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ESTUDO DO PAPEL FUNCIONAL DA CISTEÍNA SINTASE E DA CISTATIONINA β-SINTASE NA RESPOSTA AO ESTRESSE OXIDATIVO E NITROSATIVO EM *Leishmania (Viannia) braziliensis, Trypanosoma rangeli* E *Trypanosoma cruzi*

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Orientador: Prof. Dr. Edmundo Carlos Grisard

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por

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“Hay un momento en que todos los obstáculos se derrumban, todos los conflictos se apartan, y a uno se le ocurren cosas que no había soñado, y entonces no hay en la vida nada mejor que escribir”.

El olor de la guayaba 1982
Gabriel García Márquez

RESUMO

Leishmania (Viannia) braziliensis, *Trypanosoma rangeli* e o *Trypanosoma cruzi* são parasitos hemoflagelados pertencentes à Ordem Kinetoplastida, família Trypanosomatidae, capazes de infectar insetos, animais silvestres e domésticos, assim como o homem. Durante seu ciclo de vida, estes parasitos são expostos a uma grande quantidade de espécies reativas do oxigênio (ERO) e do nitrogênio (ERN), o que promove sua eliminação dentro dos hospedeiros. Por sua vez, os parasitos adotam diferentes mecanismos de defesa para lidar com essas condições de estresse, dentre dos quais, encontram-se um elaborado sistema antioxidante composto de moléculas e enzimas responsáveis pela eliminação das ERO e/ou das ERN. Nesse sentido, em tripanosomátidos a tripanotiona $T[SH]_2$ é a molécula que eficientemente regula a homeostase redox, sendo chave no mecanismo de defesa antioxidante. A síntese da $T[SH]_2$ depende da disponibilidade de seus precursores como a cisteína, um aminoácido sulfurado implicado em diversos processos celulares como a estabilidade, a estrutura e a regulação da atividade catalítica de várias proteínas. Duas vias diferentes para a biossíntese de cisteína têm sido descritas, a via de síntese *de novo*, catalisada principalmente pela Cisteína Sintase (CS), e a via de transulfuração reversa (RTS), catalisada pela Cistationina β-Sintase (CβS). Uma vez que o metabolismo da cisteína é crucial para a biossíntese da tripanotiona, a presente proposta visou determinar a função que as proteinas CS e CβS desempenham na resposta antioxidante destes parasitos. Assim sendo, realizamos a caracterização molecular e bioquímica dos genes da CS e CβS em parasitos sob condições de estresse oxidativo e nitrosativo *in vitro*. Os resultados demonstram claras diferenças na biossíntese de cisteína entre estes parasitos. Enquanto *L. braziliensis* e *T. cruzi* possuem as rotas *de novo* e RTS ativas, o *T. rangeli* possui somente a via RTS funcional. A avaliação da expressão das proteínas CS e CβS nas diferentes formas evolutivas destes parasitos revelou uma expressão estágio-dependente das duas proteínas em *L. braziliensis* e em *T. cruzi*, sendo a CS mais expressa nas formas amastigotas e a CβS nas formas promastigotas e epimastigotas, respectivamente. Por sua vez, em *T. rangeli* a CS não foi detectada e nenhum tipo de modulação foi encontrado para CβS entre as formas epimastigota e tripomastigota. Os ensaios de dose-resposta ao estresse por H_2O_2 e SNAP *in vitro* revelaram diferenças nos perfis de sensibilidade dos parasitos. O *T. rangeli* mostrou-se como o parasito mais sensível ao H_2O_2, porém o mais resistente ao SNAP, enquanto *L.*

braziliensis apresentou a maior resistência ao H_2O_2 e a maior sensibilidade ao SNAP. Para *Leishmania* houve uma associação entre o aumento da expressão e da atividade da CS e da CβS com o aumento da concentração de tióis totais em resposta à indução de estresse oxidativo e nitrosativo *in vitro*. Os resultados das análises funcionais mostraram que o aumento na expressão de CS e de CβS induz resistência dos parasitos mutantes ao estresse oxidativo em relação às cepas selvagens. Neste estudo demonstrou-se que alterações nos níveis de expressão e de atividade das proteínas CS e CβS em *L. braziliensis* e *T. rangeli* estão relacionadas com a sobrevivência destes parasitos sob condições de estresse oxidativo.

Palavras Chave: Biossíntese de cisteína, Cisteína Sintase, Cistationina β-sintase, *L. braziliensis*, Estresse oxidativo e nitrosativo, Defesa antioxidante, Tripanotiona.

ABSTRACT

Leishmania (Viannia) braziliensis, *Trypanosoma rangeli* e o *Trypanosoma cruzi*, are hemoflagellates parasites belonging to the order Kinetoplastida, family Trypanosomatidae, can infect insects, and animals as well as humans. During their complex life cycle, they are exposed to a large amount of reactive oxygen species or reactive nitrogen species (ROS and RNS) generated by the host allowing their elimination. In turn, parasites adopt various defense mechanisms to cope with oxidative or nitrosative stress, they have developed an elaborate antioxidant system composed of molecules and enzymes to scavenge these ROS and RNS. In this sense, trypanothione $T[SH]_2$ is which efficiently regulate the redox homeostasis on these parasites. The synthesis of trypanothione depends on the availability of cysteine, a sulfur-containing amino acid implicated in several processes, including the stability, structure, regulation of catalytic activity of various proteins. Two different routes for cysteine biosynthesis have been described: *de novo* or assimilatory where CS is regulated key enzyme and reverse-transsulfuration (RTS) pathways with a CβS as regulated key enzyme. Because the cysteine metabolism is considered crucial in maintaining the reducing environment for trypanosomatids due to its importance as a precursor for the biosynthesis of trypanothione, this proposal aimed to determine the role of the CS and CβS in the antioxidant response of these parasites by molecular and biochemical characterization of the genes encoding these proteins in the parasite under oxidative and nitrosative stress *in vitro*. Our results clearly demonstrated differences between these parasites in the cysteine biosynthesis. *L. braziliensis* and *T. cruzi* have active the two pathways (*de novo* and RTS), whereas *T. rangeli* only has the RTS pathway. The expression and activities analyses of CS and CβS in the different developmental forms of the parasites revealed that in *L. braziliensis* and *T. cruzi* there are a stage-specific association, where CS is more active and mostly expressed in amastigotes and CβS is associated with promastigotes and epimastigotes forms, respectively. Unlike in *T. rangeli* CS was not detected and no stage-specific association was found for CβS. The dose response assay to H_2O_2 and SNAP shown differences in susceptibility profiles for these parasites. *T. rangeli* was more susceptible to H_2O_2, but more resistant to SNAP, while *L. braziliensis* was more resistant to H_2O_2, but more sensitive to SNAP. Additionally, in *Leishmania* there was a clear association between the increased levels of protein expression and activity of LbrCS and LbrCβS with the

elevated levels of total thiols concentration in response to oxidative and nitrosative stress *in vitro*. In the functional analyses, we found an enhanced ability of parasites overexpressing CS and CβS to resist oxidative stress when compared to the control parasites. In this study we clearly demonstrated that changes in the expression protein and activity levels of CS and CβS may be related to survival to oxidative stress conditions in *L. braziliensis* and *T. rangeli.*

Key words: Cysteine biosynthesis, Cysteine synthase, Cystathionine β synthase, *L. braziliensis*, Oxidative and nitrosative stress, Antioxidant defense, Trypanothione.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Analise de variância
ATP	Adenosina trifosfato
As^V	Arsênico pentavalente
BSA	Albumina sérica bovina
CI_{50}	Concentração que inibe 50% dos parasitos
CL	*Cutaneous Leishmaniasis*
DNA	Ácido desoxirribonucléico
gDNA	Ácido desoxirribonucleico genômico
DP	Desvio Padrão
D-PBS	*Dulbecco´s buffered saline solution*
DTT	*Dithiothreitol*
EDTA	Ácido etilenodiaminotetracético
ELISA	*Enzyme-Linked Immunosorbent Assay*
EP	Erro padrão
ERO	Espécies Reativas de Oxigênio
ERN	Espécies Reativas de Nitrogênio
FBS	*Fetal Bovine Serum*
G418	*Geneticin*
GSH	Glutationa reduzida
IMAC	*Affinity chromatography*
IPTG	*Isopropyl β-D-thiogalactopyranoside*
iNOS	Óxido Nítrico-Sintase 2 induzível
MCL	*Mucocutaneous Leishmaniasis*
OD	*Optical density*
LB	*Luria– Bertani*
Lb	*Leishmania braziliensis*
LbrCS	Gene da Cisteína Sintase de *Leishmania braziliensis*
LbrCβS	Gene da Cistationina β-Sintase de *L. braziliensis*
LC	Leishmanioses Cutânea
LIT	*Liver Infusion Tryptose médium*

LMC	Leishmanioses Mucocutânea
LTA	Leishmaniose Tegumentar Americana
LV	Leishmanioses Visceral
MΦ	Macrófagos
MAPKs	Proteínas quinases ativadas por mitógenos
MCL	*Mucocutaneous Leishmaniasis*
mRNA	Ácido ribonucléico mensageiro
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NMRI	Cepa de camundongo da *Naval Medical Research Institute*
PBS	Tampão Salino Fosfato
PCR	Reação em Cadeia da Polimerase
PI3K	fosfatidilinositol 3-quinase
PKC	Proteína quinase C
PLP	*Pyridoxal phosphate*
PMA	Forbol-12-miristato-13-acetato
ROS	*Reactive Oxygen Species*
RNS	*Reactive Nitrogen Species*
RNAi	Ácido ribonucléico de interferência
RPMI	Meio de cultura *Roswell Park Memorial Institute* 1650
RTS	Transulfuração reversa
SAG	*Sodium Antimony Gluconate*
SBF	Soro bovino fetal
Sb	Antimônio
Sb^{III}	Antimonial trivalente
Sb^{V}	Antimonial pentavalente
SD	*Standard Deviation*
SDS-PAGE	*Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis*
SEM	*Standard Error of the Mean*
siRNA	*Short interfering RNA* ou *small interfering RNA*
SNAP	*S-nitroso-N-acetylpenicillamine*

THP-1	Linhagem de células humanas de leucemia monocítica aguda
TryR	Tripanotiona redutase
$T[SH]_2$	Tripanotiona
UV	Ultravioleta
VL	*Visceral Leishmaniasis*

LISTA DE SÍMBOLOS

CO_2	Dióxido de Carbono
°C	Grau Celsius
g	Gravidade
HO•	Hidroxila
H_2O_2	Peróxido de hidrogênio
H_2S	Sulfeto de hidrogênio
IU	*International units*
kDa	KiloDalton
mg	Miligrama
mL	Mililitro
NaCl	*Sodium chloride*
NaH_2PO_4	*Monosodium phosphate*
nmol	Nano moles
•NO	Oxido Nítrico
O_2	Oxigênio molecular
$O_2•^-$	Ânion superóxido
OCl^-	Ânion Hipoclorito
$ONOO^-$	Ânion peroxinitrito
pH	Potencial hidrogeniônico
RO•	Alcoxilo
RO_2•	Peroxila
UI	Unidade internacional
Na_2S	Sulfeto de sodio
nM	Nanomolar
nm	Nanômetro
µg	Micrograma
µL	Microlitro
µM	Micromolar
µmol	Micro moles
v/v	*Contration volume/volume*
WT	*Wild Type*
w/v	*Mass concentation mass/volume*

SUMÁRIO

1. INTRODUÇÃO

1.1. Os tripanosomatideos

A família Trypanosomatidae da ordem Kinetoplastida é composta por protozoários flagelados, capazes de infectar uma ampla gama de hospedeiros mamíferos (incluindo o homem), hospedeiros invertebrados e até plantas (VICKERMAN, 1994). Esta família está dividida em nove gêneros, incluindo parasitos monoxênicos (*Crithidia*, *Blastocrithidia*, *Herpetomonas*, *Wallaceina* e *Leptomonas*) e heteroxênicos (*Trypanosoma*, *Leishmania*, *Endotrypanum* e *Phytomonas*) (LOPES et al., 2010). Dentre estes, destacam-se por serem infectivos para seres humanos várias espécies do gênero *Leishmania* spp. (Subgêneros *Leishmania* e *Viannia*) e do gênero *Trypanosoma* (*Trypanosoma brucei*, *Trypanosoma cruzi* e *Trypanosoma rangeli*), das quais, somente o *T. rangeli* é considerado como não patogênico (GENOIS et al., 2014).

1.2. O gênero *Leishmania* spp. e as Leishmanioses

Leishmania spp. são parasitos intracelulares obrigatórios de grande importância médica, podendo determinar um amplo espectro de manifestações clínicas que dependem tanto da espécie do parasito quanto da resposta imune do hospedeiro. As principais formas clínicas observadas em seres humanos são a Leishmaniose Cutânea (LC), a Leishmaniose Muco-Cutânea (LMC) e a Leishmaniose Visceral (LV) (Tabela 1) (HERWALDT, 1999; KAYE e SCOTT, 2011; ANTINORI, SCHIFANELLA e CORBELLINO, 2012).

As leishmanioses são endêmicas em 98 países distribuídos nos cinco continentes. Cerca de 1,3 milhões de novos casos ocorrem anualmente, dos quais 300.000 são LV (90% das quais ocorrem em Bangladesh, Brasil, Etiópia, Índia, Nepal, Sudão do Sul e Sudão) e 1.000.000 são LC (que ocorrem principalmente no Afeganistão, Argélia, Brasil, Colômbia, República Islâmica do Irã, Paquistão, Peru, Arábia Saudita, República Árabe da Síria e Tunísia) ou LMC (que ocorrem principalmente no Brasil, Peru e do Estado Plurinacional da Bolívia). Do total de novos casos anuais, estima-se que apenas cerca de 600.000 sejam notificados (ALVAR et al., 2012; WHO, 2014b).

Nas Américas, 12 países já reportaram casos autóctones de LV, sendo que 96,5% (3.118) dos casos estão concentrados no Brasil. Historicamente a LV foi considerada no Brasil uma doença zoonótica rural, entretanto, ela tem emergido com proporções epidêmicas em áreas urbanas e periurbanas do país, devido principalmente à migração em

larga escala de pessoas de áreas rurais para as periferias das grandes cidades, gerando condições sociais precárias, além do transporte, presença e/ou acúmulo de cães domésticos como reservatórios e a adaptabilidade do vetor *Lutzomya longipalpis* à estas condições urbanas (HARHAY et al., 2011; ALVAR et al., 2012; WHO, 2014b).

Tabela 1: Principais espécies do gênero *Leishmania* que infectam seres humanos.

Espécies	Manifestações clínicas
Subgênero *Leishmania* (Velho mundo)	
L. donovani e L. infantum	Leishmaniose Visceral
L. major, L. tropica e L. aethiopica	Leishmaniose Cutânea
L. aethiopica	Leishmaniose Cutânea-Difusa
Subgênero *Leishmania* (Novo mundo)	
L. infantum	Leishmaniose Visceral
L. infantum, L. mexicana, L. pifanoi e L. amazonensis	Leishmaniose Cutânea
L. mexicana e L. amazonensis	Leishmaniose Cutânea-Difusa
Subgênero Viannia (Novo mundo)	
L. braziliensis, L. guyanensis, L. panamensis e L. peruviana	Leishmaniose Cutânea
L. braziliensis, L. panamensis	Leishmaniose Mucocutânea

O Brasil figura dentre os sete países responsáveis por cerca de 90% do total mundial dos casos de Leishmaniose cutânea (LC) em todo o mundo e dentre o total de casos registrados nas Américas para o ano 2012, 45% (24.000) foram registrados no Brasil (WHO, 2014b). Na década de 1980, apenas dezenove estados do Brasil haviam relatado casos de LTA autóctone. Já em 2003, todos os vinte e sete estados brasileiros apresentaram transmissão autóctone da LC, demonstrando a evidente e contínua expansão geográfica da doença em todo o país. No estado de Santa Catarina, a tendência de espansão não é diferente do resto do país, sendo que de 2001 até 2004, os casos foram dispersos geograficamente e na maior parte importados de outros estados. No entanto, de 2005 até 2009 houve um aumento drástico no número de casos autóctones em diferentes regiões do estado, notadamente na região nordeste, onde também foi registrado o aumento do número de casos importados (MARLOW et al., 2013).

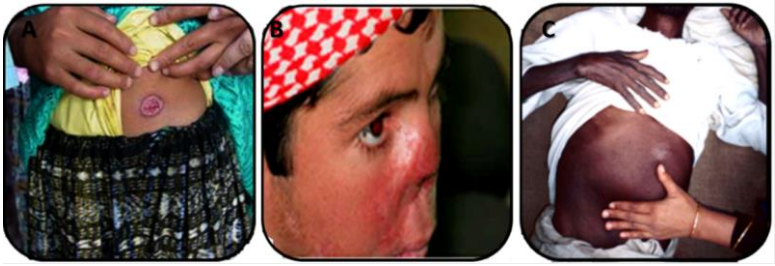
A Leishmaniose Cutânea (LC) é a forma dermotrópica mais frequente e menos grave da doença, possuindo uma variedade de agentes etiológicos, tais como *L. tropica*, *L. major* e *L. aethiopica* no Velho Mundo e *L. mexicana*, *L. amazonensis*, *L. guyanensis*, *L. panamensis* e *L. braziliensis* no novo mundo. Apresentando caracteristicamente lesões ulcerativas ou nodulares, únicas ou múltiplas,

as quais iniciam como uma pápula no local da picada do flebotomíneo, podendo evoluir para uma lesão crostosa e ulcerada (Figura 1A). Entretanto, este tipo de lesão pode evoluir para uma cura espontânea ou, em alguns casos, podem progredir para um envolvimento do tecido cutâneo-mucoso como o observado em infecções determinadas por *L. panamensis* ou *L. braziliensis* (REITHINGER et al., 2007; AMEEN, 2010).

Na Leishmaniose Mucocutânea (LMC), também chamada de espúndia, ocorre a destruição progressiva de mucosas (nariz, faringe, boca e laringe) com comprometimento desfigurante da cartilagem (Figura 1B). As lesões iniciam usualmente no septo nasal e se difundem para o palato, podendo envolver outros tecidos como lábios, faringe e traquéia. Caso o tratamento não seja ministrado em tempo, a doença pode acarretar dificuldades respiratórias e desnutrição e, eventualmente de forma mais rara, evolução para óbito em decorrência da obstrução das vias respiratórias ou por pneumonia (GOTO e LAULETTA LINDOSO, 2012; McGWIRE e SATOSKAR, 2014).

Dentre as doenças tropicais, a Leishmaniose visceral (LV) ocupa o quarto lugar em morbidade e o segundo lugar em mortalidade, determinando cerca de 20.000 a 40.000 mortes anuais. De forma global, os sintomas incluem hepatoesplenomegalia, febre alta, pancitopenia e hipergamaglobulinemia, sendo que o quadro grave da doença é quase sempre fatal se não for tratada, usualmente associado a co-infecções, sangramento massivo ou anemia severa (Figura 1C) (CHAPPUIS et al., 2007; WHO, 2014b).

Figura 1. Manifestações clínicas da Leishmaniose. A= Leishmaniose cutânea, B= Leishmaniose Mucocutânea e C = Leishmaniose Visceral.



