho beta	1.974	Plasma Membrane	Enzyme
SOD2	Superoxide dismutase 2	1.852	Cytoplasm	Enzyme
HACD3	3-hydroxyacyl-CoA dehydratase 3	1.807	Cytoplasm	Enzyme
MGST1	Microsomal glutathione S-transferase	1.733	Cytoplasm	Enzyme
CBR1	Carbonyl reductase 1	1.727	Cytoplasm	Enzyme
RRAS	RAS related	1.517	Cytoplasm	Enzyme
NRAS	NRAS proto-oncogene, GTPase	-1.521	Plasma Membrane	Enzyme
AOX1	Aldehyde oxidase 1	-2.188	Cytoplasm	Enzyme
Peptidases				
CLPP	Caseinolytic mitochondrial matrix peptidase proteolytic subunit	1.551	Cytoplasm	Peptidase
ENC1	Ectodermal-neural cortex 1	-1.996	Nucleus	Peptidase
EPHX1	Epoxide hydrolase 1	-3.291	Cytoplasm	Peptidase
Transcription regulator				
SQSTM1	Sequestosome 1	1.639	Cytoplasm	Transcription regulator
ATF4	Activating transcription factor 4	-1.639	Nucleus	Transcription regulator
BACH1	BTB domain and CNC homolog 1	-1.721	Nucleus	Transcription regulator
PMF1/PMF1-BGLAP	Polyamine modulated factor 1	-1.740	Nucleus	Transcription regulator
CREBBP	CREB binding protein	-1.743	Nucleus	Transcription regulator
MAFG	MAF bZIP transcription factor G	-1.823	Nucleus	Transcription regulator
UBE2K	Ubiquitin conjugating enzyme E2 K	-1.830	Cytoplasm	Transcription regulator
FOS	Fos proto-oncogene, AP-1 Transcription factor subunit	-3.950	Nucleus	Transcription regulator
Kinase / others				
PRKCB	Protein kinase C beta	4.026	Cytoplasm	Kinase
PRKCZ	Protein kinase C zeta	1.902	Cytoplasm	Kinase
PIK3C2B	Phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta	1.892	Cytoplasm	Kinase
DNAJB12	DnaJ heat shock protein family (Hsp40) member B12	1.794	Cytoplasm	Other
FGFR3	Fibroblast growth factor receptor 3	1.685	Plasma Membrane	Kinase
GSK3B	Glycogen synthase kinase 3 beta	1.607	Nucleus	Kinase
PIK3R3	Phosphoinositide-3-kinase regulatory subunit 3	1.597	Cytoplasm	Kinase
DNAJB14	DnaJ heat shock protein family (Hsp40) member B14	-1.532	Cytoplasm	Enzyme
DNAJC21	DnaJ heat shock protein family (Hsp40) member C21	-1.649	Other	Other
FRS2	Fibroblast growth factor receptor substrate 2	-1.665	Plasma Membrane	Kinase
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1	-1.690	Cytoplasm	Kinase
PRKCE	Protein kinase C epsilon	-1.679	Cytoplasm	Kinase
PIK3C2A	Phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 alpha	-1.716	Cytoplasm	Kinase
PIK3CB	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta	-1.761	Cytoplasm	Kinase
DNAJB4	DnaJ heat shock protein family (Hsp40) member B4	-1.843	Nucleus	Other
DNAJC18	DnaJ heat shock protein family (Hsp40) member C18	-1.897	Other	Enzyme
GAB1	GRB2 associated binding protein 1	-2.156	Cytoplasm	Kinase
MAPK14	Mitogen-activated protein kinase 14	-2.418	Cytoplasm	Kinase

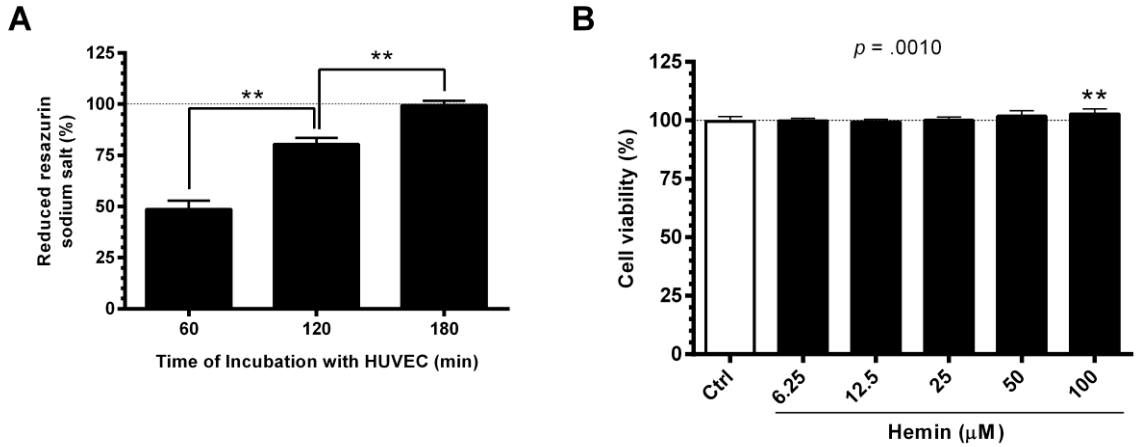
‡ Based on relative expression (log fold change > 1.5).