Fonte: (CDC, 2014; WHO, 2014b)

1.3. O tratamento quimioterápico das Leishmanioses

O antimônio pentavalente (Sb^V) na forma de antimoniato de meglumina (Glucantime®, Sanofi Aventis Farmacêutica Ltda) e o estibogluconato de sódio (Pentostam®, GlaxoSmithKline, UK) tem constituído a base do tratamento das Leishmanioses durante mais de 60 anos, ainda que sua estrutura molecular e suas formas de metabolização e de ação não tenham sido completamete elucidadas. Alguns estudos sugerem que o antimonio pentavalente atua como um pró-fármaco, que é reduzido a sua forma mais ativa e tóxica, o antimonio trivalente (Sb^{III}), para que se obtenha uma atividade leishmanicida (FRÉZARD et al., 2001; FRÉZARD et al., 2013)

Ainda que os antimoniais sejam os medicamentos de primeira linha no tratamento das Leishmanioses, estes fármacos apresentam várias limitações. Dentre estas, destacam-se o tempo prolongado e os graves efeitos secundários que usualmente dificultam ou mesmo impedem o tratamento em função da não adesão ou do abandono dos pacientes, especialmente os residentes em áreas rurais. Adicionalmente, a resistência do parasito ao fármaco também se constitue em um problema no tratamento desta doença (CROFT e YARDLEY, 2002; MURRAY et al., 2005; FRÉZARD, DEMICHELI e RIBEIRO, 2009; FRÉZARD et al., 2013).

Além dos antimoniais, outros fármacos como a anfotericina B, a pentamidina, a paromomicina e a miltefosina têm sido empregados no tratamento das Leishmanioses. Em conjunto, estes fármacos são considerados como os quimioterápicos de segunda linha, sendo particularmente úteis em casos de falha terapêutica dos antimoniais ou em pacientes que apresentam hipersensibilidade ao antimônio (CROFT e OLLIARO, 2011).

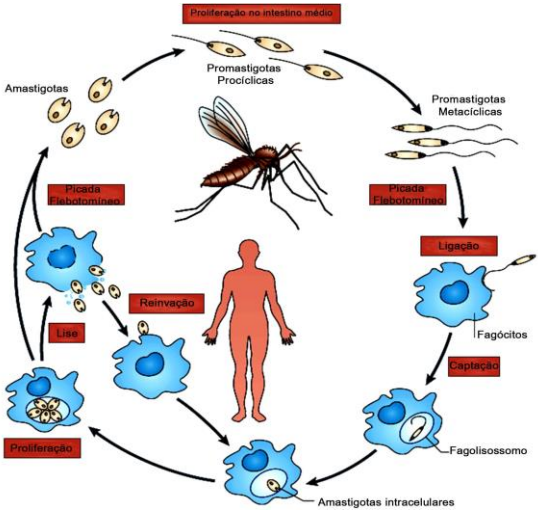
Ainda que o arsenal terapêutico para o tratamento das Leishmanioses seja reduzido e de baixa eficácia, são poucas as pesquisas visando o desenvolvimento de novos agentes terapêuticos, o que mantém as Leishmanioses dentre as 17 doenças tropicais negligenciadas (WHO, 2014b).

1.4. O ciclo biológico de *Leishmania* spp.

O ciclo de vida de *Leishmania* spp. no vetor inicia quando o inseto ingiere as formas amastigotas intracelulares durante o repasto sanguíneo. Uma vez no trato digestivo do vetor, as formas amastigotas diferenciam-se em formas promastigotas procíclicas, as quais após da multiplicação por fissão binaria, diferenciam-se em promastigotas

metacíclicas não replicativas, que se localizam próximas a válvula estomodeal (uma invaginação do intestino anterior no intestino médio), para a transmissão. Durante o repasto sanguíneo, o flebotomíneo regurgita promastigotas metacíclicas, as quais são rapidamente fagocitadas por células dos sistema fagocítico mononuclear, notadamente macrófagos. Depois de se estabelecer no interior da célula hospederia, as promastigotas metacíclicas transformam-se em amastigotas aflageladas, formas replicativas que se dividem no interior celular, produzindo dano mecânico na célula infectada (KAYE e SCOTT, 2011; McCONVILLE e NADERER, 2011). A ruptura da célula permite a reinfecção de novas células fagóciticas locais. O ciclo de transmissão se completa quando os fagócitos infectados são captados por outros flebotomíneos durante um novo respato sanguíneo, onde as amastigotas são convertidas em promastigotas no intestino médio dos flebotomíneos (SACKS e KAMHAWI, 2001; MURRAY et al., 2005) (Figura 2). A transmissão de *Leishmania* spp. é antroponótica (humano-vetor-humano) no subcontinente indiano e Ásia, enquanto que na África, Europa e as Américas, é zoonótica (reservatório-vetor-humano), onde roedores, edentados e especialmente cães tem um importante papel como reservatórios (REITHINGER et al., 2007; POSTIGO, 2010; KUMAR e ENGWERDA, 2014).

Figura 2. O ciclo de vida da *Leishmania* spp.



Adaptado de (KAYE e SCOTT, 2011).

1.5. O gênero Trypanosoma

O gênero *Trypanosoma* inclui três principais espécies transmitidas por triatomíneos capazes de infectar seres humanos, o *T. brucei*, o *T. cruzi* e o *T. rangeli*. O *T. rangeli* bem como o *T. cruzi*, ocorrem em simpatria nas Américas, onde compartilham reservatórios e vetores (D'ALESSANDRO e MANDEL, 1969; CUBA CUBA, 1998; D' ALESSANDRO e SARAVIA, 1999; GUHL e VALLEJO, 2003). Ainda que compartilhem uma extensa área geográfica, reservatórios e vetores, seus comportamentos biológicos são surpreendentemente distintos (VICKERMAN, 1985).

De acordo com o desenvolvimento nos vetores e com as vias de transmissão das formas infectantes aos hospedeiros, as espécies de *Trypanosoma* foram divididas em duas seções principais: Stercoraria e Salivaria (HOARE, 1972). A seção Stercoraria compreende espécies que se desenvolvem exclusivamente no tubo digestivo do invertebrado e sua via de transmissão é contaminativa (ou posterior), ou seja, as formas infectantes do parasito são eliminadas através das fezes do vetor e podem penetrar os hospedeiros mamíferos via soluções de continuidade como orifícios da picada ou mucosas. A seção Salivaria compreende os tripanosomas que no inseto vetor se desenvolvem no trato digestivo e completam seu desenvolvimento nas glândulas salivares, sendo sua via de transmissão inoculativa (ou anterior) pela picada do vetor (HOARE, 1972; De SANTA-IZABEL, VERMELHO e BRANQUINHA, 2004).

1.6. O *Trypanosoma rangeli*

O *T. rangeli* é a segunda espécie de tripanosomatídeo que infecta mamíferos, incluindo seres humanos, na América Latina. Originalmente descrito por Enrique Tejera em 1920 na Venezuela, o qual observou formas flageladas do parasito no conteúdo intestinal de *Rhodnius prolixus* naturalmente infectado (D'ALESSANDRO e MANDEL, 1969). A posição taxonômica de *T. rangeli* tem sido objeto de muita controvérsia ao longo do tempo. Embora seja atualmente classificado dentro do subgênero *Herpetosoma* (Seção Stercoraria), está mais do que registrado na literatura que a via de transmissão primária deste parasito é a inoculativa (anterior), sendo realizada pela picada de triatomineos infectados (CUBA CUBA, 1998; GUHL e VALLEJO, 2003)

Desde os registros originais, o *T. rangeli* tem sido relatado predominantemente na Guatemala, Panamá, Colômbia e Venezuela, e em menor medida, em El Salvador, Costa Rica, Peru, Brasil, Guiana, Paraguai, Chile e Uruguai (CUBA CUBA, 1998). Esta ampla

distribuição geográfica é em grande parte sobreposta com a do *T. cruzi*, agente etiológico da doença de Chagas, permitindo a ocorrência de infecções mistas, tanto nos insetos vetores como nos hospedeiros mamíferos (Figura 3) (GRISARD et al., 1999; GUHL e VALLEJO, 2003).

Diferentemente dos outros tripanosomas heteroxênicos, o *T. rangeli* apresenta efeitos patogênicos variados às espécies vetoras, podendo ocasionar a morte devido a uma excessiva proliferação na hemolinfa, destruição dos hemócitos e dificuldade de alimentação pela infecção das glândulas salivares, determinando processos anormais de ecdise como malformações, atraso ou interrupção do desenvolvimento das ninfas (AÑEZ e EAST, 1984; GRISARD et al., 1999; VALLEJO, GUHL e SCHAUB, 2009). De forma distinta ao que ocorre no vetor, o *T. rangeli* é inofensivo para o hospedeiro mamífero, sendo considerado como não patogênico. Entretanto, a infecção por este parasito induz no mamifero uma resposta humoral com níveis elevados de anticorpos que apresentam uma reatividade cruzada com antígenos de *T. cruzi*, o que pode interferir com o diagnóstico sorológico específico da doença de Chagas, especialmente em sua fase crônica (AFCHAIN et al., 1979; SALDAÑA e SOUSA, 1996; GRISARD et al., 1999).

Figura 3. Mapa das Américas Central e do Sul, mostrando a sobreposição geográfica da distribuição da doença de Chagas humana (sombreado) e os registros de ocorrência comprovada do *Trypanosoma rangeli* em humanos, triatomíneos ou animais silvestres (•).

Fonte: (GRISARD e STEINDEL, 2004).

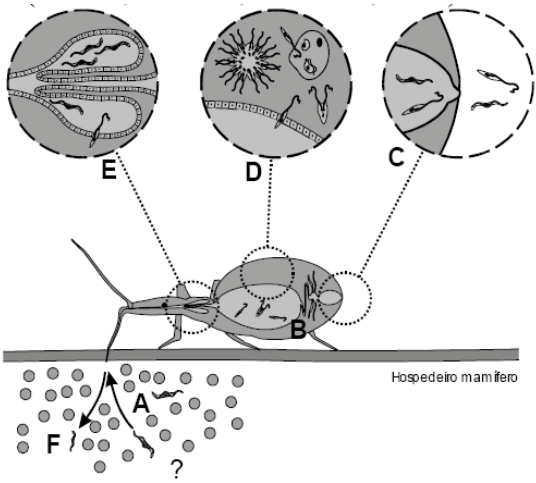
1.7. O ciclo biológico do *Trypanosoma rangeli*

Durante seu ciclo biológico o *T. rangeli* assume distintas formas evolutivas, sendo epimastigotas e tripomastigotas metacíclicos no inseto vetor e tripomastigotas sanguíneos no hospedeiro mamífero (CUBA CUBA, 1998). Os hemípteros hematófagos da subfamília Triatominae, especialmente os do gênero *Rhodnius*, representam seus principais vetores em condições naturais ou experimentais (GUHL e VALLEJO, 2003). Destacando-se nestes as espécies: *R. prolixus*, *R. brethesi*, *R. colombiensis*, *R. dalessandroi*, *R. domesticus*, *R. ecuadoriensis*, *R. nasutus*, *R. neglectus*, *R. neivai*, *R. pallescens*, *R. pictipes* e *R. robustus* (GUHL e VALLEJO, 2003; VALLEJO, GUHL e SCHAUB, 2009).

O ciclo de vida de *T. rangeli* nos triatomíneos inicia quando os insetos ingerem formas tripomastigotas sanguíneas ao realizarem o repasto sanguíneo em hospedeiros mamíferos infectados (A). Os parasitos atingem o intestino médio (B) do inseto vetor e se diferenciam em formas epimastigotas (que podem ser curtas ou longas), capazes de multiplicar-se, de se aderir ao epitélio do intestino médio, e são capazes de atravessá-lo alcançando a hemocele (C). Uma vez na hemolinfa, os parasitos multiplicam-se livremente (como formas longas) ou também podem ser observados dentro de hemócitos (D) onde a replicação não é comprovada. Após 10 a 15 dias, migram para as glândulas salivares (E) onde aderem, penetram e se diferenciam em tripomastigotas metacíclicos (F), as formas infectivas que são inoculadas nos hospedeiros mamíferos durante o próximo repasto sanguíneo (Figura 4) (D' ALESSANDRO e SARAVIA, 1992; AZAMBUJA, RATCLIFFE e GARCIA, 2005).

Cabe salientar que foi descrita uma forte relação evolutiva entre as espécies vetoras e as cepas do parasito que ocorrem em uma determinada região geográfica (VALLEJO, GUHL e SCHAUB, 2009). Desta forma, não é uma regra geral para as cepas de *T. rangeli* a evasão do trato digestivo, a proliferação na hemolinfa e a invasão das glândulas salivares de triatomíneos com consequente transmissão pela picada, sendo estas características dependentes da interação da cepa com a espécie vetora (GARCIA et al., 2012).

Figura 4: Representação esquemática do ciclo do *Trypanosoma rangeli* no hospedeiro invertebrado.



Adaptado de (GRISARD e STEINDEL, 2004).

Ao contrário de seu ciclo no hospedeiro invertebrado, o curso da infecção pelo *T. rangeli* em seu hospedeiro vertebrado é pouco conhecida, havendo muita controvérsia sobre as capacidades de replicação extracelular ou invasão das células do hospedeiro pelas formas tripomastigotas sanguíneas; bem como quanto à presença de formas amastigotas e sua multiplicação intracelular.

Relatos na literatura sobre a multiplicação do *T. rangeli* em seus hospedeiros mamíferos são escassos e inconclusivos. Isso se deve em função da utilização de cepas não caracterizadas ou a estudos realizados por grupos que igualmente manipulam o *T. cruzi*, podendo os resultados se referir a infecções cruzadas com o *T. cruzi* (GRISARD et al., 1999). Dentre estes estudos, tem sido descritas formas tripomastigotas sanguíneas em aparente divisão no sangue circulante de seres humanos e roedores e de formas intracelulares denominadas de “amastigotas-like”, no coração, fígado e baço de camundongos lactantes da cepa NMRI (URDANETA-MORALES e TEJERO, 1985; D' ALESSANDRO e SARAVIA, 1999).

1.8. O *Trypanosoma cruzi* e as manifestaçoes clínicas da doença de Chagas

Agente etiológico da doença de Chagas ou tripanosomíase americana, o *T. cruzi* foi descrito em 1909 pelo médico brasileiro Carlos Chagas, mediante observação de parasitos flagelados no sangue periférico de uma menina de dois anos (De LANA et al., 1996; COURA, 2013). Segundo a Organização Mundial da Saúde, a doença é um importante problema de saúde pública na América Latina, afetando atualmente cerca de 8 milhões de pessoas em 21 países em uma área que vai desde o sul da Argentina ao sul dos Estados Unidos (EUA) (WHO, 2014a). No entanto, a crescente emigração de pessoas oriundas de países endêmicos para a doença de Chagas para países não endêmicos da América do Norte, principalmente EUA e Canadá, da Europa, da Ásia e da Australia, fez com que a mesma ganhasse alcance global. Somente nos EUA, estima-se que vivem mais de 300.000 indivíduos infectados pelo *T. cruzi*, na sua maioria imigrantes oriundos do México e de países da América Central (BERN e MONTGOMERY, 2009; BERN et al., 2011).

A doença de Chagas é caracterizada por uma fase aguda e uma fase crônica. Durante o fase aguda da infecção, a maioria dos pacientes apresenta sintomas leves e auto-limitados como os sinais de porta de entrada (chagomas de inoculação), o sinal de Romaña (edema bipalpebral unilateral), febre, cansaço, hipertrofia de fígado, baço e/ou linfonodos. Esta fase, em geral, dura de 30 a 60 dias, apresentando parasitismo sanguíneo e tecidual detectável, o qual torna-se subpatente com a progressão da infecção e com a resposta imune do hospedeiro (RASSI e MARIN-NETO, 2010; BERN, 2011; PEREZ, LYMBERY e THOMPSON, 2014).

Na fase crônica cerca de 70% dos indivíduos soropositivos são assintomáticos (forma indeterminada), enquanto os demais evoluem para formas sintomáticas da doença com comprometimento cardíaco (forma cardíaca), digestivo (forma digestiva) ou misto. Esta fase caracteriza-se por parasitemia escassa a indetectável e um parasitismo tissular da musculatura esquelética, lisa e/ou cardíaca (RASSI e MARIN-NETO, 2010). A cardiomiopatia chagásica é a manifestação clínica mais grave e frequente da fase crônica, apresentando elevada morbidade e mortalidade e sendo considerada a principal causa de miocardite infecciosa em todo o mundo (PRATA, 2001; MONCAYO, 2003).

1.9. O tratamento quimioterápico da Doença de Chagas

Os medicamentos atualmente disponíveis para o tratamento da infecção pelo *T. cruzi* foram introduzidos para uso humano nas décadas de 1960 (Nifurtimox®, Bayer) e 1970 (Benzonidazol®, Roche). Ensaios clínicos relatam que o tratamento simultâneo ou único com estes compostos pode levar à cura até cerca de 80% dos pacientes na fase aguda (BAHIA-OLIVEIRA et al., 2000). Entretanto, na fase crônica da doença a taxa de cura é de apenas 5-20% (CANCADO, 2002). Embora o nifurtimox e o benzonidazol sejam tripanossomicidas que atuam em todas as formas do ciclo vital do parasito (RODRIQUES COURA e De CASTRO, 2002), os mesmos possuem elevada toxicidade e determinam efeitos sistêmicos adversos nos pacientes como anorexia, náuseas, vômitos, dor de cabeça, dermatites, depressão do sistema nervoso central ou sintomas neurológicos, convulsões, vertigem, parestesias, polineuropatias periféricas. Além da baixa eficácia e dos efeitos colaterais, o tratamento prolongado (em média 60 dias) dificulta ainda mais a aderência ao tratamento, diminuindo a eficácia dos fármacos e favorecendo o surgimento de cepas resistentes à quimioterapia (MAYA et al., 2007). Atualmente o medicamento utilizado para o tratamento da doença de Chagas no Brasil é o benzonidazol devido a melhor tolerância por parte dos pacientes, pois é rapidamente absorvido e metabolizado alcançando boas taxa de cura nos casos agudos (RAJÃO et al., 2014).

1.10. A transmissão e o ciclo biológico do *Trypanosoma cruzi*

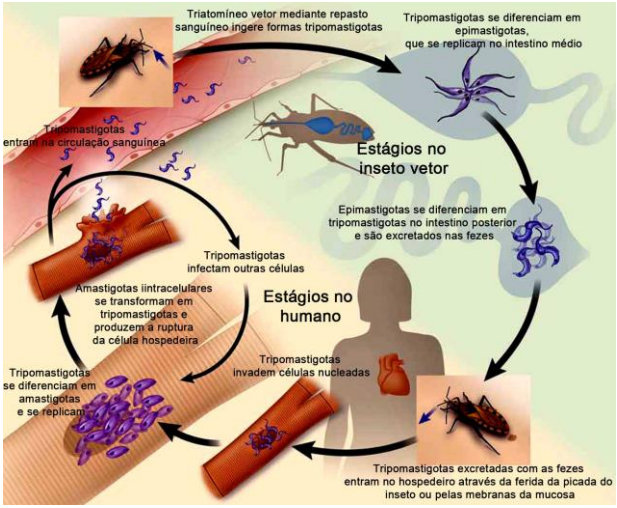
O *T. cruzi* é transmitido aos seres humanos e outros mamíferos (domésticos e selvagens) por insetos pertencente à família Reduviidae, subfamília Triatominae. Apesar de terem sido identificadas mais de 130 espécies de triatomíneos, somente algumas são vetores competentes para o *T. cruzi*, destacando-se o *Triatoma infestans*, o *Rhodnius prolixus* e o *Triatoma dimidiata* como as três espécies mais importantes na transmissão deste parasito para o homem (MARTÍNEZ et al., 2006; SCHOFIELD e GALVÃO, 2009). A Doença de Chagas também pode ser transmitida ao homem por mecanismos não vetoriais, sendo relatados a transfusão de sangue, a transmissão congênita, a ingestão de alimentos contaminados com o parasito, os transplante de órgãos, os acidentes de laboratório e, em menor porcentagem por transmissão sexual (YOSHIDA, 2009; RASSI e MARIN-NETO, 2010).

Durante o seu ciclo biológico *T. cruzi* possui formas distintas em seus hospedeiros, as formas tripomastigota sanguínea e amastigota intracelular, observadas no hospedeiro mamífero, e as formas

epimastigota e tripomastigota metacíclica, observadas no inseto vetor. O ciclo biológico de *T. cruzi* no hospedeiro mamífero inicia quando o vetor infectado, após o repasto sanguíneo, defeca sobre o hospedeiro que, ao coçar o local, permite o contato dos tripomastigotas metacíclicos presentes nas fezes do triatomíneo com as mucosas ou soluções de continuidade. Uma vez na corrente sanguínea, o *T. cruzi* pode infectar uma variedade de células do hospedeiro que, irão formar um fagossoma denominado de vacúolo parasitóforo, do qual o parasito possui a habilidade de evadir-se, passando a multiplicar-se livremente no citoplasma celular sob a forma amastigota. Após vários ciclos de divisão binária, as formas amastigotas diferenciam-se novamente para formas tripomastigotas e, com o rompimento da célula hospedeira, são liberadas no meio extracelular e podem infectar novas células como, por exemplo miocardiócitos, células musculares lisas e os astrócitos (De SOUZA, De CARVALHO e BARRIAS, 2010).

As formas tripomastigotas sanguíneas também podem ser ingeridas pelo vetor durante repasto sanguíneo, o que determina a infecção do inseto. Nestes os parasitos passam pelo estômago e, no intestino médio do inseto, se diferenciam para formas epimastigotas que se, multiplicam por divisões binárias sucessivas. Ao atingirem o intestino posterior dos triatomíneos, as alterações bioquímicas e fisiológicas do meio induzem as formas epimastigotas a diferenciarem em formas tripomastigotas metacíclicas (metaciclogênese), os quais são infectivas para os hospedeiros mamíferos, sendo liberadas junto das fezes do vetor durante um novo repasto sanguíneo (PIACENZA et al., 2008; De SOUZA, De CARVALHO e BARRIAS, 2010; RASSI e MARIN-NETO, 2010).

Figura 5. Ciclo de biológico de *Trypanosoma cruzi*.



Adaptado de (BERN, 2011).

1.11. O estresse oxidativo e nitrosativo em tripanosomatideos

As espécies reativas de oxigênio (ERO) e as espécies reativas de nitrogênio (ERN) são termos que descrevem, de forma global, radicais livres derivados de oxigênio e nitrogênio tais como o ânion superóxido (O_2•$^-$), a hidroxila (HO•), a peroxila (RO_2•), o alcoxilo (RO•), óxido nítrico (•NO) e espécies não radicalares tais como peróxido de hidrogênio (H_2O_2) e o ânion peroxinitrito ($ONOO^-$) (DRÖGE, 2002). Estas espécies são a consequência inevitável dos processos metabólicos normais em todos os organismos aeróbicos, onde a mitocôndria é uma importante fonte intracelular de ERO pois, do total de O_2 mitocondrial consumido, 1 a 2% é desviado para a formação destas, principalmente ao nível dos complexos I e III da cadeia respiratória. Outras organelas celulares como o retículo endoplasmático, os peroxissomos e o citoplasma também tem sido associadas com a produção de ERO e ERN (CIRCU e AW, 2010). Quando os níveis das ERO e ERN excede a capacidade de neutralização dos sistemas antioxidantes, o desequilíbrio gerado é chamado de estresse oxidativo ou nitrosativo, respectivamente, e incorrendo em graves danos a distintos componentes celulares tais como DNA, proteínas, lipídeos e açúcares (SIES, 2014).

Considerando que os protozoários parasitos são células eucarióticas aeróbicas, seu metabolismo também utiliza oxigênio molecular como o

aceptor final de elétrons em reações que conduzem à geração de energia na mitocôndria. Assim como em mamíferos, a geração de ERO e ERN, como subprodutos do metabolismo oxidativo na mitocôndria também tem sido documentada nos tripanosomatídeos (TURRENS, 2004). De fato, apesar das diferenças em relação a outros eucariotas, o metabolismo de todos estes parasitos (exceto nas formas sanguíneas de *T. brucei*) envolve a transferência de equivalentes redutores ao longo da cadeia respiratória, o que permite a redução de oxigênio molecular monovalente ao ânion superóxido ($O2•^-$), sendo o primeiro radical formado e o precursor do peróxido de hidrogênio (H_2O_2), além de outras espécies reativas de oxigênio (TOMÁS e CASTRO, 2013).

Apesar da diversidade biológica dos tripanosomatídeos, as diferentes espécies necessitam lidar com problemas biológicos relacionados aos ciclos de vida heteroxênicos e complexos. Além de necessitar controlar os níveis de ERO e ERN gerados a partir das fontes intrínsecas ou de forma fisiológica, os parasitos têm de lidar com o estresse oxidativo e nitrosativo imposto pela resposta imune tanto do hospedeiro mamífero quanto do inseto vetor (TURRENS, 2004; JIMENEZ, 2014). Patógenos intracelulares como *Leishmania* spp. e *T. cruzi* são confrontados com uma fonte adicional de estresse ao invadir os macrófagos, onde a explosão oxidativa e nitrosativa são parte da resposta imune inata direcionada visando a eliminação dos parasitos. Após do reconhecimento dos patógenos, o macrófago é ativado e pode fagocitar e destruir o parasito usando vários processos celulares que, incluem a produção de enzimas fagolisossomais (proteases, nucleases, fosfatases, esterases e lipases), a ativação da NADPH-oxidase associada a membrana e a indução de óxido nítrico-sintase 2 (iNOS). A explosão oxidativa provocada pela redução de oxigênio univalente catalisada pela NADPH-oxidase ocorre imediatamente após sua ativação, originando altas taxas de $O_2•^-$ durante pelo menos 90 min. A produção de radicais $O_2•^-$, conduz à formação espontânea ou enzimática de peróxido de hidrogênio (H_2O_2) no vácuolo fagocítico, assim como os radicais hidroxila (HO•), hipoclorito (OCl^-) e peroxynitrito ($ONOO^-$). O aumento dos níveis de •NO e outras ERN em macrófagos ativados são o resultado da ativação das enzimas iNOS, onde a L-Arginina atua como precursor do •NO (PELUFFO et al., 2004; NADERER e McCONVILLE, 2008; ALVAREZ et al., 2011; JIMENEZ, 2014).

Os triatomíneos possuem um sistema de imunidade inata semelhante aos mamíferos, envolvendo cascatas de ativação análogas onde as ERO e ERN são importantes na resposta frente à infecção pelos tripanosomatídeos. Em *Rhodnius prolixus* os níveis de expressão da

óxido nítrico sintase (iNOS) são modulados positivamente frente a um desafio com *T. rangeli* ou *T. cruzi*, incorrendo em uma maior atividade de •NO na parede do intestino durante a resposta à infecção por estes parasitos, sendo particularmente mais altas na infecção pelo *T. cruzi*. Esta atividade pode, segundo alguns autores, explicar por que este parasita não consegue atravessar a parede do intestino médio e atingir a hemocele como perfaz o *T. rangeli*. Além disso, níveis de (O_2•$^-$) foram significantemente incrementados durante as 12 - 24 h após da infecção com epimastigotas de *T. rangeli* na hemolinfa de *R. prolixus* indicando o alto estresse oxidativo a que estes parasitos estão expostos (WHITTEN et al., 2001; WHITTEN et al., 2007; GARCIA et al., 2009; GARCIA et al., 2012). Embora, as interações moleculares entre os parasitos do gênero *Leishmania* e os flebotomíneos sejam pouco compreendidas, os poucos estúdios indicam que ERO e ERN podem ser importantes para a defesa do flebotomíneo contra os parasitos. Estudos recentes baseados em análise transcriptômica do tecido do intestino médio de *Lutzomyia longipalpis*, demonstram a presença de enzimas antioxidantes incluindo uma catalase (LuloCAT), uma cobre-zinco superóxido dismutase (LuloSOD) e uma peroxiredoxina (LuloPRX), as quais, podem atuar na regulação da resposta ao estresse e evitar danos no intestino de flebotomíneos ocasionado pelas ERO e ERN (JOCHIM et al., 2008). Recentemente, foi desmonstrado que a administração oral de H_2O_2 para *Lutzomya* spp. infectadas com *L. mexicana* é prejudicial para a sobrevivência dos parasitos dentro do intestino do vetor. Além disso, o silenciamento gênico da catalase (enzima desintoxicante de H_2O_2) mediado por RNAi, levou a uma diminuição da população do parasito no intestino do vetor, reforçando a sensibilidade de *Leishmania* spp. a geração de ERO no intestino de vetor (DIAZ-ALBITER et al., 2012).

Interessantemente, diferentes estudos apontam que a geração de ERO e/ou ERN pode ser desencadeada por diversos compostos xenobióticos, incluindo os medicamentos de primeira escolha usados para o tratamento da leishmanioses e da Doença de Chagas. Os compostos antimoniais (Sb^V e Sb^{III}) por exemplo, tem sido associados com a geração de ERO e ERN, os quais podem auxiliar tanto na ativação de macrófagos quanto agindo diretamento sobre o parasito. Mookerjee e colaboradores (2006), mostraram que em macrófagos infectados com *L. donovani* e tratados com Sb^V, há a geração de ERO e de •NO, após 3 h e 24 h da exposição aos fármacos, respectivamente. Da mesma forma estes autores sugerem que a geração é mediada pela ativação das vias de fosforilação de fosfatidilinositol 3-quinase (PI3K), proteína quinase C (PKC) e proteínas quinases ativadas por mitógenos (MAPKs)

(MOOKERJEE BASU et al., 2006). Da mesma forma, o Sb^{III} (forma activa do fármaco), age diretamente sobre o parasito perturbando o balanço redox deste por dois mecanismos inerentemente distintos: seja pela diminuição da concentração de tióis intracelulares mediante o efluxo ao formar conjugados com o Sb^{III}, seja pela inibição da tripanotiona redutase, a enzima responsável pela manutenção do estado reduzido dos tióis intracelulares em células intactas, resultando na acumulação de formas de dissulfureto de tripanotiona e glutationa (WYLLIE, CUNNINGHAM e FAIRLAMB, 2004; MEHTA e SHAHA, 2006; BAIOCCO et al., 2009).

Da mesma forma, os medicamentos atualmente usados contra a doença de Chagas (Nifurtimox e Benzonidazol) tem sido associados com indução de estresse oxidativo contra o *T. cruzi*. O Benzonidazol forma conjugados com os tióis (cisteína, glutationa, γ-glutamilcisteína, glutathionilspermidina, tripanotiona) o que determina a depleção endógena de estas moléculas perturbando o balanço redox dos parasitos expostos ao fármaco (TROCHINE et al., 2014). Em contraste, nifurtimox tem sido associado com a geração de $O_2\bullet^-$, e H_2O_2 depois da redução intracelular do grupo nitro pela ação da NADPH-citochromo P-450 reductase, também podendo formar conjugados com os tióis e levar à perturbação do balanço redox (MAYA et al., 2007; RAJÃO et al., 2014).

1.12. O sistema antioxidante em tripanosomatídeos

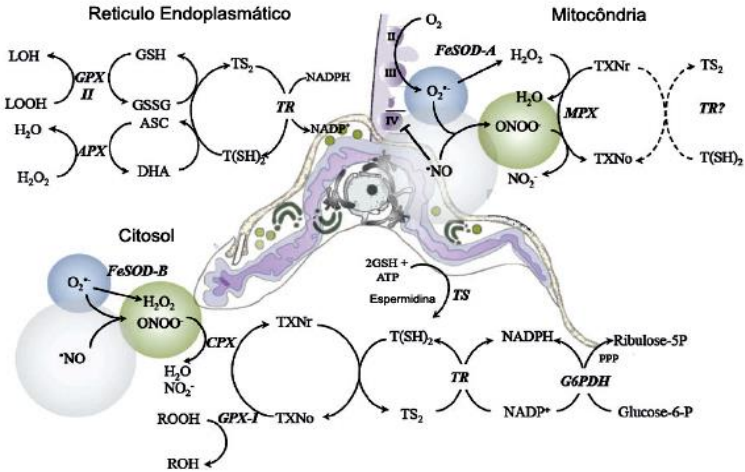
Todos os organismos aeróbios desenvolveram ao longo de sua história evolutiva uma série de vias metabólicas no intuito de minimizar danos oxidativos e nitrosativos, as quais são coletivamente conhecidas como o sistema de defesa antioxidante. Os tripanosomatídeos não são exceção à esta regra, ainda que não possuam enzimas como a catalase, as glutationas peroxidases-selênio dependentes e glutationa redutase presentes nos mamíferos. Estes parasitos possuem um sistema de defesa antioxidante único e eficiente que lhes permite suportar com sucesso a explosão oxidativa e/ou nitrosativa a que são expostos durante a infecção do hospedeiro mamífero, assim como se adaptar às diferentes condições metabólicas e ambientais impostas pelo seu ciclo de vida digenético (TURRENS, 2004; KRAUTH-SIEGEL, COMINI e SCHLECKER, 2007).

Ao contrário de outros organismos eucariotos e procariotos, os tripanosomatídeos possuem um sistema antioxidante baseado em tripanotiona $T[SH]_2$, um tiol de baixo peso molecular que, juntamente

com um arsenal de enzimas óxido-redutases composto pela tripanotiona redutase (TryR), triparedoxina (TXN), triparedoxina peroxidase (TNXPx), Glutationa peroxidase-like (GPx), ascorbato-peroxidase (APX) e superóxido dismutase dependente de ferro (SOD), as quais estão distribuídas em diferentes compartimentos subcelulares dos parasitos, participam da resposta ao estresse (IRIGOÍN et al., 2008; TOMÁS e CASTRO, 2013).

A defesa antioxidante depende de uma série de cascatas de oxidação e redução encadeadas, nas quais os elétrons são transferidos desde o NADPH (derivado da via das pentoses fosfato; PPP) a uma variedade de sistemas enzimáticos através da $T[SH]_2$ e do TXN, permitindo a neutralização das espécies reativas de oxigênio e/ou nitrogênio geradas nos diferentes compartimentos subcelulares. Em *T. cruzi*, por exemplo, foi descrito que a detoxificação de H_2O_2 a nível do retículo endoplasmático é realizada pela APX, usando o ascorbato como doador de elétrons, em uma reação mediada pela tripanotiona. Especificamente na mitocôndria, esta função é realizada pela triparedoxina ou peroxiredoxina mitocondrial (MPx), utilizando a TXN como doador de elétrons e, no citosol, as responsáveis também são as isoformas citosólicas das triparedoxinas (Figura 7) (KRAUTH-SIEGEL, BAUER e SCHIRMER, 2005; KRAUTH-SIEGEL, COMINI e SCHLECKER, 2007; PIACENZA et al., 2009; PIACENZA et al., 2013).

Figura 6. Distribuição subcelular da rede antioxidante em *T. cruzi*.



Adaptado de (PIACENZA et al., 2009; PIACENZA et al., 2013).

1.13. Os principáis tióis antioxidantes em tripanosomatídeos

1.13.1. A triopanotiona - $T[SH]_2$

A tripanotiona $T[SH]_2$ é o principal tiol de baixo peso molecular, sendo constituído por duas moléculas de glutationa (GSH) ligadas covalentemente à espermidina. Descrita há quase 30 anos em tripanosomatídeos, a tripanotiona substitui a GSH na maioria das reações de oxidação e redução das células (FAIRLAMB et al., 1985).

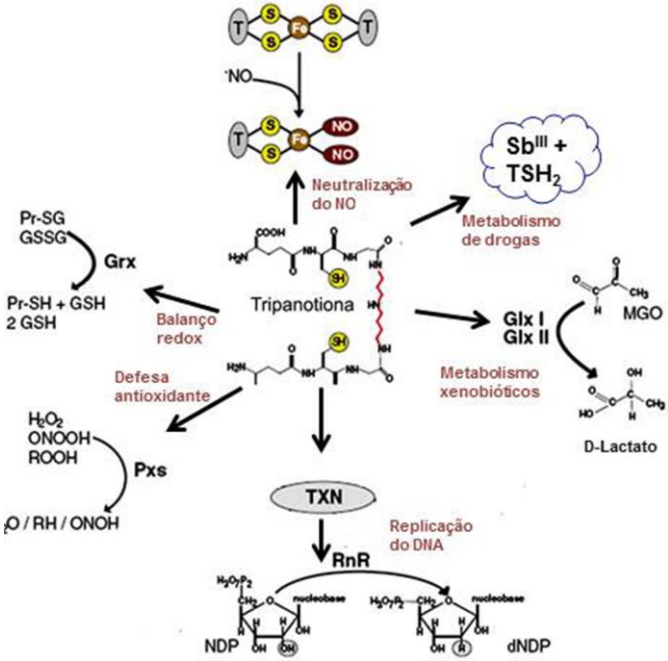
Esta molécula está envolvida em diferentes processos celulares dos tripanosomatídeos como doadora de elétrons em uma série de diferentes vias metabólicas que resultam na decomposição dos oxidantes tais como H_2O_2, peroxinitrito e hidroperóxidos. Em todos os casos, a $T[SH]_2$ oferece equivalentes redutores para moléculas intermediárias, tais como GSSG, dehidroascorbato (DHA), ou a proteína ditiol triparedoxina (TXN) que, em seus estados reduzidos, podem em seguida, transferir elétrons para as peroxidases. A tripanotione pode ainda desempenhar um papel na síntese de DNA ao fornecer equivalentes redutores para a ribonucleotídeo-redutase (RR), enzima que catalisa a síntese de precursores de DNA, reagindo diretamente com a RR quando em concentrações altas ou pelo fluxo de elétrons através da TXN. A $T[SH]_2$ também tem sido implicada na detoxificação de xenobióticos, transformando um cetoaldeído altamente tóxico (metilglioxal, MGO) derivado a partir do catabolismo da glicose em ácidos orgânicos inócuos à célula (D-lactato) pelo sistema glioxalase, assim como tem sido implicada na detoxificação de metais pesados e fármacos. A maioria destas evidências foi revelada com estudos envolvendo diferentes espécies do gênero *Leishmania* resistentes a compostos antimoniais com os quais a $T[SH]_2$ forma conjugados que são facilmente secretados. Recentemente, Bocedi e colaboradores (2010) demonstraram que a $T[SH]_2$ é capaz de interceptar o óxido nítrico e o ferro lábil, formando um complexo estável e inofensivo para o parasito, sendo que esta capacidade peculiar para sequestrar e neutralizar NO• pode explicar a predominância deste tiol em parasitas regularmente expostos a NO• (Figura 7) (ASHUTOSH, SUNDAR e GOYAL, 2007; IRIGOÍN et al., 2008; BOCEDI et al., 2010; KRAUTH-SIEGEL e LEROUX, 2012; MANTA et al., 2013).

A biossíntese de tripanotiona pode ser dividida em três etapas envolvendo duas vias metobólicas presentes em todos os organismos eucariotos; a via de síntese de espermidina e a via de síntese da GSH. Em tripanosomatídeos, a conjugação das duas moléculas de GSH com a

espermidina ocorre através de uma reação ATP-dependente que, dependendo da espécie do parasito, é catalisada pela tripanotiona sintetase (Trys) como no *T. brucei* ou pela ação de uma enzima adicional, a glutathionilespermidina sintetase (GSPS), capaz de dirigir o primeiro passo da união de uma molécula de GSH com a espermidina formando a glutathionilespermidina e, posteriormente, a Trys une a segunda molécula de GSH como acontece em algumas espécies de *Leishmania* e no *T. cruzi* (KRAUTH-SIEGEL e LEROUX, 2012; MANTA et al., 2013).