SUPPLEMENTARY FIGURE 1. Phenotypic and morphological characterization of HUVEC by flow cytometry and light microscopy. The dot-plots and histogram summarize the labeling of HUVEC with anti-CD142 (tissue factor) expressed after activation with 200 µM H_2O_2 after 24 h of treatment. Mean Fluorescence Intensity (MFI) of unlabelled HUVEC = 37.6; MFI HUVEC + CD142 = 125.0. The photomicrographs show the typical cobblestone morphology the oval nucleus and monolayer growth of the HUVEC cell line used in the assays viewed under light microscopy (magnification 40X). Cells newly adhered (above) to the beginning of confluence and confluent cells (below) and monolayer formation (40X magnification).



SUPPLEMENTARY FIGURE 2. Scavenging activity of 50% of free radical DPPH 100 µM of hydroxyurea after 30 and 60 min of incubation. Each value corresponds to the mean concentration ± standard deviation (n = 4). BHT and L-acorbate were used as reference antioxidant drugs. HU: hydroxyurea; L-Asc: L-ascorbate; BHT: butylated hydroxytoluene. Wilcoxon test, *p < .05.



SUPPLEMENTARY FIGURE 3. Kinetic standardization of resazurin sodium salt reduction and cytotoxicity of HUVEC at different hemin concentrations and cytotoxicity of HUVEC at different hemin concentrations. A) Kinetics of resazurin sodium salt reduction in resofurin 12.5 μM by HUVEC after 60, 120 and 180 min. Wilcoxon test, ***p* < .01. **B)** Determination of heme cytotoxicity at different concentrations ranging from 6.25 to 100 μM after 24 h of challenge. In Each value corresponds to the mean concentration ± standard deviation (n = 8). One-way ANOVA, *p* < .05; Tukey's post-hoc: Heme vs medium, ***p* < .01.

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6. DISCUSSÃO

Tendo em foco as repercusões oxidativas e inflamatórias como elementos chave relacionados à gravidade da DF, este trabalho consistiu em avaliar os efeitos do heme em pacientes com AF, e investigar propriedades terapêuticas da HU visando a elucidação de mecanismos alternativos distintos daquele relacionado a indução de HbF.

No manuscrito 1, foi investigada a associação do heme com marcadores laboratoriais comumente utilizados no acompanhamento clínico de pacientes com AF, com objetivo de identificar os efeitos dessa molécula em diferentes contextos clínicos. Inicialmente, demonstrou-se que o aumento dos níveis de heme livre está associado ao aumento da HbS, e à diminuição dos níveis de HbF. Uzunova e colaboradores (2010) demonstraram in vitro que concentrações de heme superiores a 66 μM, aumentam em duas ordens de magnitude a formação de aglomerados metaestáveis de HbS, sugerindo que os picos de concentração de heme livre nos eritrócitos de pacientes com AF podem contribuir para a complexidade das manifestações clínicas da doença. Por outro lado, a HbF apresenta estabilidade e alta afinidade pelo oxigênio, impedindo o início da polimerização da HbS e, consequentemente, os eventos vasoclusivos e hemolíticos (AKINSHEYE et al., 2011; STEINBERG et al., 2014). De acordo com esses achados, a relação inversa entre heme e HbF sugere que níveis mais elevados de heme circulante se devem ao aumento das crises hemolíticas em função da diminuição dos níveis de HbF. Embora a liberação de heme esteja diretamente relacionada à hemólise na AF, a correlação inversa e significativa encontrada entre os níveis plasmáticos de heme livre e HbF no presente estudo não foi previamente descrita, reforçando a importância dos níveis de HbF na liberação de heme, e que esta molécula pode ser um indicador da gravidade da AF.

Em seguida, constatou-se que o aumento da concentração plasmática de heme nesses pacientes está associado com o aumento no número de monócitos. A função eritróide está intimamente relacionada à biologia dos macrófagos que são derivados da maturação de monócitos, podendo desempenhar papéis importantes na captura de heme livre, reciclagem de ferro, eritropoiese e inflamação (ALAM; DEVALARAJA; HALDAR, 2017). Os produtos de degradação da hemólise podem estimular os subtipos de monócitos que reconhecem os complexos de Hb/heme-

haptoglobina e hemopexina, via CD163 e CD91, respectivamente, receptores de alta afinidade presentes na superfície de monócitos/macrófagos (KRISTIANSEN et al., 2001; MOESTRUP e MOLLER, 2004; HVIDBERG et al., 2005). Níveis elevados de heme também estimulam a diferenciação de monócitos em macrófagos de reciclagem de ferro no baço via fator de transcrição Spi-C (HALDAR et al., 2014) e atuam como uma alarmina (ou DAMP), estimulando a resposta pró-inflamatória via TLR4, ativando o inflamassoma, e induzindo a secreção de citocinas pró-inflamatórias (FIGUEIREDO et al., 2007; DUTRA et al., 2014; DUTRA e BOZZA, 2014; LI et al., 2014).

Como esperado, a concentração de heme está fortemente associada com marcadores de hemólise e função hepática, assim como o aumento da produção de bilirrubina total e frações (direta e indireta) e AST. O heme é catabolizado pela HO-1, gerando biliverdina, monóxido de carbono e Fe^{2+}. Após esta etapa, a biliverdina sofre ação da bilirrubina redutase para formar a bilirrubina (TENHUNEN; MARVER; SCHMID, 1968). Tanto a biliverdina como a bilirrubina possuem propriedades antioxidantes, protegendo os tecidos contra insultos oxidativos (ZIBERNA et al., 2016). O aumento da bilirrubina indireta está relacionado ao aumento da degradação da Hb proveniente da hemólise, acúmulo de bile e aumento do risco de colelitíase (STEPHENS e SCOTT, 1980; COATS; GARDNER; THEIN, 2014; MARTINS et al., 2017). Por outro lado, a bilirrubina direta pode estar relacionada à lesão hepática ou obstrução biliar. Aliado a isso, o aumento da AST geralmente está relacionado com lesões hepáticas crônicas, embora não seja um marcador exclusivamente hepático, podendo ser encontrado em outros tecidos como coração, rins, cérebro e músculos (BOTROS and SIKARIS, 2013). Um estudo de caso-controle demonstrou que pacientes com AF apresentam níveis aumentados de heme e mediadores inflamatórios (CARVALHO et al., 2017). Em conjunto, esses achados sugerem que o heme pode contribuir sistemicamente para a disfunção hepática, gerando estresse oxidativo, dano tecidual e inflamação, promovendo danos em diferentes órgãos.

Também foi encontrada a associação forte do heme com marcadores lipídicos, sugerindo que as suas propriedades pró-oxidantes podem interferir no metabolismo lipídico na AF. O heme apresentou associação positiva muito forte com triglicérides, e associação negativa com HDL-C. Vários estudos demonstraram distúrbios no metabolismo lipídico na AF e DF (DJOUMESSI et al., 1994; SHORES et al., 2003; SEIXAS et al., 2010; ZORCA et al., 2010; OZTURK et al., 2013;

ALELUIA et al., 2017), e têm sido proposto a existência do subfenótipo dislipidêmico (SEIXAS et al., 2010; ALELUIA et al., 2017), embora o mecanismo dessa disfunção seja pouco compreendido. Shalev e colaboradores (2007) sugeriram que a hipocolesterolemia observada em pacientes com anemia crônica se deve ao aumento da atividade eritropoiética da medula, que mobiliza o *pool* de colesterol plasmático para produzir novas células eritróides. O HDL-C exerce propriedades antioxidantes e anti-inflamatórias, removendo o excesso de colesterol dos tecidos periféricos e depositado nas artérias, por transporte reverso para o fígado a ser eliminado (NOFER et al., 2002; SEIXAS et al., 2010), conferindo pacientes com menor risco de hemólise, disfunção endotelial e inflamação, diminuindo o risco de complicações cardiovasculares (DJOUMESSI et al., 1994; NAVAB et al., 2004) e hipertensão pulmonar (ZORCA et al., 2010). Em contraste, uma abordagem antagônica mostrou que em ambientes oxidantes ou inflamatórios, o HDL-C pode sofrer alterações funcionais e exercer propriedades pró-inflamatórias (p-HDL) exacerbando o estresse oxidativo (NAVAB et al., 2004; MCMAHON et al., 2006; WATANABE et al. al., 2007; NAVAB et al., 2009; OZTURK et al., 2013; ATAGA et al., 2015). Níveis aumentados de Hb/heme livre na hemólise, dentre outros fatores pró-inflamatórios na DF, atuam como um desafio oxidativo ao HDL-C e contribuem substancialmente para a formação de sua forma inflamatória (WATANABE et al., 2007; JI et al., 2016).

Níveis aumentados de triglicérides e VLDL-C estão associados a muitas dessas complicações clínicas (ZORCA et al., 2010). Triglicerídeos e fosfolipídios de membrana são os alvos primários de ataque de radicais hidroxila ($^{\bullet}OH$) gerados por reações de Fenton, mediadas por Fe^{2+} e H_2O_2 (GUTTERIDGE, 1984; GARDNER, 1989) com a formação de radicais lipídicos causando lipotoxicidade (BIELSKI; ARUDI; SUTHERLAND, 1983; GUTTERIDGE, 1984), sendo correlacionados com biomarcadores de hemólise, ativação endotelial, inflamação e alterações cardiovasculares (ZORCA et al., 2010). Além disso, as reações oxidativas em cadeia podem provocar o encurtamento das cadeias acil gerando moléculas reativas como aldeídos poliinsaturados α e β, promovendo alterações na arquitetura e nas propriedades funcionais desses lipídios, incluindo transporte iônico, atividade enzimática ligada à membrana, e a função de receptores celulares (SPECTOR; YOREK, 1985; HAUCK; BERNLOHR, 2016). Soupene e colaboradores (2016) demonstraram que o ambiente inflamatório altamente oxidativo da DF interfere na

esterificação do colesterol e na função do HDL-C, o que pode explicar a diminuição dos seus níveis circulantes. Neste contexto, pode-se sugerir que o microambiente oxidante gerado pelos produtos da hemólise, pode contribuir para mudanças estruturais nesses lipídios e em algum ponto-chave do metabolismo lipídico, incluindo reações de esterificação, transporte e/ou reconhecimento, modificando a função normal destas moléculas na homeostasia metabólica, resultando no acúmulo de triglicerídeos, diminuição do HDL-C, ou geração de p-HDL.