Desde a descoberta da tripanotiona, as evidências sobre sua importância biológica é inquestionável, sendo que outros tióis de alta importância como a cisteína e a glutationa foram relegados por serem considerados precursores biossintéticos secundários deste específico ditiol, único de tripanosomatídeos.

Figura 7. Principáis funções dependentes da T[SH]$_2$ em tripanosomatídeos.



Adaptado de (MANTA et al., 2013).

1.13.2. A Glutationa – GSH

A glutationa (GSH) é um tripeptídeo composto de glutamato (Glu), cisteína (Cys) e glicina (Gly), o qual está presente em todos os mamíferos onde desempenha várias funções vitais incluindo a defesa antioxidante, detoxificação de xenobióticos, armazenamento de cisteína e a manutenção do balanço redox, dentre outras. Em tripanosomatídeos, é um importante precursor para biossíntese da $T[SH]_2$ (LU, 2013). A biossíntese da GSH é um processo de duas etapas altamente conservadas entre uma gama de organismos. A primeira etapa é catalisada pela γ-glutamilcisteína sintetase (GSH1) que liga o L-glutamato e a L-cisteína para produzir a γ-glutamilcisteína. Na segunda etapa, a glutationa-sintetase (GSH2) forma uma ligação entre γ-glutamilcisteína e L-glicina para gerar a GSH (MANTA et al., 2013; MORRIS et al., 2013). A GSH1 tem sido considerada a enzima chave da biossíntese de GSH, o que foi demonstrado por diversos estudos funcionais. Em *T. brucei* ou *L. tarentolae*, por exemplo, a indução do aumento de expressão de GSH1 está associado com um aumento significativo dos níveis de GSH e $T[SH]_2$. Da mesma forma, a regulação negativa de GSH1 mediada por RNAi em *T. brucei* determinou o declínio das reservas celulares de glutationa e tripanotiona. De forma similar, em *L. infantum* com níveis deficientes de GSH1 induzido por mutação, os parasitos foram mais suscetíveis ao estresse oxidativo e xenobióticos, apresentando uma redução da taxa de sobrevivência dentro de macrófagos ativados (MUKHERJEE et al., 2009).

1.13.3. A cisteína

A cisteína é um aminoácido sulfurado, presente em todos os seres vivos, implicado em vários processos celulares, incluindo a estabilidade, a estrutura, a regulação da atividade catalítica para várias proteínas. Este aminoácido é considerado o bloco básico de construção de todos os tióis por conseguinte o reagente limitante na síntese da GSH e $T[SH]_2$ e é o componente que fornece a atividade antioxidante da GSH, pois confere a esta molécula um grande poder de doar ou receber elétrons que são utilizados para neutralizar as espécies reativas de oxigênio e manter o equilíbrio redox intracelular (NOZAKI, ALI e TOKORO, 2005; KRAUTH-SIEGEL e LEROUX, 2012).

Duas vias diferentes para a biossíntese de cisteina são descritas: a via de transulfuração reversa (RTS) e a via *de novo* ou via assimiladora. A via *de novo* comum em plantas, bacterias e algumos protozoários, mas ausente em mamíferos; em esta via a cisteina é formada através de duas

etapas, a primeira catalisada pela Serina Acetiltransferase (SAT) para formar O-Acetil-Serina (OAS) a partir de Serina e Acetil-CoA e a segunda na qual OAS reage com sulfeto gerando a cisteina, reação catalisada pela Cisteína Sintase (CS) (Figura 8, Tabela 2). Em contraste, a via RTS usa metionina como doador de enxofre e envolve uma série de reacções espontâneas e catalisadas por enzimas, para se obter a cisteína como o produto final. Os dois últimos passos, exclusivos da via RTS, são catalisados pelas enzimas cistationina-β-sintase (CβS), a qual condensa a homocisteína com serina para gerar a cistationina que por sua vez é convertida em cisteína pela ação da cistationina-γ-liase (CGL). Essa via foi originalmente demonstrada em mamíferos e fungos e, na sequencia, análises *in silico* indicaram a presença de sequências codificantes para CβS e CGL no genoma dos TriTryps (*T. cruzi*, *T. brucei* e *L. major*) (WALKER e BARRETT, 1997; NOZAKI et al., 1999; NOZAKI et al., 2001; WILLIAMS, WESTROP e COOMBS, 2009; GIORDANA et al., 2014).

Figura 8. Biossíntese de cisteína em tripanosomatídeos e sua relação com o metabolismo de T[SH]$_2$. A via de assimilação ou *de novo* em vermelha e a via de transulfuração reversa em cor azul.

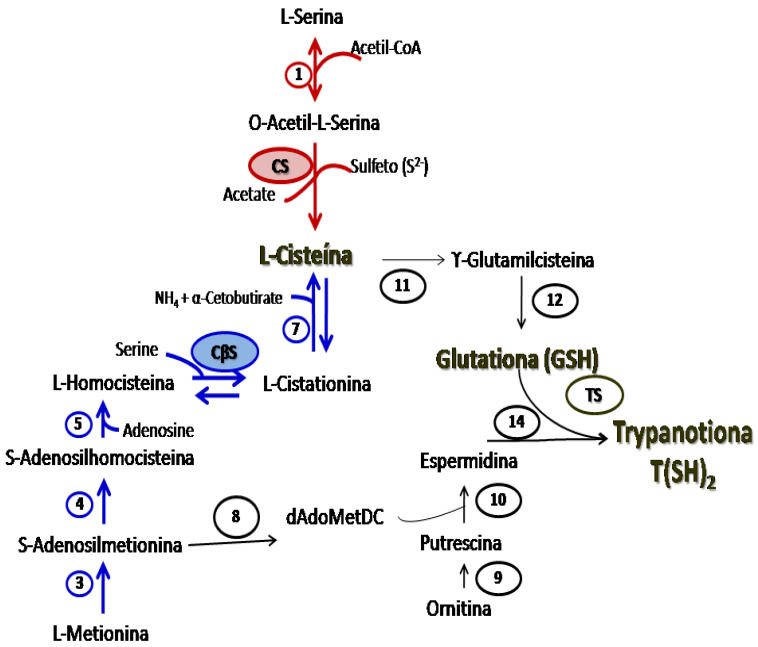


Tabela 2. Enzimas envolvidas na biossíntese de Cisteína e Tripanotiona $T[SH]_2$

EC código	Número na figura	Nome da enzima
2.3.1.30	1	Serina acetil-transferase
2.5.1.47	2	Cisteína sintase
2.5.1.6	3	S-adenosil-L-metionina sintetase
2.1.1.37	4	DNA (citosina-5-)-metiltransferase
3.3.1.1	5	S-adenosilhomocisteina sintase
4.2.1.22	6	Cistationina beta-sintase
4.4.1.1	7	Cistationina gamma-liase
4.1.1.50	8	S-adenosilmetionina decarboxilase
4.1.1.17	9	Ornitina descarboxilase
2.5.1.16	10	Espermidina sintase
6.3.2.2	11	Gamma-glutamilcisteina sintetase
6.3.2.3	12	Glutationa sintase
6.3.1.8	13	Tripanotiona sintase
6.3.1.9	14	Glutationil-espermidina sintase

Dependendo da espécie, os protozoários podem obter a cisteína partir de fontes endógenas (biossíntese) ou exógenas (captação). *Plasmodium falciparum* e *Giardia duodenalis*, por exemplo, dependem exclusivamente de cisteína exógena para seu crescimento e defesa contra agentes oxidativos, não possuíndo os genes para ambas as vias de síntese (NOZAKI, ALI e TOKORO, 2005). Já *Entamoeba histolytica* e *Trichomonas vaginalis* utilizam a via *de novo* como fonte primordial de cisteína, mas alçando a captação exógena de cisteína uma vez que este aminoácido é crucial para o balanço redox destes parasitos (NOZAKI et al., 1999; WESTROP et al., 2006; HUSAIN et al., 2011). De forma distinta, o *T. brucei* possui apenas a via RTS, sendo considerado um organismo auxotrófico para cisteína, (DUSZENKO, MÜHLSTÄDT e BRODER, 1992; BACCHI et al., 1995; CREEK et al., 2013). Interessantemente, *T. cruzi* e *L. major* parecem ser os únicos dentre os parasitos a possuir as duas vias para a biosíntese de cisteína, além de manter a capacidade de realizar a captação do aminoácido, porém de forma menos eficiente se comparado com o *T. brucei* (NOZAKI et al., 2001; WILLIAMS, WESTROP e COOMBS, 2009).

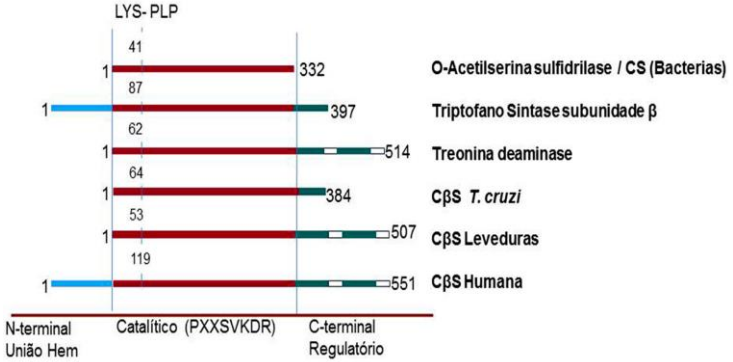
Essa aparente redundância torna-se intrigante pois muitas células lidam muito bem com apenas uma única fonte de cisteína, podendo esta capacidade estar ligada à necessidade de quantidades de cisteína maiores

a fim de abastecer o aumento da síntese de $T[SH]_2$ e outros tióis em função da necessidade variável de resposta antioxidante durante seu ciclo de vida (NOZAKI et al., 2001). Outra hipótese a considerar é a grande disponibilidade de nutrientes exógenos, a qual difere consideravelmente entre os ambientes (no vetor e nos hospedeiros mamíferos). Por exemplo, nos mamíferos os aminoácidos de enxofre e seus intermediários estão facilmente disponíveis para o parasito, enquanto que esses compostos são escassos no trato digestivo do vetor, podendo influenciar a regulação estágio-específico da síntese de cisteína, onde a via *de novo* dependente da CS ocorre em um estágio e via de transulfuração dependente da CβS ocorre em outro (WILLIAMS, WESTROP e COOMBS, 2009).

1.14. A Cisteina Sintase (CS) e a Cistationina β-Sintase (CβS): Enzimas PLP-dependentes

A CS e a CβS pertencem à família β ou tipo II da superfamília de enzimas dependentes de piridoxal 5-fosfato (PLP). Estas enzimas são evolutivamente relacionadas, apresentam um domínio de união à PLP altamente conservado (PXXSVKDR), além dos 3-4 resíduos de lisina importantes para a atividade catalítica. Análises das sequencias proteicas entre vários membros desta família mostraram uma homologia na região central ou catalítica e poucas semelhanças nas regiões N- e C-terminais das proteínas (Figura 9) (ALEXANDER et al., 1994; MILES e KRAUS, 2004; EL-SAYED e SHINDIA, 2011)

Figura 9. Organização dos domínios catalíticos e reguladores de enzimas PLP na família β ou tipo II.



Adaptado de (MILES e KRAUS, 2004).

2. JUSTIFICATIVA

A cisteína é um aminoácido indispensável para a sobrevivência de uma ampla gama de organismos vivos, desde bactérias até eucariotos superiores. Este aminoácido está implicado em muitos processos celulares cruciais, incluindo a defesa antioxidante, no qual a cisteína é o reagente central na síntese dos principais tióis antioxidantes (GSH e $T[SH]_2$) para a manutenção da homeostase redox nas células. Interessantemente, em alguns tripanosomatídeos como *L. major* e *T. cruzi*, a biosíntese de cisteina pode ocorrer por duas vias, a via *de novo* e a via de RTS. Esta estratégia redundante para a biosíntese de cisteína deve prover a quantidade necessária do aminoácido quando da necessidade de aumento da síntese de $T[SH]_2$, permitindo a sobrevivência dos parasitos nos diferentes ambientes intracelulares e/ou extracelulares ao qual os mesmos estão expostos a estresse oxidativo e/ou nitrosativo, tanto no hospedeiro mamífero quanto no inseto vetor. Sendo assim, estudos mais aprofundados destas vias são fundamentais para a compreensão dessa aparente redundância de vias metabólicas e de sua importância nos mecanismos de resposta do parasito sob condição de estresse.

Desta forma, o presente estudo focou especificamente na CS e na CβS, enzimas chaves para a biossíntese da cisteína, visando compreender o papel funcional destas enzimas na sobrevivência dos parasitos sujeitos à estresse oxidativo/nitrosativo, considerando que estas vias possuem um considerável potencial como alvos terapêuticos. Esse potencial é corroborado por outros fatores como i) a inexistência da via *de novo* em mamíferos; ii) as significativas diferenças bioquímicas/estruturais entre as CS e a CβS dos tripanosomatídeos e seus homólogos em mamíferos; iii) a essencialidade da cisteína como precursor na síntese de $T[SH]_2$. Neste sentido, a presente proposta visa determinar a função que a CβS e a CS desempenham na resposta antioxidante de tripanosomatídeos avaliando a hipótese de que "**as enzimas CβS e CS são componente essencial da resposta antioxidante e, portanto da sobrevivência de *L. braziliensis, T. rangeli* e *T. cruzi* ao estresse oxidativo e nitrosativo *in vitro***".

3. OBJETIVOS:

3.1. Objetivo Geral:

Determinar o papel funcional da cisteína sintase e da cistationina β-sintase na resposta ao estresse oxidativo e nitrosativo *in vitro* em *L. (Viannia) braziliensis*, *T. rangeli* e *T.* cruzi.

3.2. Objetivos Específicos:

3.2.1. Caracterizar molecularmente a CS e CβS de *L. braziliensis, T. rangeli e T. cruzi*.

3.2.2. Comparar os níveis de expressão e atividade enzimática da CS e CβS em diferentes estágios evolutivos dos parasitos *L. braziliensis, T. rangeli e T. cruzi***.**

3.2.3. Determinar se a sensibilidade *in vitro* de *L. braziliensis, T. rangeli e T. cruzi* ao estresse oxidativo e nitrosativo está associada com os níveis de tióis totais nestes parasitos.

3.2.4. Avaliar a expressão das enzimas CS e CβS em os três parasitos sob condições de estresse oxidativo e nitrosativo *in vitro*.

3.2.5. Determinar se o aumento de expressão das enzimas CS e CβS afetam o perfil de sensibilidade ao estresse oxidativo e nitrosativo, favorecendo a sobrevivência dos parasitos *in vitro.*

4. RESULTADOS

Neste estudo, foi usado um repertório de abordagens bioquímicas e moleculares para analisar o papel funcional da cisteína sintase e da cistationina β-sintase na resposta ao estresse oxidativo e nitrosativo em *L. (Viannia) braziliensis*, *T. rangeli* e *T. cruzi*, com o intuito de identificar, semelhanças ou diferenças entre estes parasitos. Os resultados obtidos neste trabalho estão descritos em dois artigos apresentados a seguir.

4.1. Artigo 1: "Transsulfuration is an active pathway for cysteine biosynthesis in *Trypanosoma rangeli*".

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Transsulfuration is an active pathway for cysteine biosynthesis in *Trypanosoma rangeli*

Ibeth Romero[1*†], Jair Téllez[1†], Lais Eiko Yamanaka[1], Mario Steindel[1], Alvaro José Romanha[1,2] and Edmundo Carlos Grisard[1*]

Abstract

Background: Cysteine, a sulfur-containing amino acid, plays an important role in a variety of cellular functions such as protein biosynthesis, methylation, and polyamine and glutathione syntheses. In trypanosomatids, glutathione is conjugated with spermidine to form the specific antioxidant thiol trypanothione ($T[SH]_2$) that plays a central role in maintaining intracellular redox homeostasis and providing defence against oxidative stress.

Methods: We cloned and characterised genes coding for a cystathionine β-synthase (CβS) and cysteine synthase (CS), key enzymes of the transsulfuration and assimilatory pathways, respectively, from the hemoflagellate protozoan parasite *Trypanosoma rangeli*.

Results: Our results show that *T. rangeli* CβS (TrCβS), similar to its homologs in *T. cruzi*, contains the catalytic domain essential for enzymatic activity. Unlike the enzymes in bacteria, plants, and other parasites, *T. rangeli* CS lacks two of the four lysine residues (Lys^{26} and Lys^{184}) required for activity. Enzymatic studies using *T. rangeli* extracts confirmed the absence of CS activity but confirmed the expression of an active CβS. Moreover, CβS biochemical assays revealed that the *T. rangeli* CβS enzyme also has serine sulfhydrylase activity.

Conclusion: These findings demonstrate that the RTS pathway is active in *T. rangeli*, suggesting that this may be the only pathway for cysteine biosynthesis in this parasite. In this sense, the RTS pathway appears to have an important functional role during the insect stage of the life cycle of this protozoan parasite.

Keywords: Cysteine biosynthesis, Cystathionine β-synthase, Cysteine synthase, *T. rangeli*, Thiol metabolism, Antioxidant defence

Background

L-cysteine, a sulfur-containing amino acid, is indispensable for the survival of virtually all living organisms, from bacteria to higher eukaryotes. This amino acid is implicated in several processes, including the stability, structure, regulation of catalytic activity, and post-translational modification of various proteins [1]. Due to the ability of its thiol group to undergo redox reactions, L-cysteine forms the basic building block of all thiol antioxidants, acting as a direct antioxidant and also as a precursor for the biosynthesis of glutathione, trypanothione, or ovothiol [2]. In addition, cysteine is also essential for the synthesis of biomolecules, including coenzyme A, hypotaurine, taurine, and ubiquitous iron-sulphur (Fe-S) clusters, which are involved in electron transfer, redox regulation, nitrogen fixation, and regulatory process sensing [3,4].

Two different routes for cysteine biosynthesis have been described: reverse-transsulfuration (RTS) and *de novo* or assimilatory pathways. RTS has been demonstrated in fungi and mammals and includes the complete process leading to cysteine from methionine via the intermediary formation of cystathionine [5]. These reactions are catalysed by two enzymes, CβS (cystathionine β-synthase), which synthesizes cystathionine from homocysteine and serine, and CGL (cystathionine γ-lyase), which forms cysteine from cystathionine [6]. The *de novo* pathway is also catalysed by two steps starting with serine acetyltransferase (SAT) to form O-acetylserine (OAS) from L-serine and acetyl-coenzyme A. Subsequently, OAS

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reacts with sulfide to produce cysteine in an alanyl-transfer reaction by cysteine synthase (CS) [7]. This *de novo* pathway for cysteine biosynthesis is found in plants, bacteria, and some protozoa, such as *Entamoeba histolytica*, *Entamoeba dispar* [8], *Leishmania major* [9], and *Leishmania donovani* [10], but is absent in mammals [11]. Both CβS and CS are PLP-dependent enzymes that are evolutionary-related and in most cases some CS activity has been demonstrated for the CβS enzymes described to date [12].

It is well established that the antioxidant defence system plays a key role in the host-parasite interaction for intracellular pathogenic trypanosomatids such as *T. cruzi* and *Leishmania* spp., promoting the protection of the parasite against macrophage-derived oxygen and nitrogen-reactive species [13,14]. Among trypanosomatids, the mammalian-infective and non-pathogenic *Trypanosoma rangeli* is of growing interest because its intracellular life stage within mammalian hosts is still unknown and its sympatric occurrence with *T. cruzi* [15].

Because *T. rangeli* is required for a response to a variety of oxidative stresses in both mammalian and invertebrate hosts, the present study characterised genes encoding key enzymes of cysteine biosynthesis, a crucial precursor of trypanothione.