O próximo passo consistiu em avaliar a contribuição dos haplótipos ligados ao grupo de genes da globina beta na produção de HbF, e se eles interferem na liberação do heme livre, uma vez que encontramos anteriormente a associação negativa entre heme e HbF. Os pacientes com genótipo BEN/BEN apresentaram níveis mais elevados de HbF, e os CAR/CAR apresentaram níveis mais baixos, sendo que nossos resultados são consistentes com outros estudos (NAGEL et al., 1987; GREEN et al., 1993; GONÇALVES et al., 2003). Deste modo, sugere-se que a presença de pelo menos um alelo BEN tenha efeito benéfico, mesmo na presença do alelo CAR, apresentando níveis equivalentes de HbF comparado aos homozigotos para BEN. Ao avaliar a influência desses haplótipos na liberação de heme, não foram observadas diferenças estatisticamente significativas entre os haplótipos estudados, embora tenhamos observado que indivíduos com genótipo BEN/BEN possuem níveis absolutos de heme livre mais elevados em comparação ao CAR/CAR, sendo que os CAR/BEN apresentam níveis intermediários.

Foi demonstrado também que as concentrações de heme estão significativamente elevadas em pacientes com histórico clínico de AVC prévio. O heme contribui sistemicamente para inflamação gerando estresse oxidativo e inflamação por várias vias que favorecem o estado de hipercoagulabilidade e disfunção endotelial, que constitui um microambiente plenamente favorável para o desenvolvimento do AVC (MULLER-EBERHARD et al., 1968; REITER et al., 2002; SWITZER et al., 2006; FORTES et al., 2012; DUTRA et al., 2014; DUTRA and BOZZA, 2014; KATO; STEINBERG; GLADWIN, 2017). Novos estudos são necessários para entender se os níveis de heme estão elevados antes do desenvolvimento do AVC, ou se esses níveis elevam-se após a sua ocorrência. Deste modo, considerando-se que a ocorrência de AVC é um fator predisponente para ocorrência de novos eventos, e a contribuição do heme para inflamação sistêmica e disfunção endotelial, sugere-se que os níveis elevados de heme constitui

um fator de risco para o desenvolvimento do AVC, merecendo atenção como possível marcador de predição de gravidade da doença, especialmente para os defechos clínicos de AVC.

No manuscrito 2 foi investigado o papel de hemácias falciformes íntegras e lisadas na ativação do inflamassoma NLRP3, e se os efeitos do tratamento desses pacientes com HU interfere na formação de componentes e ativação do inflamassoma, assim como a liberação de LTB_4. O inflamassoma NLRP3 é bem caracterizado e possui papel importante na modulação de processos inflamatórios sendo ativado por vários sinais, como por exemplo, os produtos de hemólise, muito frequentes na AF (SCHRODER e TSCHOPP, 2010; DUTRA et al., 2014; MENDONÇA et al., 2016).

Inicialmente foi demonstrado que hemácias SS (SS-RBCs) podem atuar como DAMPs, induzindo a expressão de componentes do inflamassoma NLRP3 em PBMC. Ao comparar os efeitos de SS-RBCs íntegras e lisadas, foi observada maior indução de *CASP1* e *IL18* em SS-RBCs lisadas. Por outro lado, a comparação entre hemácias íntegras e lisadas de voluntários sadios (AA-RBC) mostrou que apenas AA-RBCs lisadas foram capazes de induzir a expressão de componentes do inflamassoma NLRP3. É descrito que SS-RBCs apresentam uma série de alterações estruturais como a perda de simetria lipídica com expressão aumentada de fosfatidilserina e moléculas de adesão como ICAM-4, CD44 e CD47, perda da fluidez da membrana e maior susceptibilidade a danos oxidativos (DE OLIVEIRA e SALDANHA, 2010). Ren e colaboradores (2006) demonstraram previamente que SS-RBCs possuem níveis aumentados de ácido aracdônico, e níveis diminuídos de ácido eicosapentaenóico e ácido docosahexaenóico, em comparação com eritrócitos de voluntários sadios. Isso sugere que alterações na membrana eritrocitária podem justificar o contraste observado entre indivíduos saudáveis e com AF na ativação de vias do inflamassoma.

Por outro lado, observou-se um comportamento diferente para *IL1B*, uma vez que PBMC desafiadas com SS-RBCs íntegras apresentaram níveis de expressão mais elevados de *IL1B* do que PBMC desafiadas com SS-RBCs lisadas. Apesar dos efeitos observados em AA-RBCs lisadas na expressão de *IL1B*, *CASP1* e *NLRP3*, é importante considerar que a hemólise intravascular ocorre fisiologicamente em níveis reduzidos em voluntários sadios, o que contrasta com os eventos hemolíticos frequentes em pacientes com AF (TAYLOR et al., 2008; VILAS-BOAS et al., 2010).

Assim, a inflamação observada em indivíduos com AF pode ser consequência da exposição constante a DAMPs originados de SS-RBCs íntegras e lisadas durante a hemólise intravascular, que leva ao aumento da expressão de receptores semelhantes ao NOD (NLRs) e pode promover o recrutamento de leucócitos por mecanismo dependente de componentes do inflamassoma (DUTRA et al., 2014).

Após isso, foi investigado se SS-RBCs íntegras e lisadas eram capazes de estimular a produção de IL-1β. Os resultados demonstraram que SS-RBCs íntegras e lisadas promoveram a produção de níveis significativos de IL-1β, diferentemente de AA-RBCs que não demonstraram esse efeito. Este achado sugere que a secreção de IL-1β pode estar relacionada a DAMPs encontrados apenas em SS-RBCs.

Além disso, tanto os AA-RBCs quanto SS-RBCs foram capazes de induzir a produção de LTB_4, mas a indução foi mais pronunciada em PBMC desafiados com SS-RBCs íntegras. O LTB_4 é um mediador inflamatório e seus níveis estão aumentados na AF (SETTY e STUART, 2002; CARVALHO et al., 2017; PITANGA, et al., 2016). O LTB_4 atua como mediador quimioatrativo que aumenta a migração leucocitária e a adesão ao endotélio vascular, contribuindo para o desenvolvimento do ambiente inflamatório observado na AF. A análise conjunta desses dados permite sugerir que apesar das preocupações iniciais acerca dos produtos liberados durante a hemólise, especialmente o heme (MONTEIRO et al., 2011), os componentes presentes na membrana de SS-RBCs têm papel fundamental na produção de IL-1β e LTB_4.

Uma vez que os marcadores hematológicos e bioquímicos são conhecidos por diferirem substancialmente entre indivíduos com AF e voluntários sadios, avaliamos o efeito do do uso de HU em relação a estes parâmetros. Na AF, o principal alvo terapêutico consiste em tentar substituir a produção de HbS por HbF. A HU produz benefícios terapêuticos pelo aumento dos níveis de HbF, com diminuição da polimerização de HbS, o que melhora a reologia dos eritrócitos com redução de sua adesão (KUMKHAEK et al., 2008; RAMIREZ-RAMIREZ et al., 2013). No entanto, no presente estudo, não houve diferença significativa na concentração de HbF entre os grupos tratados e não tratados. Os haplótipos ligados ao grupo de genes da globina β^S têm papel importante na síntese da HbF, sendo que alguns estudos mostraram que esta hemoglobina é encontrada em concentrações maiores em pacientes com haplótipo BEN em comparação com o haplótipo CAR (ADORNO

et al., 2004; REZENDE et al., 2016), fato que pode justificar a maior responsividade ao tratamento a HU em pacientes com apenas o haplótipo CAR em comparação com CAR/BEN ou BEN/BEN. Considerados em conjunto, estes resultados demonstram a capacidade do tratamento com HU em melhorar a produção de HbF em pacientes com AF e o haplótipo CAR.

Como esperado, os níveis de heme e proteína C-reativa foram mais elevados nos pacientes com AF em comparação aos voluntários sadios (REITER et al., 2002; MOHAMMED et al., 2010; REES e GIBSON, 2012). Além disso, foi demonstrado que o tratamento com HU foi capaz de reduzir os níveis de heme e aumentar os de proteína C-reativa. Sabe-se que o heme livre tem propriedades pró-oxidantes e pró-inflamatórias e que os níveis de PCR estão associados à lesão endotelial e ao estado inflamatório crônico observado em pacientes com AF (REES; GIBSON, 2012). Assim, esse achado pode sustentar a hipótese de que pacientes com AF podem ter inflamação persistente mesmo sob tratamento com HU, uma vez que esse tratamento não interfere em todos os mecanismos inflamatórios.

A ferritina é um biomarcador que se encontra elevado na inflamação e nas doenças autoimunes, como o lúpus eritematoso sistêmico (VANARSA et al., 2012), nas CVOs e infecções em pacientes com AF (AL-SAQLADI; BIN-GADEEM; BRABIN, 2012). A variação nos níveis de ferritina em pacientes tratados com HU e não tratados foi muito alta, sendo que indivíduos submetidos à terapia com HU costumam apresentar histórico clínico mais grave, que em geral, melhora significativamente após a terapia. Entrentanto, o ferro liberado nas crises hemolíticas prévias associado ao regime transfusional ao longo da vida, promovem acúmulo de ferro com aumento nos níveis plasmáticos da ferritina (BOURBON FILHO et al., 2011; BARCELLINI e FATTIZZO, 2015), o que pode explicar a grande variação existente entre os dois grupos, e o aumento observado em pacientes tratados com HU. Adicionalmente, essa grande variação observada entre pacientes que tomam HU ou não, pode ser explicada pela heterogeneidade clínica da AF.

Com relação à análise do inflamassoma NLRP3 foi observado que o tratamento com HU não interferiu na expressão de *CASP1*, *IL1B* e *IL18*, sugerindo que a HU não afeta a inflamação condicionada a esse inflamassoma, cuja ativação é observada em pacientes com AF. Entretanto, nossos resultados sugerem que pacientes com AF tratados com HU diminuem a expressão do gene *NLRP3*. O tratamento com HU não alterou os níveis de IL-1β em pacientes com AF, mas

diminuiu os níveis séricos de LTB_4. Assim, a redução da expressão do gene *NLRP3* e os níveis de LTB_4 podem sugerir um melhor prognóstico, demonstrando que a terapia com HU pode diminuir a inflamação por essas vias.