Methods

Parasites and culture

Epimastigotes of *T. rangeli* Choachí strain and *T. cruzi* Y strain were grown at 26.5°C in liver infusion tryptose medium (LIT) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin by weekly passaging [16]. Parasites were harvested at the late log phase for DNA or protein extraction as well as for thiol profiling and *in vitro* oxidative and nitrosative stress testing. Trypomastigotes of *T. rangeli* were obtained *in vitro* under conditions previously described [17].

T. cruzi culture-derived trypomastigotes and amastigotes were obtained from THP-1 differentiated macrophage-like cells (ATCC) infected with Y strain metacyclic trypomastigotes [18]. Briefly, THP-1 cells (ATCC) were cultured in RPMI 1640 medium supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere and transformed to adherent macrophages using phorbol myristate acetate (50 ng/mL) for 72 h at 37°C and 5% CO_2 prior to experiments. THP-1 macrophage-like cells were infected with *T. cruzi* trypomastigotes for 2 h at a 3:1 parasite-cell ratio and then washed to remove the extracellular parasites. After 72 h at 37°C under 5% CO_2, the trypomastigotes were collected from the culture supernatant, centrifuged at 600 × *g* for 30 min, and then left under the same conditions for 3 h to separate the trypomastigotes from the amastigotes and cellular debris. The supernatant containing the trypomastigotes was used for protein extraction.

Identification of *T. rangeli* CβS and CS

Both the *T. rangeli* genome and transcriptome databases (http://www.rangeli.lncc.br) [19] were searched using the TBLASTN algorithm with the protein sequences of cystathionine β-synthase (CβS) and cysteine synthase (CS) from bacteria, yeast, plants, and parasitic protozoa as queries to identify putative *T. rangeli* proteins involved in transsulfuration and assimilatory pathways. Other coding sequences for potential enzymes comprising the two biosynthetic pathways were also searched in the genome and transcriptome databases. *T. rangeli* genomic DNA (gDNA) was isolated by the phenol–chloroform method following a standard protocol [20]. The open reading frames (ORFs) of the *CβS* and *CS* genes were amplified by PCR using gene-specific primers: CBTrXhoI (5′-**CTC GAG** ACC ATG GCT CAA ACC CAC-3′) and CBTrBamHI (5′-**GGA TCC** GCG CAC CTG CTT TTT ATC C-3′) for *CβS* and CSTrNdeI (5′-**CAT ATG** GAA GCT CTC ATC GGG G-3′) and CSTrXhoI (5′- **CTC GAG** CCA GCA CCA CGG GAA GC-3′) for *CS*. Sites for restriction enzymes (included in the primer name; bolded nucleotides) were included to allow cloning. All PCR assays were carried out using a Mastercycler® Gradient (Eppendorf, Hamburg) for 30 cycles of denaturation (94°C, 1 min), annealing (60°C, 45 sec), and extension (72°C, 1 min), followed by a final extension step (72°C) for 5 min. The PCR products were cloned into the pGEM-T-Easy vector (Promega), and the resulting constructs were verified by sequencing using a Megabace 1000® DNA Analysis System with the DYEnamic ET terminators kit (GE Healthcare) according to the manufacturer's conditions. Both DNA strands were sequenced for each clone obtained; after analysis using the Phred/Phrap/Consed package [21], only high-quality DNA sequences (Phred ≥ 20) were compared with the public databases using the GenBank BLAST algorithm.

Protein expression and purification

The inserts corresponding to the *CβS* and *CS* ORFs cloned into pGEM-T-Easy (Promega) were excised and subcloned into the pET14b expression vector (Novagen) pre-digested with the appropriate restriction enzymes (included in the PCR primers). The resulting plasmids containing the *CβS* and *CS* genes were named pET14-TrCβS and pET14-TrCS, respectively, and re-sequenced for confirmation as described above.

The pET14-TrCβS plasmid was used to transform *E. coli* BL21 (DE3) for recombinant protein expression. Pre-inoculum was grown overnight in LB (Luria–Bertani) broth supplemented with 100 μg/mL ampicillin at 37°C and then used to inoculate fresh LB until an

O.D.$_{600}$ of 0.6 was reached. The expression of recombinant CβS (rTrCβS) was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C. The cells were harvested and resuspended in 5 mL of buffer A [50 mM sodium phosphate, 0.3 M NaCl, pH 8.0, and 25 μM pyridoxal phosphate (PLP)] containing 5 mM imidazole and then disrupted by sonication. The soluble and insoluble fractions were recovered by centrifugation at 16,000 × *g* for 30 min at 4°C [9]. rTrCβS was purified from insoluble fractions by affinity chromatography on a Ni^{2+}-nitrilotriacetic (NTA) column (Qiagen) following standard procedures. Briefly, the insoluble fraction was resuspended in a buffer containing 8 M urea, 10 mM Tris, and 100 mM NaH_2PO_4, pH 8.0, and incubated for 1 h at 65°C to dissolve the inclusion bodies and then centrifuged (10,000 × *g* for 30 min at 4°C). The supernatants were then applied to the Ni^{2+}-nitrilotriacetic (NTA) resin (Qiagen) pre-equilibrated with the same buffer and incubated for 1 h at 4°C under continuous agitation. The resin was washed three times using washing buffer (100 mM NaH_2PO_4, 100 mM Tris/HCl, and 8 M urea, pH 6.3), and rTrCβS elution was carried out using an appropriate buffer (100 mM NaH_2PO_4, 100 mM Tris/HCl, and 8 M urea, pH 4.5). The eluted proteins were dialysed using 50 mM NaH_2PO_4 pH 7.4, 300 mM NaCl and 150 mM imidazole overnight at 4°C. The purity of the recombinant protein was then assessed by SDS-PAGE, and its concentration was determined by the Bradford method (Bio-Rad) using BSA as a standard. The protein was stored at −20°C.

To obtain recombinant CS (rTrCS), different approaches were assessed. pET14-TrCS was introduced into *E. coli* BL21 (DE3), BL21 (DE3)pLysS, and Rosetta strain, and expression was induced using different IPTG concentrations (0.2, 0.5, or 1.0 mM) and temperatures (15°C, 25°C, or 37°C). Despite the number of experimental conditions tested, it was not possible to obtain recombinant TrCS.

Production of α-rTrCβS mouse polyclonal antibodies

Approximately 50 μg of purified rTrCβS (44 kDa) was subcutaneously inoculated into Balb/C mice using Alu-Gel (Serva) as an adjuvant. Each mouse received four consecutive inoculations at 12-day intervals, with monitoring for an antibody response using enzyme-linked immunosorbent assay (ELISA) with rTrCβS as the antigen.

Comparative analysis of CβS expression by *T. rangeli* and *T. cruzi*

Quantification of CβS expression was performed using soluble protein fractions from *T. rangeli* and *T. cruzi*. A total of 1×10^8 epimastigotes or trypomastigotes were washed once with D-PBS and lysed by repeated aspiration in ice-cold lysis buffer (0.25 M sucrose, 0.25% Triton X-100, and 10 mM EDTA) containing a protease inhibitor cocktail (Sigma-Aldrich). Cellular debris was removed by centrifugation at 12,000 × *g* for 20 min at 4°C [22]. The protein concentrations in the extract were determined by the Bradford method (Bio-Rad) using BSA as a standard and stored at −20°C.

Soluble protein extracts (30 μg) of the different life cycle stages of *T. rangeli* and *T. cruzi* were fractionated on 12% SDS-PAGE and electroblotted onto nitrocellulose membranes (GE Healthcare) in an appropriate buffer (25 mM Tris; 192 mM glycine; 20% v/v methanol, pH 8.3). The membranes were then blocked with 5% non-fat milk in blotting buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, and 0.1% Tween-20) overnight at 4°C [23]. After blocking, the membranes were incubated for 1 h with an anti-rTrCβS mouse polyclonal antiserum (1:4,000) or anti-α tubulin monoclonal antibody (1:10,000) used as a loading control. After washing, the membranes were incubated with anti-mouse IgG conjugated to horseradish peroxidase (1:10,000), followed by washing and detection on radiographic films using an ECL kit (Pierce) according to the manufacturer's recommendations. The western blots were digitally analysed using the software package Image J 1.463r, subtracting the background of each blot prior to measuring the intensity of specific bands. Integrated densities for each band were determined for each protein of interest and its corresponding loading control. The ratio of the band intensity of the protein of interest versus the band intensity of the corresponding loading control was used as the relative protein expression level and allowed the comparison with other samples.

Enzymatic assays for CβS and CS activities

Cystathionine β-synthase

The assay method described by Walker and Barret was used [24]. Briefly, the reaction mixture contained 70 μmol Tris–HCl buffer (pH 8.4), 0.4 mM PLP, and 1.5 μg/μL of total protein extract from parasites or 0.1 μg/μL of rTrCβS (as a positive control) in a final volume of 100 μL. In the case of the protein extract, the mixture also contained 0.1 mM $CuSO_4$ to inhibit cystathionase activity. All components were equilibrated for 2 min at 37°C, and the reaction was initiated by the addition of 40 mM D,L-homocysteine and 20 mM L-serine. The reaction was stopped 45 min later by the addition of 100 μL 50% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation at 12,000 × *g* for 5 min, and the amount of cystathionine was determined by adding 1 mL of acid-ninhydrin reagent (1 g ninhydrin dissolved in 100 mL concentrated acetic acid and 1/3 volume of phosphoric acid) to 100 μL of the assay supernatant fraction. The mixture was then boiled for 5 min, cooled for 2 min on ice, and incubated for 20 min at room temperature (25°C) for

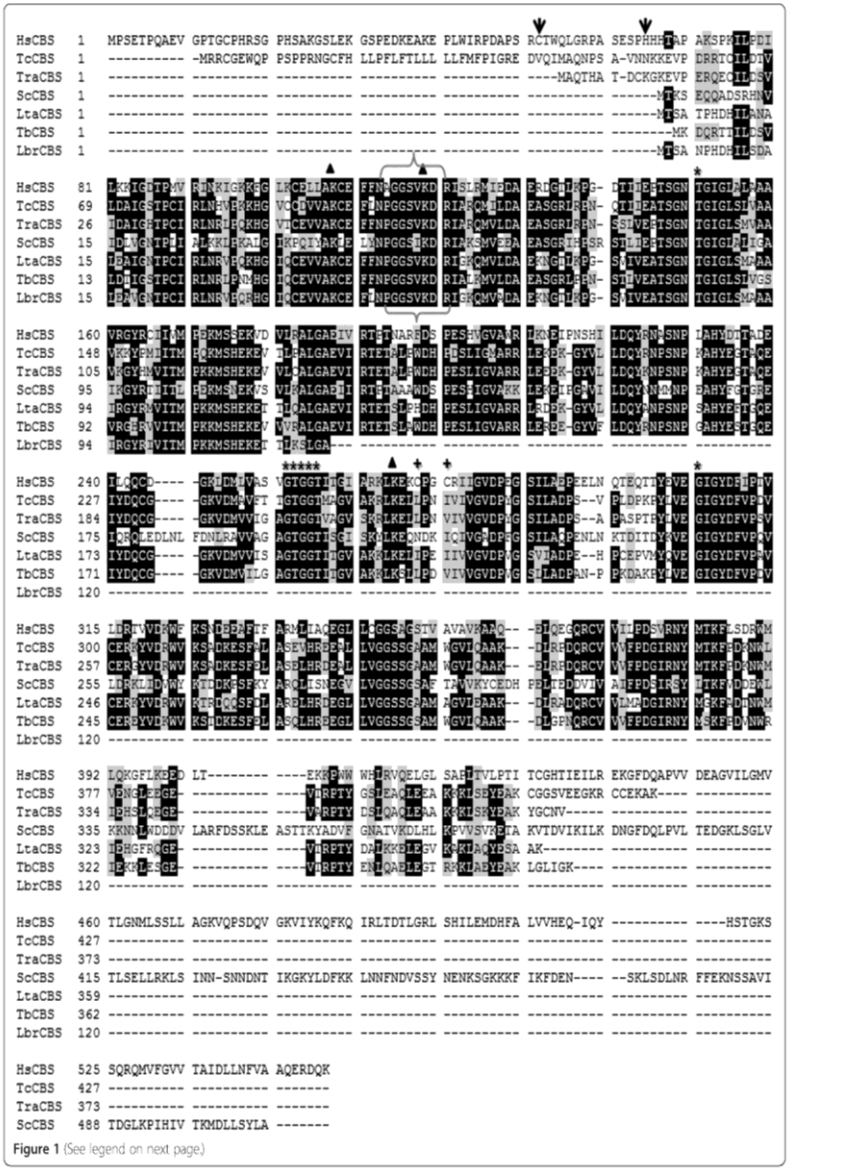

Figure 1 (See legend on next page.)

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Figure 1 Multiple alignment of deduced amino acid sequences of CβS from *T. rangeli* (TrCβS) and other representative organisms. The identity (black background) and conservation (grey background) of the amino acid residues are shown. Brackets indicate the consensus amino acid residues of the putative pyridoxal phosphate-binding motif (PXXSVKDR), and other motifs vital for CβS activity are indicated with asterisks (*). The oxido-reductase motif of HsCβS is highlighted with (+). The lysine residues required for CS catalytic activity are marked with triangles. The positions of the heme-binding residues within the heme domain of the human CβS enzyme (Cys^{52} and His^{65}) are marked with (↓). HsCβS: Human (P35520); TcCβS: *Trypanosoma cruzi* (Tc00.1047053511691.20); ScCβS: *Saccharomyces cerevisiae* (P32582); LtaCβS: *Leishmania tarentolae* (LtaP17.0270); TbCβS: *Trypanosoma brucei* (Tb11.02.5400); LbrCβS: *Leishmania braziliensis* (LbrM.17.0230).

colour development. The absorbance was measured at 455 nm. Each enzymatic assay was performed including negative controls (all reagent components without enzyme or without substrate). A standard curve was prepared using 0–3 μmol of cystathionine dissolved in acid-ninhydrin reagent and treated as described above to quantify the amount of cystathionine formed [25].

Cysteine synthase

The CS activity in the total protein extracts from parasites (1.5 μg/μL) or bacteria (positive control) was determined by measuring cysteine production at 37°C in a 500 μL reaction containing 200 mM potassium phosphate buffer (pH 7.5), 10 mM DTT, 0.2 mM PLP, 6.5 mM *O*-acetylserine (OAS), and 4 mM sodium sulfide (Na_2S). All the components except sodium sulfide were pre-incubated for 5 min at 37°C; the reaction was initiated by the addition of sodium sulfide and incubated for another 30 min and then stopped using 50 μL of 20% trichloroacetic acid (w/v). The mixture was centrifuged for 5 min at 12,000 × *g*, and the supernatant was used for cysteine analysis, as previously described with some modifications [26]. Briefly, an aliquot (500 μL) of the supernatant was added to 500 μL of ninhydrin reagent (250 mg ninhydrin dissolved in 10 mL concentrated acetic acid: concentrated HCl, 60 ~ 40 v/v). The mixture was boiled for 10 min and immediately cooled on ice before the addition of 500 μL of 95% (v/v) ethanol. The amount of cysteine formed was determined by measuring the absorbance of the reaction mixture at 560 nm [27]. Each enzymatic assay was performed including negative controls (all reagent components without enzyme or without substrate). A standard curve was prepared with L-cysteine (0–1 μmol) dissolved in ninhydrin reagent and treated as described above to quantify the amount of cysteine formed. The serine sulfhydrylase activity of CS was determined in the same way as described for the CS assay above, except that 6.5 mM serine was used instead of OAS.

Cellular thiol contents

The total thiol content of *T. rangeli* and *T. cruzi* epimastigotes was determined using deproteinised parasite extracts prepared as formerly described [28]. Epimastigotes in the exponential phase (1×10^8 parasites/mL) were harvested, washed with D-PBS, and suspended in 0.6 mL of 25% trichloroacetic acid. After 10 min on ice, the denatured proteins and cell debris were removed by centrifugation at 13,000 × *g* for 10 min at 4°C. The thiol content of the supernatant solution was determined by Ellman's method [29] using 0.6 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) in 0.2 M sodium phosphate buffer (pH 8.0). The concentration of DTNB derivatives of thiols was estimated spectrophotometrically at 412 nm. Calibration curves were performed with known amounts of cysteine.

Epimastigote susceptibility to oxidative and nitrosative stress *in vitro*

Parasite susceptibility to oxidative or nitrosative stress was assessed using Alamar blue (AB) assays, as described elsewhere [22,30] with minor modifications. Briefly, 5×10^5 *T. rangeli* and *T. cruzi* epimastigotes were incubated for 48 h with 100 μL parasite culture in quadruplicate in 96-well plates. Aliquots of 100 μL of 30% hydrogen peroxide (Sigma-Aldrich) or S-nitroso-N-acetylpenicillamine (SNAP, Molecular Probes®- Life Technologies) prepared at different dilutions (0–100–150–300–500–1000–1500 μM and 0–5–20–50–150–300–500–1000 μM, respectively) were added, as reported [22,31]. After incubation at 26°C for 24 h, 20 μL of AB reagent (Invitrogen) was added to each well to assess parasite viability via fluorescence emission at 600 nm. Data from treated and non-treated cultures were used to calculate the IC_{50} by a sigmoidal regression analysis (with variable slope) using GraphPad Prism v.5.0. Untreated control parasites and reagent blanks were included in each test plate.

Statistical analysis

All experiments were performed in triplicate, and the results are presented as the mean and the standard deviation (SD) or standard error of the mean (SEM). Normalised data were analysed by a one-way ANOVA followed by Bonferroni post-tests or Student's *t*-test, as indicated in the figure legends, using the software GraphPad Prism v.5.0.

Ethical approval

The procedures involving animals were previously approved by the UFSC Ethics Committee on Animal Use – CEUA (Reference number: 23080.025618/2009-81).

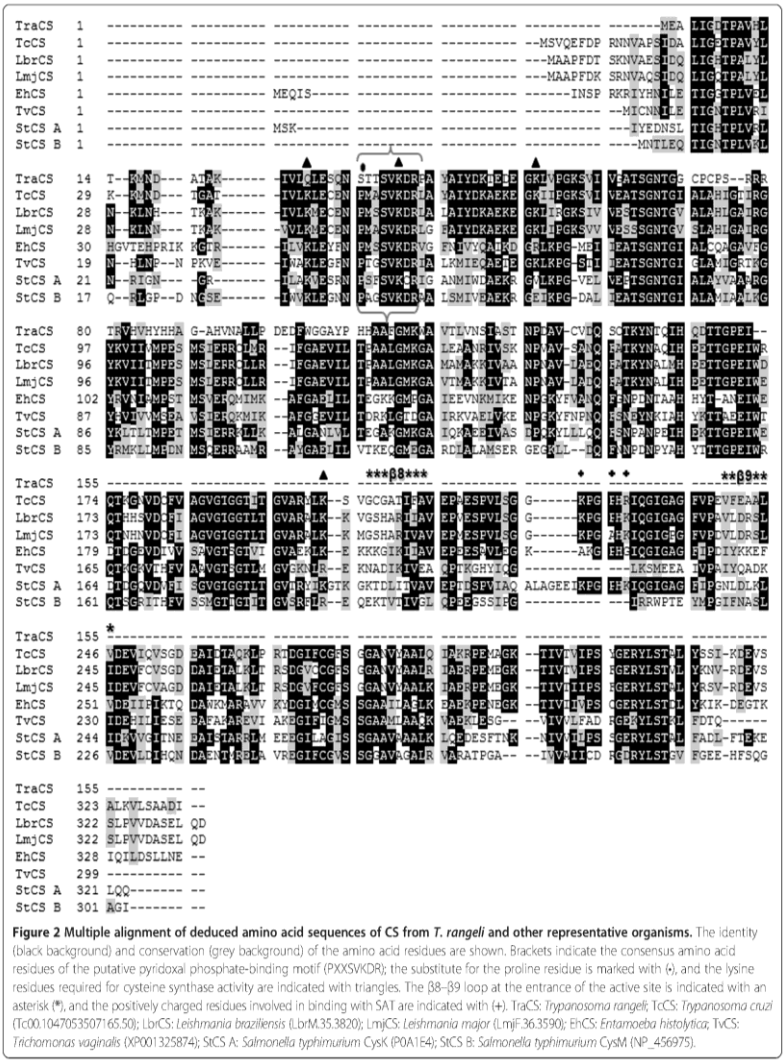

Figure 2 Multiple alignment of deduced amino acid sequences of CS from *T. rangeli* and other representative organisms. The identity (black background) and conservation (grey background) of the amino acid residues are shown. Brackets indicate the consensus amino acid residues of the putative pyridoxal phosphate-binding motif (PXXSVKDR); the substitute for the proline residue is marked with (•), and the lysine residues required for cysteine synthase activity are indicated with triangles. The β8–β9 loop at the entrance of the active site is indicated with an asterisk (*), and the positively charged residues involved in binding with SAT are indicated with (+). TraCS: *Trypanosoma rangeli*; TcCS: *Trypanosoma cruzi* (Tc00.1047053507165.50); LbrCS: *Leishmania braziliensis* (LbrM.35.3820); LmjCS: *Leishmania major* (LmjF.36.3590); EhCS: *Entamoeba histolytica*; TvCS: *Trichomonas vaginalis* (XP001325874); StCS A: *Salmonella typhimurium* CysK (P0A1E4); StCS B: *Salmonella typhimurium* CysM (NP_456975).