No manuscrito 3, a hipótese de que a HU atua na redução do estresse oxidativo induzindo a resposta antioxidante in vitro em HUVEC e PBMC foi testada. Para demonstrar isso, foram investigados os efeitos da HU em concentrações equivalentes àquelas encontradas no plasma de pacientes tratados pela droga em HUVEC e PBMC. A hemina também foi utilizada para tentar mimetizar, pelo menos em parte, o microambiente inflamatório presente na AF.

Inicialmente, foram realizados ensaios para avaliar se a HU era capaz de atuar diretamente via atividade sequestradora de radicais livres após incubação com o radical DPPH. Mesmo apresentando um CI_{50} superior comparado aos controles antioxidantes avaliados, a HU apresentou atividades sequestradoras de DPPH consideráveis em concentrações de 100 e 200 µM (73,5 e 90%). Interessantemente, essas variações de concentração são consistentes com as concentrações plasmáticas encontradas em indivíduos com AF tratados com HU, e que são também amplamente utilizadas em estudos in vitro (ELIAS et al., 2014; LOPES et al., 2014; ALMEIDA e SOUZA, 2015). A partir desses achados, decidiu-se utilizar essas concentrações de HU para os demais ensaios. O potencial de “captura” de radicais livres da HU pode ser explicado por sua capacidade de atuar como doador de elétrons ou átomos de hidrogênio para neutralizar o composto DPPH (BLOIS, 1958). Estes resultados são indicativos de que a HU pode agir diretamente neutralizando radicais livres no microambiente extra e intracelular. Essa propriedade é de grande importância, pois pode conferir efeito protetor importante às células contra ataques oxidativos diretos em fosfolipídios de membrana, prevenindo o desencadeamento de respostas de ativação envolvidas no início da cascata oxidativa e estabelecimento de inflamação (HALLIWELL e GUTTERIDGE, 1984; GRIJALBA et al., 1998; NOOR et al., 2005).

O próximo passo foi avaliar se os tratamentos promoveriam toxicidade em HUVEC e PBMC. A estratégia consistiu em investigar separadamente os tratamentos com HU e heme em células endoteliais, que representam a interface entre os tecidos e o microambiente vascular; e em células mononucleares do sangue periférico (monócitos e linfócitos), importantes na regulação da resposta imune inata e adaptativa interagindo em uma via dupla com o endotélio vascular. A

concentração de hemina 70 μM foi escolhida com base nos achados de Carvalho e colaboradores (2017), de acordo com as concentrações plasmáticas médias de heme livre encontradas em indivíduos com DF em estado de crise. Curiosamente, não foi observada a diminuição da viabilidade de PBMC e HUVEC após os tratamentos com hemina, a HU ou o tratamento combinado com ambos, após 24 h de desafio. A ausência de toxicidade pode ser explicada pela resiliência celular apresentada por HUVEC e PBMC ao microambiente pró-oxidante condicionado pela hemina. O estímulo da HO-1 pelo heme demonstrou efeito citoprotetor em monócitos por inibição da apoptose, apesar de induzir positivamente a caspase-3 (LANG et al., 2005). O heme livre também promove a diferenciação de monócitos em macrófagos de reciclagem de ferro por indução do fator de transcrição Spi-C (HALDAR et al., 2014). Outros estudos mostraram que o heme inibe a morte de células neutrofílicas por apoptose por um mecanismo dependente de fosfatidilinositol-3-quinase (PIK3), MAPK e NF-kB, com a geração de EROs por indução de PKC e aumento da secreção de IL-8 (GRACA-SOUZA et al., 2002; ARRUDA et al. 2004). Além disso, a hemina promoveu a expressão elevada de *HMOX1* em ambos os tipos celulares, o que pode explicar o efeito protetor observado em resposta ao estresse ao heme prevenindo a morte celular.

Em seguida, ensaios funcionais foram desenvolvidos para demonstrar in vitro se os tratamentos com HU eram capazes de inibir a produção de $O_2^{\bullet-}$ em PBMC. Os resultados demonstraram que a HU foi capaz de diminuir a produção de $O_2^{\bullet-}$, embora a redução não tenha sido estatisticamente significativa. Além disso, os tratamentos com HU e hemina diminuíram a produção de $O_2^{\bullet-}$ em PBMC quando comparados com o tratamento com a hemina. Sugere-se três hipóteses: *(i)* que esta inibição ocorreu devido às propriedades sequestradoras da HU, abolindo a formação de $O_2^{\bullet-}$; *(ii)* devido à indução de SOD, que atua na dismutação do $O_2^{\bullet-}$ em H_2O_2 e O_2; e *(iii)* devido a inibição mediada por NO pré-formado. De fato, foi observado que tratamentos combinados com HU e hemina induziram produção de nitrato/nitrito, diferentemente do observado em PBMC com hemina. Da mesma forma, HUVEC tratadas simultaneamente com HU e hemina também mostraram o aumento significativo na produção de nitrato/nitrito em relação ao controle e HUVEC desafiadas com hemina. Estudos prévios demonstraram que a HU reage com a Hb para produzir hemoglobina ferro nitrosilo (HbNO), nitrito e nitrato, o que pode explicar o aumento na produção de NO (HUANG et al., 2002; LOCKAMY et al.,

2004). Isso sugere que o tratamento combinado induziu a produção de NO, que é uma das propriedades mais notáveis e conhecidas da HU, atuando na regulação do tônus vascular (COKIC et al., 2003; ALMEIDA; SOUZA, 2015).