Results

The *T. rangeli* genome contains genes encoding CβS and CS enzymes

Using the nucleotide and protein sequences of CβS and CS orthologs from plants, bacteria, yeast, and parasitic protozoa as queries, a search of *T. rangeli* genome and transcriptome databases allowed the identification of genes encoding CβS and a partial gene sequence for CS. Additionally, the *T. rangeli* genome contains a single copy of the cystathionine γ-lyase (CGL) gene of the RTS

pathway but lacks the genes encoding serine acetyltransferase (SAT) present in the *de novo* biosynthetic pathway of other trypanosomatids. The sequences for CβS and CS were then back-searched using the SWISSPROT and NCBI databases, which confirmed the identity of both genes. These results suggest that, as in other trypanosomatids, *T. rangeli* possesses genes coding for the enzymes involved in these two cysteine biosynthetic routes: CβS in the transsulfuration pathway and CS in the *de novo* biosynthesis pathway.

After cloning and sequencing, it was found that *T. rangeli* CβS (*TrCβS*) predicts a protein of 373 amino acids (44 kDa) that reveals high sequence identity with CβS from *T. cruzi* (84%), *T. brucei* (78%), and *L. major* (75%) compared to human CβS (50%). Multiple sequence alignment confirmed that TrCβS contains three out of the four lysine residues (Lys ^{53}, Lys^{64}, Lys^{213}) required for CS activity; the consensus sequence for the putative cofactor pyridoxal phosphate-binding domain is highly conserved. rTrCβS, as well as CβS from other trypanosomatids, differs from *H. sapiens* CβS (HsCβS) by lacking the haem-binding (redox sensor) and oxidoreductase motifs (Cys XX Cys) at the N- and C-termini, respectively (Figure 1).

The *T. rangeli* CS gene (TrCS) encodes a protein of 155 amino acids (~16.8 kDa) that is 53% identical to the *T. cruzi* ortholog but exhibits lower identity with *L. major* (46%) and *L. infantum* (45%). Although CβS and CS are evolutionarily related enzymes, we found a low identity between TrCβS and TrCS (≤13%) when compared to the TrCS identity with the other orthologues from plants and bacteria (~31-33%). An analysis of the predicted amino acid sequences of TrCS revealed an amino acid change of Pro^{32} → Ser within the putative pyridoxal phosphate-binding domain (PXXSVKDR). Unlike other CSs, TrCS has only two of the four lysine residues (Lys^{37}, Lys ^{53}) shown to be important for the catalytic activity of the enzyme. Furthermore, TrCS does not have the canonical β8-β9 loop described in CS enzymes, which is important for access to the active site, and neither of the positively charged residues (Lys-His-Lys) involved in binding with serine acetyl-transferase (SAT) (Figure 2).

Stage-specific expression of CβS in *T. rangeli*

The relative abundance of the CβS protein was evaluated in *T. rangeli* epimastigote and trypomastigote forms by western blotting, showing no significant differences between the forms. The absence of TrCβS stage-specific expression contrasts with the homologous protein in *T. cruzi* (TcCβS), for which the expression level of CβS was found to be significantly increased in epimastigotes (Figure 3A, B).

CβS is active in *T. rangeli*

The enzymatic studies on *T. rangeli* extracts showed that CβS activity is detectable in both epimastigotes (0.13 μmol min^{-1} mg^{-1}) and trypomastigotes (0.079 μmol min^{-1} mg^{-1} of protein) (Figure 4A), whereas CβS activity was 1.9 times higher in the extracts from *T. cruzi* epimastigotes versus trypomastigotes. Conversely, CS activity was undetectable in the protein extracts from both *T. rangeli* forms (Figure 4B).

rTrCβS showed CβS activity of 2.2 ± 0.2 μmol min^{-1} mg^{-1} of protein (Figure 4A), with a *km* of 1.702 ± 0.11 mM for L-serine and a *Km* of 7.301 ± 1.9 mM for L-homocysteine, indicating a high binding affinity for L-serine and a weak binding affinity for L-homocysteine. rTrCβS was also capable of generating L-cysteine from serine and sodium sulfide, but with a very low specific activity (serine sulfhydrylase activity of 0.013 μmol min^{-1} mg^{-1} of protein). Different from *T. cruzi* CβS, rTrCβS did not show any CS activity (data not shown).

Total thiol content in *T. rangeli* and *in vitro* oxidative/nitrosative stress phenotyping

A comparative analysis of the total thiol levels of *T. rangeli* and *T. cruzi* revealed significant differences between these

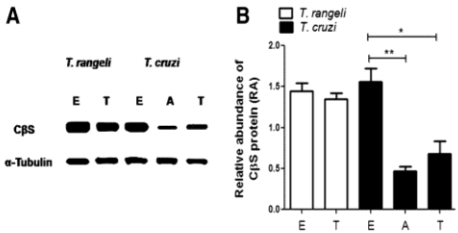

Figure 3 Protein expression levels of CβS in *T. rangeli* and *T. cruzi*. A. Western blot analysis of soluble extracts obtained from epimastigotes (E), and trypomastigotes (T) of *T. rangeli* and *T. cruzi*, and amastigotes (A) of *T. cruzi*. **B**. Densitometric analysis of CβS expression using ImageJ software and significant differences in CβS expression between epimastigotes and trypomastigotes, as determined by the t-test (*P < 0.05, **P < 0.01). The normalisation of protein loading was performed by the immunodetection of α-tubulin.

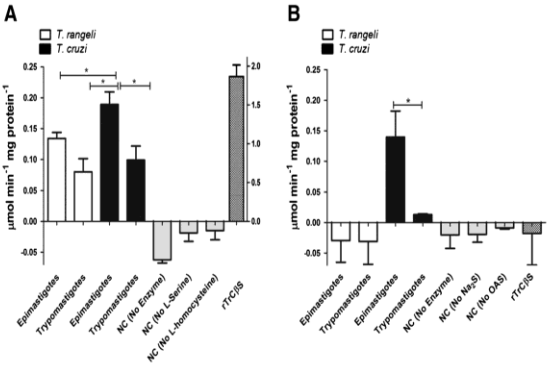

Figure 4 Detection of CβS and CS activities in protein extracts of *T. rangeli* and *T. cruzi* epimastigotes and trypomastigotes. A. The activities of CβS were determined in soluble extracts from trypanosomes using the recombinant enzyme rTrCβS as a positive control (axis Z). The results represent the average of five independent experiments performed in triplicate ± SD. **B**. The activities of CS were determined in soluble extracts from trypanosomes. The data represent the mean of five independent experiments performed in triplicate ± SD. Significant difference (*P < 0.05). NC = negative controls.

parasites. *T. cruzi* showed a thiol content of 7.8 nmoles/10^8 parasites, whereas *T. rangeli* had a thiol content that was almost seven times less (1.1 nmoles/10^8 parasites) (Figure 5A).

Based on these results, the *T. rangeli* susceptibility to oxidative and nitrosative stress was evaluated by subjecting epimastigotes to stress conditions *in vitro* with H_2O_2 or SNAP. This parasite was found to be more sensitive than *T. cruzi* to oxidative stress (H_2O_2), showing an IC_{50} of 53 μM, which is significantly less (P < 0.01) than the IC_{50} obtained for *T. cruzi* epimastigotes (188.3 μM). Nevertheless, the difference between these parasites was less pronounced under nitrosative stress conditions (SNAP), with *T. rangeli* being more resistant than *T. cruzi* (IC_{50}: 312 μM and 240.7 μM, respectively) (Figure 5B).

Discussion

Our results indicate that RTS appears to be the only pathway for cysteine biosynthesis in *T. rangeli*. At the

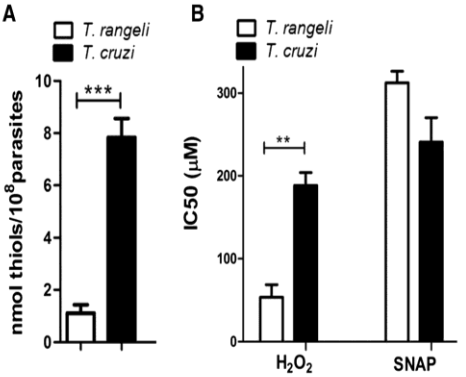

Figure 5 Total thiol content and effects of oxidative and nitrosative stress on *T. rangeli* and *T. cruzi* viability. A. The total thiol content was determined in soluble extracts obtained from the epimastigote form. Error bars represent the SEM of three independent experiments. **B**. *In vitro* susceptibility of epimastigotes of *T. rangeli* and *T. cruzi* exposed to oxidative stress by hydrogen peroxide (H_2O_2) or nitrosative stress by S-nitroso-N-acetylpenicillamine (SNAP). Error bars represent the SEM of three independent experiments, performed in quadruplicate. Significant differences were detected by a one-way ANOVA, followed by Bonferroni post-tests (**P < 0.01, ***P < 0.001).

genomic level, *T. rangeli* contains single copies of genes coding for the CβS and CGL (cystathionine γ-lyase) enzymes of the RTS pathway but lacks genes encoding a protein of the cysteine *de novo* biosynthetic pathway (SAT). Additionally, a partial gene sequence for CS was found that has an A-G nucleotide transition at position 470, which generates a stop codon (TAG) (data not shown); thus, the truncated protein encoded lacks two of the four lysine residues required for CS activity.

A biochemical analysis of rTrCβS showed a higher CβS activity compared to hsCβS for generating cystathionine via the condensation of L-serine and L-homocysteine, though rTrCβS is less active than TcCβS [32-34]. In spite of this, the binding substrate affinity was comparable to the affinity of the CβS enzyme from *L. major* and humans [9]. Similar to other CβSs, rTrCβS can also form cysteine from L-serine and sodium sulfide, but is unable to utilise OAS and sulfide to catalyse the production of cysteine. Nevertheless, inter-species variations in other CβS catalysed reactions [24] could explain the absence of CS activity mediated by TrCβS.

The presence of a truncated CS gene as revealed by high-quality sequencing (Phred ≥50), and the absence of CS activity in both epimastigote and trypomastigote extracts suggests that the *de novo* cysteine biosynthetic pathway is absent or not functional in *T. rangeli*. Nevertheless, *T. rangeli* possesses a functional RTS pathway, a characteristic shared with *T. brucei*, for which only CβS activity has been reported in bloodstream trypomastigote extracts but at a very low level [35]. This result indicates that similarities in the metabolism of sulfur-containing amino acids exist between *T. rangeli* and *T. brucei*, another parasite that does not possess an intracellular mammalian host stage. Such findings may suggest that the extracellular stage of the life cycle of parasitic protozoa and the RTS biosynthetic pathway are causally connected.

No stage-specific association was found for *T. rangeli* CβS activity and protein levels, contrasting with *T. cruzi*, with epimastigotes (insect-form) that present significantly higher activity and protein levels. Other studies on the RTS pathway in *T. cruzi* have demonstrated the same stage-specific regulation of this pathway and have shown a likely association with the complex life cycle of this parasite and the availability of sulfur-containing amino acids in different parasite environments [33,34].

We found significantly lower levels of total thiol content in *T. rangeli* compared to *T. cruzi* epimastigotes. Based on the fact that cysteine forms the basic building block of all thiol antioxidants [2], one possible explanation for the lowest thiol levels observed may be because *T. rangeli* only uses the RTS pathway as a cysteine biosynthesis source. Another important aspect is related to the fact that exogenous organic sulfur-containing amino acids can be supplied by transporters [3,9,36]. However, such a mechanism and its possible influence on the total thiol levels in *T. rangeli* remain to be explored.

Different from *T. cruzi*, which faces oxidative stress in the mammalian host and within the triatomine vector's digestive tract, *T. rangeli* is exposed to further oxidative and nitrosative stress while reaching the triatomine hemolymph and salivary glands [37]. Recently, studies have demonstrated the activation of the vector immune system during *T. rangeli-Rhodnius prolixus* interactions, including the generation of nitric oxide and superoxide free radicals [38-40]. The greater resistance of *T. rangeli* to SNAP compared to *T. cruzi* could be explained by the ability of *T. rangeli* to modulate insect immune/cellular factors [38,41], especially those related to nitrosative production, thus allowing the parasite to survive and multiply freely in the insect's hemolymph and to invade and complete its development within the salivary glands [42].

Because thiols have been demonstrated to be the central metabolites in the redox metabolism of several parasite species [43], thus playing an important role in protection against oxidative stress, the higher *T. rangeli* susceptibility to hydrogen peroxide may be due its reduced total thiol content. In addition, the absence of an active CS enzyme potentiates the *T. rangeli* susceptibility to hydrogen peroxide, leading to the death of the parasite. Such findings are in agreement with reports in amoebae, whereby the overexpression of CS increases the total cellular thiol content and the resistance to oxidative stress due to hydrogen peroxide [8].

Conclusion

These findings demonstrate that the RTS pathway is active in *T. rangeli*, suggesting that this may be the only pathway for cysteine biosynthesis in this parasite because no CS activity was detected in epimastigotes and trypomastigotes and the CS genes are truncated due to the presence of stop codons. In this sense, the RTS pathway would have an important functional role during the insect stage of the life cycle of this protozoan parasite.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IR and JT participated in the conception and design of the study and wrote the manuscript. LY was involved in cloning CβS. MS, AR, and EG were involved in the conception of the study and wrote the manuscript. All authors read and approved the final version of the manuscript.

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4.2. Artigo 2: "Upregulation of Cysteine Synthase (CS) and Cystathionine-β-Synthase (CβS) proteins contributes to *Leishmania braziliensis* survival under oxidative stress".

Artigo em processo de revisão final para ser submetido e considerado para publicação na revista "Plos Pathogens"

Upregulation of Cysteine Synthase (CS) and Cystathionine-β-Synthase (CβS) proteins contributes to *Leishmania braziliensis* survival under oxidative stress

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ABSTRACT

Background: Cysteine metabolism is considered crucial in maintaining the reducing environment in trypanosomatids due to its importance as a precursor of trypanothione biosynthesis. **Methods**: Expression, activity, functional rescue and overexpression of cysteine synthase (CS) and cystathionine beta synthase (CBS) were evaluated in *Leishmania braziliensis* promastigotes and intracellular amastigotes under hydrogen peroxide (H_2O_2), SNAP and antimonial compound stress conditions *in vitro*. **Principal findings**: Our results demonstrated a stage-specific increase in the level of protein expression and activity of LbrCS and LbrCβS, leading to elevated levels of total thiols concentration in response to oxidative and nitrosative stress *in vitro*. Our functional characterization results allowed the rescue of the CS activity in *T. rangeli* and showed epimastigotes expressing CS had increased parasite survival after exposure to different concentrations of H_2O_2 when compared to WT epimastigotes. We also found an enhanced ability of *L. braziliensis* overexpressing LbrCS and LbrCβS to resist oxidative stress when compared to the controls. In amastigotes overexpressing LbrCS and LbrCβS, our findings clearly demonstrated a significant loss of susceptibility to Sb^V treatment *in vitro*. **Conclusion:** Modifications in the protein expression and activity levels of LbrCS and LbrCβS may alter parasite survival under oxidative stress conditions and can influence the antimony drug efficacy in the New World *Leishmanias.*

Key words: Cysteine biosynthesis, Cysteine synthase, Cystathionine β synthase, *L. braziliensis*, oxidative stress, antimonial compounds, antioxidant defense.

Introduction

The intracellular protozoan parasite *Leishmania* spp. causes a neglected infectious disease commonly referred to as leishmaniasis. Depending on infecting species of parasite and the immune status of the host, the disease can manifest in a variety of clinical conditions with cutaneous (CL), mucocutaneous (MCL) or visceral (VL) involvement [1-3]. The species *Leishmania* (Viannia) *braziliensis*, the most prevalent species in the Americas, is the causative agent of CL and MCL [4,5].

Leishmania spp. have a digenetic life cycle, alternating between flagellated promastigotes forms in the mid-gut of the sand fly insect and non-motile amastigotes inside the macrophage of the mammalian host [6,7]. During its complex life cycle, these parasites are exposed to a large amount of reactive oxygen species or nitrogen species (ROS and RNS) generated by the host to create unfavourable conditions for the invading pathogens that allow their elimination [8,9]. The antimonial compounds, such as sodium stibogluconate (SAG, PENTOSTAN®) and meglumine antimonniate (GLUCANTIME®), continue to be the mainstay drugs for leishmaniasis chemotherapy [10]. SAG has been reported to induce indirectly oxidative and nitrosative stress by stimulating infected macrophages (MΦ) to generate ROS and Nitric Oxide (•NO) via phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs) activation [11]. Additionally, the reduced form of the drug $Sb(^{III})$, acts directly on the parasite survival perturbing its redox-balance and imposes an oxidative/nitrosative stress by binding to thiols and inhibiting the enzyme TR [12,13].

In turn, the parasites have developed an elaborate antioxidant defense system composed of molecules and enzymes to scavenge the ROS and RNS stresses [14,15]. Furthermore, the low-molecular-mass thiols, glutathione and trypanothione, are essential in the defence mechanism systems since they regulate redox homeostasis in parasites [16]. The synthesis of glutathione, and thus, trypanothione depends on the availability of cysteine, a sulfur-containing amino acid implicated in several biological processes, as well as the stability, structure, regulation of catalytic activity, and posttranslational modification of various proteins [17,18].

Two different routes for cysteine biosynthesis have been described: *de novo* or assimilatory and reverse-transsulfuration (RTS) pathways. RTS has been demonstrated in fungi and mammals and includes the complete

process leading to cysteine from methionine via the intermediary formation of cystathionine [19]. These reactions are catalysed by two enzymes, CβS (cystathionine β-synthase), which synthesizes cystathionine from homocysteine and serine, and CGL (cystathionine γ-lyase), which forms cysteine from cystathionine [20]. The *de novo* pathway is also catalysed by two steps, starting with serine acetyltransferase (SAT) to form O-acetylserine (OAS) from L-serine and acetyl-coenzyme A. Subsequently, OAS reacts with sulfide to produce cysteine in an alanyltransfer reaction by cysteine synthase (CS) [21]. This *de novo* pathway for cysteine biosynthesis is found in plants, bacteria, and some protozoa, such as *Entamoeba histolytica*, *Entamoeba dispar*, *Leishmania major* and *Leishmania donovani*, but is absent in mammals [22-25].