A expressão de genes de resposta antioxidante foi avaliada para verificar se a HU era capaz de modular respostas antioxidantes citoprotetoras. Um perfil heterogêneo de expressão foi observado nos diferentes tipos celulares utilizados após 4 h de tratamento com HU e/ou hemina. PBMC tiveram níveis mais acentuados de expressão do que as HUVEC, o que pode ser explicado pela grande capacidade de reconhecimento e respostas efetoras de fagócitos/linfócitos, especialmente monócitos. Essas células geralmente são mais sensíveis a perturbações ambientais (CHAPLIN, 2010). Tratamentos com HU ou combinados com hemina, promoveram indução significativa de SOD em PBMC e HUVEC. Normalmente, sob condições de estresse, o acúmulo de $O_2^{\bullet-}$ contribui para a iniciação da resposta antioxidante induzindo a expressão de SOD pela ativação dependente de Nrf2. PBMC tratados com HU mostraram aumento na expressão de *GPx*, independentemente do tratamento combinado com hemina. Não foram observadas diferenças na expressão de *GPx* em HUVEC. Outros estudos descreveram os benefícios da HU na indução de *GPx* pela via de sinalização de GMPc dependente de NO em indivíduos com AF (CHO et al., 2010; EK MD et al., 2016). Além disso, os tratamentos com HU induziram aumento da expressão de *GSR* na presença de hemina nos tipos celulares estudados. Duas hipóteses são propostas: *(i)* a HU pode atuar induzindo a ação de fatores transcricionais e modulando a expressão de *GPx*; ou *(ii)* os metabólitos da HU podem promover a geração de H_2O_2 controlada pela expressão da *GPx* usando glutationa reduzida (GSH) sintetizada pela GSR para manter a homeostase (MALEC et al., 1984; IYAMU et al., 2001; SAKANO et al., 2001; NAGAI et al., 2003; HUANG; KIM-SHAPIRO; KING, 2004). Também foi demonstrado que a HU não interfere na expressão de *HMOX1*, sugerindo que a indução da resposta antioxidante mediada por ela envolve mecanismo de ativação distinto.

O aumento estatisticamente significante na indução de *GSR* foi observado em tratamentos de HUVEC e PBMC desafiadas com heme na presença de HU. O *GSR* usa glutationa dissulfeto (GSSG) como substrato à custa de NADPH para formar o sulfidril GSH, que é um importante antioxidante celular. Por outro lado, as glutationa S-transferases (GSTs), isoenzimas metabólicas de fase II, atuam catalisando a redução da glutationa (GSH) em substratos xenobióticos com o propósito de

desintoxicação (HODGES e MINICH, 2015). Diferentes estudos mostraram que a GST funciona fisiologicamente como uma proteína de ligação e/ou transporte de heme, para que este possa dempenhar várias funções celulares importantes (HARVEY e BEUTLER, 1982; KIRSCHNER-ZILBER; LAUFER; SHAKLAI, 1989; BOYER e OLSEN, 1991; KHAN e QUIGLEY, 2011).

De acordo com os resultados alcançados, sugere-se que o aumento na expressão de *GSR* observado em PBMC e HUVEC submetidas ao tratamento combinado de HU e heme seja devido a diminuição na biodisponibilidade de GSH, resultantes das reações de transferência de GSH mediadas por GST para redução do heme. Assim, com a diminuição dos níveis basais de GSH, a indução de GSR é necessária para restaurar os seus níveis basais.

Por fim, análises preliminares de microarranjos foram realizadas em HUVEC para investigar os efeitos do tratamento da HU em vias relacionadas à indução de resposta antioxidante. As HUVEC tratadas com HU mostraram aumento na taxa de expressão de enzimas detoxificantes e antioxidantes como *SOD2*, *GSR*, *GSTM2*, *CR1* e *MGST1*. Também foi observado aumento na expressão de *p62/SQSTM1* e diminuição de *BACH1* e *UBE2K*. A expressão de *p62/SQSTM1* é regulada positivamente de forma dependente de Nrf2 sob certas condições de estresse químico/oxidativo (ISHII et al., 2000; WARABI et al., 2007). Sob condições de ativação de Nrf2 induzidas por resposta antioxidante ou eletrofílica, a expressão elevada de *p62/SQSTM1* pode conduzir a fragmentação de Keap1 na via de degradação autofágica, possibilitando a translocação de Nrf2 para o domínio ARE/EpRE que dará início à indução dos sistemas antioxidantes (COPPLE et al., 2010; FURFARO et al., 2016; TANIGUCHI et al., 2016). Por outro lado, BACH1 e UBE2K exercem efeitos opositores à indução da resposta antioxidante. BACH1 atua como regulador negativo de Nrf2 que se liga ao ARE/EpRE, impedindo a indução da resposta antioxidante; e UBE 2K (E2) faz parte do complexo de poliubiquitinação (E1, E2 e E3), sendo responsável por catalisar a conjugação da ubiquitina para o complexo Cul3-Keap1-E3, que direciona o Nrf2 para degradação via sistema de proteassoma ubiquitina (UPS) (CULLINAN et al., 2004; HE et al., 2006; COPPLE et al., 2010). Assim, a diminuição da expressão de *BACH1* e *UBE2K* constitui uma condição plenamente favorável para a indução de resposta antioxidante pela via de sinalização de Nrf2.