Given the putative importance of thiol metabolism in *Leishmania* parasites to survive oxidative attack inside the different hosts during their life cycle as well as the base of L-cysteine forming the basic building block of all thiol antioxidants, in the present study, we characterised genes encoding key enzymes of cysteine biosynthesis (CS and CβS) to determine whether these enzymes may be involved in the antioxidant defense in response to H_2O_2, SNAP and Sb compounds.

Methods

Parasites

Promastigotes of *L. (Viannia) braziliensis* (MHOM/BR/75/M2904) were grown at 26 °C in Schneider's' Drosophila medium (Sigma) containing 10% heat-inactivated fetal bovine serum, 1% glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin [26]. Intracellular amastigotes were obtained from THP-1 differentiated macrophages cells as described below. Epimastigotes of the *T. rangeli* Choachí strain were cultured in liver infusion tryptose medium (LIT) under conditions previously described [27]. Parasites were harvested at the exponential and late log phase for DNA or protein extraction as well as for thiol profiling and *in vitro* oxidative and nitrosative stress testing.

Human THP-1 macrophages

The human acute monocytic leukemia cell line, THP-1 (ATCC) was cultured at 37 °C in a 5% CO_2 atmosphere, using RPMI 1640 medium containing 1% glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin [28,29]. Monocytes were harvested during the logarithmic stage of growth and

transferred to a medium containing 50 ng/mL phorbol myristate acetate (PMA; Sigma) for the induction of adherence and differentiation into macrophages; samples containing 1 x 10^5 cells (for infection with *Leishmania braziliensis*) were transferred to a 6-well plate and incubated for 72 h at 37 °C, 5% CO_2 [30].

Macrophages infections

Stationary-phase parasites were harvested at 6 days after subculture, opsonized by treatment with RPMI 1640 containing 10% human AB+ serum for 1 h as described [30]. Parasites were added to each sample of 1 x 10^6 differentiated macrophages using a parasite-to-cell ratio of 20:1, and infection was allowed to proceed for 2 h (34 °C, 5% CO_2). Free parasites were removed by washing 2 times with PBS 1X. Following a further 24 h incubation (34 °C, 5% CO_2) to allow the complete development of amastigotes, intracellular amastigotes were collected by disrupting THP-1 macrophages by repeated passage through a 27-gauge needle connected to a 3 mL syringe to lyse macrophages and release parasites. Cell debris was removed by centrifugation (800 x *g* 10 min, 4 °C) and amastigotes in the supernatant collected by a second centrifugation step (4000 x *g* 10 min, 4 °C). Amastigotes were washed in sterile PBS and used for protein extraction.

Identification of *L. braziliensis* CS and CβS

The integrated databases TriTrypDB, version 6.0 (http://tritrypdb.org/tritrypdb), were searched to identify putative sequences of Cysteine synthase (CS) and Cystathionine β-synthase (CβS) in *L. braziliensis* MHOM/BR/75/M2904. Based on possible CS and CβS sequences deposited in the *L. braziliensis* genome, the open reading frame (ORF) of CS gene (1002 pb) and a partial gene of CβS (360pb) were amplified by PCR from genomic DNA (gDNA) isolated by the phenol–chloroform method following a standard protocol [31] and using gene-specific primers modified with appropriate restriction sites (to facilitate cloning into their respective vectors) as detailed in Supplementary Table S1. All PCR assays were carried out using a Mastercycler® Gradient (Eppendorf, Hamburg) for 30 cycles of denaturation (94 °C, 1 min), annealing (45/62 °C, 45 sec), and extension (72 °C, 30 sec), followed by a final extension step (72 °C) for 5 min. PCR products were cloned into the pGEM-T-Easy vector (Promega), and the resulting constructs were verified by sequencing using a Megabace 1000® DNA Analysis System with the DYEnamic ET terminators kit (GE Healthcare), according to the manufacturer's

conditions. Both DNA strands were sequenced for each clone obtained; after analysis using the Phred/Phrap/Consed package [32], only high-quality DNA sequences (Phred ≥ 20) were compared with the public databases using the GenBank BLAST algorithm.

Protein expression and purification

The insert corresponding to the CS ORF cloned into pGEM-T-Easy (Promega) was excised and subcloned into the pET21a expression vector (Novagen) pre-digested with the appropriate restriction enzymes included in the PCR primers. The resulting plasmid containing the CS gene was named pET21-LbrCS and re-sequenced for confirmation as described above. The pET21-LbrCS plasmid was used to transform *E. coli* BL21 (DE3) for recombinant protein expression. Pre-inoculum was grown overnight in LB (Luria– Bertani) broth supplemented with 100 μg/mL ampicillin at 37 °C and then used to inoculate fresh LB until an $O.D._{600}$ of 0.6 was reached. The expression of recombinant CS (rLbrCS) was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h at 37 °C. Cells were harvested and resuspended in 5 mL of buffer A [50 mM sodium phosphate (NaH_2PO_4), 0.3 M NaCl, pH 8.0, and 25 μM pyridoxal phosphate (PLP)] containing 5 mM imidazole and then disrupted by sonication. The soluble and insoluble fractions were recovered by centrifugation at 16,000 x *g* for 30 min at 4 °C [23]. rLbrCS was purified from soluble fractions by immobilized metal affinity chromatography (IMAC) on a TALON Metal Affinity Resin (Clontech) following standard procedures. Briefly, the soluble fraction was applied to the TALON Metal Affinity Resin (Clontech) pre-equilibrated with the equilibration buffer (50 mM NaH_2PO_4, 0.3 M NaCl, pH 7.4) and incubated for 20 min at 4 °C under continuous agitation. The resin was washed three times using washing buffer (50 mM NaH_2PO_4, 0.3 M NaCl, and 10 mM imidazole, pH 7.4), and rLbrCS elution was carried out using the appropriate buffer (50 mM NaH_2PO_4, 0.3 M NaCl, and 150 mM imidazole, pH 7.4). Eluted proteins were dialyzed using 50 mM NaH_2PO_4, 150 mM NaCl and 25 μM PLP, pH 8.0 overnight at 4 °C. Purity of the recombinant protein was then assessed by SDS-PAGE, and its concentration was determined by the Bradford method (Bio-Rad) using BSA as a standard. The protein was stored at −20 °C until used.

Production of α-rLbrCS mouse polyclonal antibodies

Approximately 50 μg of purified rLbrCS (35 kDa) was subcutaneously inoculated into Balb/C mice using Alu-Gel (Serva) as an adjuvant. Each

mouse received four consecutive inoculations at 12-day intervals, with monitoring for an antibody response using enzyme-linked immunosorbent assay (ELISA) with rLbrCS as the antigen. The procedures involving animals were previously approved by the UFSC Ethics Committee on Animal Use – CEUA (Reference number: 23080.025618/2009-81).

Comparative analysis of CS and CβS expression

Quantification of CS and CβS expression was performed using soluble protein fractions from promastigotes and amastigotes of *L. braziliensis*, as well as from epimastigotes of *T. rangeli*, following the protocol described previously [27]. Briefly, soluble protein extracts (30 μg) of parasites were subjected to Western blot analysis using polyclonal immune mouse antisera raised against recombinants proteins rLbrCS (from *L. braziliensis*) and rTrCβS (from *T. rangeli*) diluted 1:500 (v/v), 1:250 (v/v), respectively, in blotting buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, and 0.1% Tween-20) containing 2% (w/v) non-fat milk. Bound antibody was detected using anti-mouse IgG conjugated to horseradish peroxidase (1:10,000), and the enhanced chemiluminescence ECL kit (Pierce) according to the manufacturer's recommendations. Anti-α tubulin monoclonal antibody (1:10,000) was used as a loading control. The antisera against rTrCβS were used to detect CβS, as described previously, and the western blots were digitally analysed using the software package Image J 1.463r, as also described in [27]. Integrated densities for each band were determined for each protein of interest and its corresponding loading control. The ratio of the band intensity of the protein of interest versus the band intensity of the corresponding loading control was used as the relative protein expression level and allowed the comparison with other samples.

Enzymatic assays for CS and CβS activities

The CS and CβS activities in the total protein extracts (1.5 μg/μL) from *L. braziliensis* (promastigotes and amastigotes) and *T. rangeli* (epimastigotes) were assayed by measuring cystathionine or cysteine production at 37 °C as described in Romero et al 2014. Each enzymatic assay performed included positive controls (rLbrCS or rTrCβS) and negative controls (all reagent components without enzyme or without substrate).

Parasite susceptibility to oxidative and nitrosative stress *in vitro*

Parasite susceptibility to three different oxidative/nitrosative stresses was assessed using Alamar blue (AB) assay, as reported previously in [27,33]. Briefly, 5×10^5 parasites were exposed for 48 h at 26°C to 30% hydrogen peroxide (Sigma-Aldrich), S-nitroso-N-acetylpenicillamine (SNAP, Molecular Probes®- Life Technologies) potassium antimonyl tartrate trihydrate (Sb^{III}, Sigma-Aldrich) prepared at different dilutions. After treatment, parasite viability was evaluated via fluorescence emission at 600 nm. Data from treated and non-treated cultures were used to calculate IC_{50} by a sigmoidal regression analysis with normalized response -- variable slope using GraphPad Prism v.5.0. Untreated control parasites and reagent blanks were included in each test plate. To evaluate the effect of oxidative and nitrosative stress on CβS and CS expression and activity in *L. braziliensis*, 1 x 10^8 promastigotes were incubated in the presence of the IC_{50} dose of H_2O_2 (550 µM) and SNAP (195 µM) at 26 °C. Aliquots of treated parasites were collected at 1, 2 and 4 h intervals and were used for protein expression and enzymatic assays.

Intracellular total thiol contents

The total reduced thiol content of *L. braziliensis* promastigotes was determined by Ellman's method, using deproteinised parasite extracts as formerly described [27].

Functional rescue of CS activity and heterologous expression of CβS in *T. rangeli*

The pTEXCSeGFP construct, obtained as described below, which contained *L. braziliensis CS* gene was introduced into epimastigotes of *T. rangeli* by electroporation via Amaxa Nucleofector® system (Lonza), using the program U-033. Briefly, 5 x10^7 logarithmic phase promastigotes suspended in Human T Cells Nucleofector (Lonza) were transfected with 10 µg DNA. Parasites were immediately mixed with 1 mL of culture medium and grown at 26 °C without selective drug for 24 h. Antibiotic selection of stable transfectants by incremental exposure to geneticin (G418) was initiated at 20 µg mL^{-1} and continued up to a final concentration of 400 µg mL^{-1}. Additionally, the pTEXCβSeGFP (carrying the *L. braziliensis CβS* partial gene) was also inserted within *T. rangeli* by electroporation as described above.

Overexpression of CS and CβS genes in *L. braziliensis*

The *CS* and *CβS* genes from the *L. braziliensis* strain were amplified by PCR as described above. The amplified DNA fragments were first cloned in pGEMTEasy vector and then subcloned into pTEXeGFP shuttle vector in the EcoRI/EcoRV sites to create pTEXCSeGFP or pTEXCβSeGFP constructs containing the neomycin phosphotransferase gene (G418) antibiotic marker. All constructs were confirmed by DNA sequencing. Each construct was transfected into promastigotes of *L. braziliensis* by electroporation as described above. The transfectants were selected for resistance to G418 (initiating with 10 µg mL^{-1}); growth of stable transfectants highly resistant to G418 (60 µg mL^{-1}) was observed after 60 days. Stable transfectants were harvested at the exponential and late log phase for protein extraction as well as *in vitro* oxidative and nitrosative susceptibility assays as described above. Transfectants with the pTEXeGFP vector alone and wild type parasites were used as controls.

Susceptibility of intracellular amastigotes to pentavalent antimony

Susceptibility assays were performed using differentiated human THP-1 macrophages infected with transfected *L. braziliensis* as described above, however, using 8 x 10^5 cells/well of 8-well chamber slides (Nunc) and a parasite-to-cell ratio of 10:1. Cultures were treated with Glucantime (Sb^V antimony) over a dose range from 8 to 256 µg mL^{-1} for 72 h at 34 °C, 5% CO_2 with one change of drug-containing medium at 48 h. After the medium was removed, the cells were washed twice in PBS and subsequently fixed with methanol and stained with Giemsa. A drug-free control was included in all assays. The percentage of infected cells and the number of intracellular amastigotes (burden parasite) were determined by random counting of 300 cells per well under a 100X objective microscope (Olympus IX70). Susceptibility was expressed as percent of viability determined by comparing parasite burden of infected cells exposed to Sb^V versus that of infected cells without Sb^V. The IC_{50} value was estimated from two independent experiments by a sigmoidal regression analysis with normalized response -- variable slope using GraphPad Prism v.5.0.

Statistical analysis

All experiments were performed in duplicate or triplicate, and the results are presented as the mean and the standard deviation (SD) or standard error of the mean (SEM). Normalised data were analysed by a one-way or two-way ANOVA followed by Bonferroni post-tests, as indicated in

the figure legends, and correlation analyses using the software GraphPad Prismv.5.0.

Results

L. braziliensis has putative genes encoding for CS and CβS

The search of *L. braziliensis* genome allowed identification of genes encoding CS (LbrM.35.3820) and a partial gene sequence for CβS (LbrM.17.0230) present in single copy in the MHOM/BR/75/M2904 strain. After cloning and sequencing, the *L. braziliensis* CS gene (*LbrCS*) was found to encode a protein of 333 amino acids (~35.4 kDa) that exhibited higher identities with CS of other protozoa, namely *L. major*, *L. infantum*, *L. donovani* (90-91%) and *T. cruzi* (75%), but lower with *T. vaginalis* and *E. histolytica* (50%), when compared to other CS orthologous from plant and bacteria (~50-55%). Analysis of the predicted amino acid sequences confirmed LbrCS has the four lysine residues (Lys^{40}, Lys^{51}, Lys^{67}, Lys^{199}) shown to be important for the catalytic activity of enzyme and the consensus sequence for the putative cofactor pyridoxal phosphate-binding domain (PXXSVKDR) is highly conserved. On the other hand, LbrCS has the positively charged residues (Lys^{222}- His^{226}- Lys^{227}) involved in binding with Serine Acetyl-transferase (SAT) and a short β8-β9 loop which is important for access to the active site (Supplementary Figure S1).

The *L. braziliensis* CβS partial gene (*LbrCβS*) from M2904 strain predicts a 120 amino acid (~12.7 kDa) protein that revealed high sequence identities with CβS from *L. infantum*, *L. donovani*, *L. major* (89%), *T. rangeli* (71%), *T. cruzi* (70%) and *T. brucei* (69%), compared to the human CβS (54%). Multiple sequence alignment confirmed LbrCβS contains two out of four lysine residues (Lys^{42}, Lys^{53}) required for CS activity and the consensus sequence for the putative cofactor pyridoxal phosphate-binding domain is highly conserved. LbrCβS as well as CβS from other trypanosomatids differs from *H. sapiens* CβS (HsCβS) by lacking the heme-binding (redox sensor) and oxidoreductase motifs (Cys XX Cys) at the N- and C-terminal, respectively (Supplementary Figure S2).

Stage-specific modulation of CS and CβS in *L. braziliensis*

To examine whether protein expression and activities of CS and CβS are stage-specific, the relative abundance and specific activity of these proteins were evaluated in soluble protein extracts from promastigotes and intracellular amastigotes of *L. braziliensis.* Both the protein

expression and activity of CS were 1.3-1.7 times higher in the lysate of intracellular amastigotes than those in promastigotes. In contrast, the protein expression and activity of CβS showed to be more abundant (1.5-1.7 times) in the extracts from promastigotes than from amastigotes (Figure 1).

Addition of H_2O_2 and SNAP increased the protein expression levels and activity of CS and CβS in *L. braziliensis* promastigotes

Expression protein level and specific activity of the CS and CβS were investigated in soluble extracts from promastigotes of *L. braziliensis* exposed to H_2O_2 (550 µM) and SNAP (195 µM). A time-dependent increase protein expression and activity of CS was observed in the extracts obtained from parasites exposed to both H_2O_2 and SNAP. Interestingly, significant increases of 2.0-2.3 fold in protein expression levels and specific activity was observed within the first 2 h, and at 4 h, values were below those observed at one hour of exposure to H_2O_2 (Figure 2A and 2B). In contrast, when parasites were exposed to SNAP, a significant 1.8 fold increase was only detected at 4 h post treatment (Figure 2A and 2B). Additionally, we detected a significant 1.51 fold increase in protein expression levels of CβS at 2 h in response to oxidative stress, whereas under nitrosative stress, the highest protein level was observed at 4 h. The increase in protein expression levels of CβS under both stresses were lower than those observed in CS (Figure 2A i and 2Aii). Furthermore, during exposure to H_2O_2 and SNAP, the specific activity of CβS showed slight increases without significant variations over the time course (Figure 2B).

Total intracellular thiols content was altered in response to oxidative and nitrosative stress in *L. braziliensis* promastigotes

Comparative analysis of total thiols content in *L. braziliensis* under oxidative and nitrosative stress revealed changes in treated parasites versus untreated control. In promastigotes exposed to H_2O_2, total thiols increased slightly after the first hour of exposure (1.53 fold), but showed a significant increase (2.25 fold) after 2 h; followed by a 0.72 fold decrease when compared to untreated control (Figure 2C). However, during the first hour of exposure to SNAP, we observed an increase of total thiols of 2.13 fold with a progressive decrease from 9.2 to 6.35 nmol $(10^8 \text{ cells})^{-1}$ after 4h of treatment, remaining even higher when compared to the untreated control 3.79 nmol $(10^8 \text{ cell})^{-1}$ (Figure 2C).

Functional rescue of CS activity with *LbrCS* and heterologous expression of *LbrCβS* in *T. rangeli*

In order to investigate the activity of LbrCS *in vitro*, we examined its ability to rescue the activity of CS in a CS-deficient parasite like *T. rangeli*. As expected, introduction of a plasmid containing *LbrCS* gene in epimastigotes of *T. rangeli* allowed rescue the activity of CS. The results revealed *T. rangeli* carrying pTEXCSeGFP not only expressed CS protein, but also showed higher activity equivalent to that observed in lysates from wild type promastigotes of *L. braziliensis* used as control (0.072 µmol min^{-1} mg $protein^{-1}$) (Figure 3A, 3Bi and 3Ci). Additionally, in *T. rangeli* transfected with pTEXCβSeGFP, we observed a significant increase (1.62 fold) in the CβS activity compared with wild type strain or with the parasites carrying the empty vector (Figura 3A, 3Bii and 3Cii).

Susceptibility of *T. rangeli* transfect with pTEXCSeGFP and pTEXCβSeGFP to H_2O_2 and SNAP *in vitro*

In order to evaluate the susceptibility of transfected *T. rangeli* parasites to oxidative or nitrosative stress, epimastigotes were exposed to different concentrations of H_2O_2 and SNAP for 48h and the parasite viability under this exposure was assessed. *T. rangeli* epimastigotes overexpressing *LbrCS* showed a 2.42-fold increase in their IC_{50} value in response to H_2O_2 when compared to the wild type and pTEXeGFP parasites (Table 1). Interestingly, parasites overexpressing CβS protein were also more resistant (2.3 times) to H_2O_2 than wild type parasites (Table 1). The dose-response curves showed a marked resistance to H_2O_2 between *T. rangeli* wild type and mutant strains at the lower concentration, where clear differences were observed from 100-150 µM of H_2O_2. However, the major difference in the percentage of viability was observed at 150 µM H_2O_2 with a 45% increase in the viability of mutant parasites when compared to the wild type parasites. In contrast, parasite viability at higher H_2O_2 doses (>300 µM) was inhibited in all *T. rangeli* strains (Figure 3Di). Unlike to H_2O_2, CS or CβS transfected parasites did not confer protection against nitrosative stress (Figure 3Dii) and showed the IC_{50} values after exposure to SNAP were quite similar between transfected parasites and WT control (Table 1).