Adicionalmente, foi observado o aumento na expressão das subunidades fosfatidilinositol-4-fosfato 3-quinase (*PIK3C2B* e *PIK3R3*) e proteínas quinases (*PRKCB* e *PRKCZ*) envolvidas na fase de indução de resposta antioxidante; e *GSK3B* que atua na fase pós-indutória de resposta antioxidante, reforçando as evidências de que se trata de uma indução da resposta antioxidante via sinalização de Nrf2 (NAKASO et al., 2003; NITURE; KHATRI; JAISWAL, 2014). Na fase pós indutória a *GSK3B*, quando ativada, fosforila membros da família Src e estes entram no núcleo para fosforilar o Nrf2Tyr568, que desencadeia a sua exportação do núcleo seguida pela degradação do Nrf2 e inibição da resposta antioxidante (JAIN; JAISWAL, 2006; NITURE et al., 2017).

Upstream analysis demonstrou a ativação de miR-155-5p, um inibidor específico de BACH1 (GU et al., 2017); miR-141-3p, que exerce efeito supressor em Keap1 (CHENG et al., 2017); e Jun, um regulador transcricional da proteína AP-1 que está envolvido na ativação da via antioxidante mediada por Nrf2 (NITURE; KHATRI; JAISWAL, 2014; PULLARKAT et al., 2014).

Esses resultados sugerem que a HU pode estar induzindo resposta antioxidante dependente de Nrf2 e ativando p62/SQSTM1, que pode atuar regulando a integridade do complexo Keap1-Nrf2 (COPPLE et al., 2010). Deste modo, sugere-se que a HU ou os produtos eletrofílicos provenientes da sua degradação metabólica estão induzindo resposta antioxidante/eletrófilo via Nrf2, mediada por regulação do p62/SQSTM1. Esses mecanismos estão envolvidos na desintoxicação de eletrófilos endógenos e exógenos que podem reagir com componentes celulares, como o DNA (MALEC et al., 1984; HAYES et al., 2000; NAGAI et al., 2003; NITURE; KHATRI; JAISWAL, 2014; HODGES; MINICH, 2015; FURFARO et al., 2016). Estudos clássicos com HU mostraram que após a sua adminstração oral, esta é absorvida e convertida em radical nitróxido in vivo, e é transportada para o sítio ativo da subunidade M2 da proteína ribonucleotídeo redutase, inativando a enzima e gerando supressão citotóxica, que sugere a indução da resposta antioxidante por essa via (YARBRO, 1992; ZHOU et al., 2001).

No entanto, a coleta desses dados nos permite sugerir que o HU pode atuar como *(i)* eliminador de radicais extra/intracelulares devido a suas propriedades redutoras e ser facilmente absorvido; *(ii)* fonte de NO no microambiente oxidante (na presença de hemina); *(iii)* estimula resposta celular antioxidante por indução eletrofílica, devido à indução do domínio ARE/EpRE mediada por Nrf2 sob regulação

do p62/SQSTM1; *(iv)* induzindo a expressão heterogênea de genes antioxidantes em PBMC e HUVEC; *(v)* não interfere na expressão da *HMOX1*.

7. CONCLUSÕES

Considerando-se a grande contribuição do heme para o estabelecimento e manutenção do estado inflamatório na AF, a análise combinada destes dados sugere que a liberação excessiva de heme a partir de eventos hemolíticos, contribui substancialmente para a gravidade da doença aumentando a auto-oxidação da HbS, o estresse oxidativo e a inflamação crônica, que contribuem para danos teciduais em diferentes órgãos, alteram o metabolismo lipídico, e aumentam o risco de AVC.

Também foram demonstradas novas evidências de que o inflamassoma NLRP3 é uma via inflamatória importante na AF. A administração de HU não alterou a produção da citocina pró-inflamatória IL-1β, embora tenha sido comprovada a diminuição da indução do componente NRLP3 do inflamassoma e LTB_4. Assim, estudos adicionais são necessários para avaliar a associação biológica de moléculas de superficie eritrocitária e produtos de hemólise na ativação de vias inflamatórias. Este conhecimento pode levar ao desenvolvimento de novos alvos para intervenção terapêutica, preferencialmente, DAMPs de hemácias falciformes ou moléculas associadas ao inflamassoma NLRP3.

Poucos estudos têm dado enfoque distinto à ação da HU em mecanismos alternativos que ampliem o campo do conhecimento acerca de seus efeitos sistêmicos nas doenças hemolíticas, especialmente na AF. Neste contexto, os resultados apresentados sugerem que a HU pode contribuir para a redução do estresse oxidativo na AF por apresentar propriedades antioxidantes neutralizando radicais livres; ou induzindo o sistema enzimático de defesa antioxidante pela via Nrf2 regulado pelo p62/SQSTM1. No entanto, faze-se necessário a realização de estudos adicionais voltados para a investigação dos reflexos causados pela ativação do p62/SQSTM1 na AF, embora o seu papel como indutor de resposta antioxidante tenha sido demonstrado em vários estudos.

Em conjunto, esses achados podem auxiliar no desenvolvimento de terapias combinadas visando minimizar os efeitos oxidantes e pró-inflamatórios que contribuem mutuamente para cronicidade inflamatória da AF.

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