Protein expression and activity characterization of *L. braziliensis* overexpressing LbrCS and LbrCβS

To evaluate whether the overexpression of CS and CβS proteins in *L. braziliensis* results in increased tolerance to oxidative stress, we initially

generated stable promastigotes overexpressing LbrCS and LbrCβS using the episomal vector pTEXeGFP carrying the genes *LbrCS* and *LbrCβS* and analyzed the response of transfected promastigotes overexpressing CS or CβS proteins to H_2O_2 and SNAP treatment. The relative abundance and specific activity of CS and CβS in the parasites carrying pTEXCSeGFP or pTEXCβSeGFP was evaluated in *L. braziliensis* promastigote forms by western blotting and activity assays, showing significant differences between the mutant lines and wild type or empty vector carrying parasites (Figure 4A, 4Bi-ii, 4C). We observed a 2.02 fold increase in protein expression level in parasites carrying pTEXCSeGFP, and in pTEXCβSeGFP parasites, this increase was 1.72 fold when compared to wild type and empty vector carrying parasites. In addition, the CS and CβS activity was verified using soluble extracts from overexpressing parasites; as expected, the specific activity of CS or CβS was found to be significantly increased in overexpressing parasites (Figure 4C). As shown in Figure 4Ci, the pTEXCSeGFP transformant parasites showed 2.7-fold higher CS activity, and in pTEXCβSeGFP parasites, a 2.0-fold higher CβS activity was revealed when compared to the control parasites (Figure 4Cii).

Overexpression of LbrCS and LbrCβS increases survival against oxidative stress in promastigotes of *L. braziliensis*

LbrCS and LbrCβS overexpression showed significantly higher resistance against H_2O_2 but not SNAP (Figure 4Di and Dii). Under oxidative stress, promastigotes overexpressing LbrCS and LbrCβS showed a 2.42 and 2.7 fold increase in their IC_{50} value respectively, compared to the wild type and pTEXeGFP parasites (Table 1). As shown in Figure 4Di, the dose-response curves showed a quite similar profile for both overexpressing parasite lines, suggesting a decrease in the susceptibility to H_2O_2 at lower doses. This was particularly apparent for parasites overexpressing CS, in which the percentage of viability remained higher (88%) even at 1000 µM of exposure when compared to the pTEXeGFP viability value of 47% and to the wild type parasites of 32%. We found a direct relationship between LbrCS overexpression, increased activity and IC_{50} viability values with H_2O_2 exposure. This finding was supported by the high correlations seen between the CS expression or specific activity when compared to the IC_{50} parasite viability value under H_2O_2 treatment ($r= 0.91$; $p= 0.0018$ and $r= 0.90$; $p= 0.0026$, respectively). A clear positive correlation was also found between overexpression or specific activity of LbrCβS with IC_{50} viability value to H_2O_2 exposure, as evidenced by the high correlation

coefficients obtained (r= 0.94; p= 0.0005 and r= 0.77; p= 0.02, respectively). In contrast, there was no detectable correlation between CS and CβS activities and the parasite susceptibility values to nitrosative stress treatment (data not shown).

Decreased susceptibility to Sb^{III} by overexpression of LbrCS and LbrCβS in *L. braziliensis*

Our analyses of dose-response curves suggested that viability percentage of overexpressing LbrCS and LbrCβS strains only was reduced by 3% at 2 µg mL^{-1} of Sb^{III} treatment. However, the main difference between overexpressing strains and controls was observed at a dose of 7 µg mL^{-1} with a reduction in the percentage of viability of 18% and 32.5% for pTEXCSeGFP and pTEXCβSeGFP transfected parasites, respectively. In the controls pTEXeGFP and WT parasites, these values were between 50-65% (Figure 5A). Interestingly, parasites overexpressing LbrCS showed a 43% reduction in the percentage of viability at 20 µg mL^{-1} dose concentration of Sb^{III} (Figure 5A). In addition, we found an increase in IC_{50} values in the overexpressing LbrCS and LbrCβS *L. braziliensis* parasites treated with Sb^{III}, where promastigotes overexpressing LbrCS were 4.8 fold more resistant to Sb^{III} exposure when compared to the wild type parasites, increasing their tolerance from 4.8 to 23.2 µg mL^{-1} (Table 2).

Decreased susceptibility to Glucantime (Sb^{V}) by overexpression of LbrCS and LbrCβS in *L. braziliensis*

In the light of above results, we evaluated whether overexpression of CS and CβS also could modulate the Sb^{V} susceptibility profile in intracellular amastigotes, which is the clinically relevant stage of the parasite. THP-1 derived macrophages were infected with stationary-phase WT or transfected promastigotes carrying the empty or CS/ CβS vectors and then subjected to Sb^{V} treatment for 72h. As predicted, the sensitivity patterns of transfected parasites exposed to Sb^{III} and Sb^{V} were similar. Results demonstrated pTEXCSeGFP and pTEXCβSeGFP transfectants amastigotes displayed lesser percentage of viability reduction in response to Sb^{V} as compared with WT or pTEXeGFP amastigotes even in the higher dose tested (256 µg mL^{-1}) (Figure 5B). Then, when the infected macrophages were exposed to 32 µg mL^{-1} dose of Sb^{V} (close to IC_{50} intracelluar value for the WT strain), the results seen in Figure 5Ci clearly showed the overexpressing CS parasites exhibited a significant increase in the percentage of parasite survival (78.3%) when compared to the WT or pTEXeGFP (∽41%). This

difference in parasite survival was evidenced by the increase in the number of amastigotes per macrophage after treatment (Figure 5Cii). Similarly, the IC_{50} values increased 3.7 and 4.7 fold in intracellular amastigotes overexpressing LbrCβS and LbrCS, respectively (Table 2).

Discussion

In the present study, we have reported for the first time that CS and CβS play an important role in protecting *L. braziliensis* against oxidative stress as demonstrated by genomic, biochemical and functional analyses. Bioinformatics analyses established *L. braziliensis* contains single copies of genes coding for the CS and CβS key enzymes in the *de novo* and RTS L-cysteine biosynthetic pathways. Although these enzymes are evolutionarily related to human CβS, our genomic analyses showed LbrCS and LbrCβS lack the catalytically essential heme-binding site and regulatory carboxyl terminal domain typically found in human CβS [34-36].

Additionally, we found LbrCS possesses the SAT binding domain described in other CS from plants and type CS A from bacteria and Old World *Leishmania* spp.; however, it is missing the related CβS enzymes from humans [23,37,38]. The SAT binding domain is important to form the enzymatic complex CS-SAT, which has been reported to have an antagonist regulatory functional role in these enzymes, whereas SAT is required to be bounded to CS for their activity and CS-SAT complex formation outcomes in the CS inhibition [23,39,40]. Crystallography and biochemical studies of CS from Old World *Leishmania* have confirmed the interaction between SAT and CS to form the regulatory complex CS-SAT and also allowed the design of peptides based on the C-terminus of SAT as possible selective inhibitors for the CS enzyme [24,41,42].

Biochemical analysis confirmed that, similar to other trypanosomatids, *L. braziliensis* has two pathways for generating cysteine and revealed for the first time that the CS and CβS are active in both stages of the parasite [23,43,44]. Additionally, our results demonstrated stage-specific protein levels and activity association for LbrCS and LbrCβS, where in the intracellular amastigote stage (mammalian form), LbrCS showed an increase in protein expression levels and activity. This same behavior was observed for LbrCβS in the promastigotes stage (insect-form). Our findings are consistent with proteomic studies conducted in axenic amastigotes of *L. panamensis*, in which CS protein expression levels have been found to increase exclusively in the axenic amastigotes

form when compared to promastigotes [45]. Moreover, our results in *L. braziliensis* are in agreement with our previous studies in other intracellular parasite like *T. cruzi* as well as in other studies working with this parasite where epimastigotes (insect-form) have been demonstrated to exhibit significantly higher protein levels and activity of CβS [27,43,46].

On the other hand, in our previous findings working with *T. rangeli* as a model, no stage-specific modulation was found in CβS [27]. The stage-specific regulation of CS and CβS are likely due to an association between the cysteine biosynthetic pathways with the complex life cycle of *Leishmania* and *T. cruzi* parasites. For instance, *de novo* pathway via CS may occur predominantly in the intracellular mammalian form and reverse-transsulfuration (RTS) through CβS in the insect form. This observation is consistent with the fact that *T. rangeli* and *T. brucei*, parasites with unknown or without an intracellular mammalian host stage, only possess the RTS pathway for cysteine biosynthesis [27,47]. Whereas, in parasites with only mammalian hosts life cycles, such as *Entamoeba spp.*, solely possess *de novo* or assimilatory pathway [17]. In contrast, herein we reported for the first time both CS and CβS active pathways in the New World *L. braziliensis* species that is in agreement with others studies done in the Old World *L. major* species [23]. Interestingly, in *T. cruzi*, another intracellular parasite, the activity of these two enzymes has also been reported [27,43]. These findings reinforce the hypotheses of the possible association between the cysteine biosynthetic pathways with the complex life cycle, particularly for *Leishmania* spp. and *T. cruzi* parasites. The redundancy of having the two routes for the cysteine synthesis observed in *Leishmania* spp. and *T. cruzi* remains an interesting question to be resolved. In this sense, it has been proposed that it may be due to the availability of exogenous nutrients, which differs considerably between the invertebrate and mammalian hosts' parasite environments [23]. For instance, promastigotes reside in glucose rich and slightly alkaline environments but where amino acids are scarce in the sand fly vector alimentary tract, while in human macrophage phagolysosomes, amastigotes cope with an acidic surroundings where glucose is scarce and amino acids are abundant [48].

Furthermore, the presence of two active biosynthetic pathways for cysteine synthesis, especially in *Leishmania*, should be explained by the cysteine requirement of Trypanothione $T[SH]_2$ synthesis and others antioxidants thiols, necessary to endure the oxidative environment within the host macrophage [15,49]. Interestingly, we found increased

levels of protein expression and activity of LbrCS and LbrCβS leading to elevated levels of total thiols concentration in response to *in vitro* oxidative and nitrosative stress in *L. braziliensis.* These findings are in agreement with several studies that have clearly demonstrated an association between cysteine biosynthesis and oxidative and nitrosative stress. In this sense, in bacterial upon oxidative stress, an increase of cysteine and GSH levels has been shown by induction of the expression of cysB regulon genes [50,51]. Similar findings have been reported in amoeba parasites when treated with nitric oxide, where a correlation between the increased in CS mRNA and higher CS activity has been found upon exposure to sodium nitroprusside (SNP) [52]. Additionally, the increased activity of CS and SAT has been found to be positively correlated with cysteine synthesis in arsenic tolerant plants, and also the major thiol glutathione (GSH) was found to increase in response to oxidative stress upon exposure to As^V [53,54].

Likewise, the transsulfuration pathway has also been considered as an alternative source for supplying redox potential to the cells under oxidative stress [55]. Indeed, metabolic labelling studies have revealed an increment in the level of cystathionine formation catalysed by CβS, which in turn lead to an increase in glutathione levels in human hepatic cells [55,56]. Consistently, in human lens epithelial cells and astrocytes, both the expression and activity of CβS were gradually induced after exposure to increased H_2O_2 concentration and also after exposure to an inductor of oxidative stress, such as tert-butylhydroperoxide [57,58]. Our findings in the regulation of LbrCS and LbrCβS have established the first report of the activation of these enzymes under oxidative and nitrosative stresses. However, the underlying molecular mechanism of this regulation remains unclear and further studies are needed to investigate these associations.

In order to better understand the role of the CS and CβS under stress conditions, the functional characterisation of *LbrCS* and *LbrCβS* genes was undertaken using a genetic complementation assay to rescue the CS activity and heterologous protein expression of CβS in *T. rangeli*. This parasite was selected to study the functional role of LbrCS, since we recently demonstrated *T. rangeli* does not have the *de novo* pathway for cysteine biosynthesis, possibly due to the presence of a stop codon on the *CS* gene sequence and also because no CS activity in the different evolutionary forms of the parasite was found [27]. Another important characteristic in this parasite is associated with their higher oxidative stress susceptibility and lower total thiols content found in the epimastigotes form when compared to *T. cruzi* [27]. Our functional

characterization results allowed the rescue of the CS activity in *T. rangeli* and showed epimastigotes expressing CS have an increased survival after exposure to increasing concentrations of hydrogen peroxide when compared to WT epimastigotes. Similarly, the LbrCβS heterologous expression studies showed that overexpression of CβS also confers resistance to oxidative stress in epimastigotes overexpressing this protein. Taken together, these results indicate not only that *T. rangeli* is a valid model for functional studies, but also highlights the significant role of LbrCS and LbrCβS in response to oxidative stress but not to nitrosative stress.

The protective relationship between higher protein and activity levels of LbrCS and LbrCβS and parasite survival under stress conditions was further demonstrated by the enhanced ability of *L. braziliensis* overexpressing LbrCS and LbrCβS to resist oxidative stress when compared to the control parasites. These data support the above findings in *T. rangeli* expressing LbrCS and LbrCβS, in which we observed an enhance parasite survival under oxidative and nitrosative stresses and provide strong argument that these proteins may play a major role in the defense of *L. braziliensis* against oxidative stress. Interestingly, our functional results are consistent with previously studies in amoebas, where CS overexpression and increased activity enhanced resistance to oxidative stress when exposured to hydrogen peroxide [22]. Likewise, overexpression of CS in transgenic plants also has been reported to induce an increased tolerance to oxidative stress generated by metals such as cadmium or herbicides like methyl viologen (paraquat) [59-61].

Unfortunately, there are few available data on overexpression of CβS and its role under oxidative stress; it is worthy to mention that our study is the first to shed new light on this function of CβS in *L. braziliensis.* In this sense, we demonstrated CβS overexpression may provide an advantage to parasite survival under conditions of oxidative stress. Recently, overexpression of CβS and CSE (cystathionine γ-lyase) has been shown to allow an increase in cysteine production. Additionally, normalized H_2S generation lead to inhibition of NADPH oxidase expression, which prevents the O2•- generation and consequently the oxidative damage in mouse glomerular mesangial cells [62]. Furthermore, other functional studies have reported that using aminooxyacetate (AOAA) and D, L-propargylglycine (PAG) to inhibit CβS and CSE, respectively, or siRNA-mediated protein knockdown increased cellular death induced by oxidative stress in mesenchymal progenitor cells [63].

The ability of the LbrCS and LbrCβS transfectants to withstand oxidative stress better than wild-type or empty vector transfectants raised the question as to whether they would also survive other oxidative pressure, such as those induced by antimonial compounds [11,12,64]. Our results showed a dose-dependent response in the decrease parasite susceptibility to Sb^{III} exposure, evidenced by the increase in IC values observed in both LbrCS and LbrCβS transfectant parasites. These findings were supported by the positive correlation found between the expression or specific activity of CS and CβS when compared to the parasite survival after exposure to Sb^{III}.

Having found that promastigotes overexpressing LbrCS and LbrCβS were more tolerant to Sb^{III}, we assessed whether overexpression of these enzymes increases tolerance in intracellular amastigotes in THP-1 human macrophages exposed to Glucantime® (Sb^{V}). Our findings clearly demonstrated that, in amastigotes overexpressing LbrCS and LbrCβS, there was a significant loss of susceptibility to Sb^{V} treatment. Correlation analysis also corroborated a relationship between protein expression and activity of these enzymes with the parasite survival after exposure to Sb^{V} (data not shown). Together, our data suggest the increased tolerance to antimonial compounds mediated by CS and CβS enzymes observed may be due to increased activities that lead to the production of cysteine to support the needs for glutathione and trypanothione synthesize required for detoxification of antimony [29,65].

As demonstrated in other works, increasing thiols levels has been considered one of the major mechanisms for Sb^{III} detoxification observed in laboratory resistant *Leishmania* lines [66]. For instance, in *L. amazonensis* promastigotes laboratory resistant to Sb^{III}, significantly increased levels in cysteine, glutathione and trypanothione have been reported, while in *L. infantum* Sb^{III} resistant lines, only cysteine levels showed a significant increase [67,68]. In addition, in clinical isolates of *L. donovani*, GSH and $T[SH]_2$ thiols levels have been found to be significantly lower in sensitivity compared to resistant lines [69]. Surprisingly, and contrary to these findings, another study has demonstrated that there were no differences in the $T[SH]_2$ levels between sensible and resistant clinical isolates, although there was a significant increase in levels of cysteine and glutathione in the resistant isolates [70]. These authors explained that $T[SH]_2$ levels were not increased in the resistant isolates, most likely due to increased efflux of this thiol by conjugation with Sb^{III} that has also been reported in *L. donovani* [65]. Similarly, some studies have shown a clear increase in

the expression of others genes, such as γ-glutamylcysteine synthetase, ornithine decarboxylase, trypanothione synthetase, trypanothione reductase and spermidine synthase, are involved in the glutathione and trypanothione synthesis pathways in both antimony-resistant clinical isolates and laboratory induced lines [68,70]. In summary, these reports, together with our findings, demonstrate the complex regulation on the thiols biosynthetic pathways where cysteine may be crucial in this regulation.

Consistent with this statement, *in vitro* studies using *L. tarentolae* and *L. infatum* laboratory resistant parasites lines have shown an increased expression of S-adenosyl homocysteine hydrolase gene (SAHH) to be involved in the conversion of S-adenosyl homocysteine homocysteine, as well as an important precursor of cysteine in the RTS pathway [67,71]. Additionally, in naturally resistant Sb^V clinical isolates, an increase expression levels of genes coding for a CβS were observed, which reinforces the hypothesis that cysteine can be involved directly or indirectly in resistance phenotypes [33]. The enhanced tolerance to antimonial compounds (trivalent and pentavalent Sb form) found in LbrCS and LbrCβS overexpressing promastigotes and amastigotes clearly demonstrated changes in the levels of these enzymes can induce alterations in the antimony susceptibility in *L. braziliensis* parasites and opens the question that this also may occur in other *Leishmania* species.

Since cysteine forms the basic building block of all thiols, our results showed that CS and CβS have an important role in *Leishmania* survival to oxidative stress conditions. Interestingly, we were able to clearly demonstrate that changes in the protein expression and activity levels of LbrCS and LbrCβS may be related to antimony efficacy. Overall, our findings, together with the fact that mammals lack the pathway for *de novo* biosynthesis of cysteine, make CS a good exploitable drug target where the SAT-binding domain represents an excellent candidate for the rational design of selective inhibitors in the New World *Leishmanias*. However, additional studies are required to confirm these suggestions.

Competing interests

The authors declare no competing interests.

Authors' contributions

IR and JT participated in the conception, design of the study and manuscript writing. MS and EG were involved in the conception of the study and manuscript development. All authors read and approved the final version of the manuscript.

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Table 1 Differential susceptibility of wild-type and pTEXeGFP, pTEXCS or pTEXCβS transfected parasite to H_2O_2 and SNAP challenge

		IC_{50} (µM)	
Parasite	**Cell line**	**H_20_2**	**SNAP**
T. rangeli	WT	66 ± 4	353 ± 10
	pTEXeGFP	69 ± 0.3	316 ± 17
	pTEXCSeGFP	160 ± 19**	402 ± 49
	pTEXCβSeGFP	153 ± 18**	345 ± 3
L. braziliensis	WT	560 ± 4	99 ± 13
	pTEXeGFP	558 ± 3	96 ± 3
	pTEXCSeGFP	1368 ± 7***	93 ± 6
	pTEXCβSeGFP	1551 ± 33***	80 ± 17

Results are presented as mean IC_{50} ± SEM, significant differences detected by one-way ANOVA, followed by Bonferroni's Multiple Comparison Test (**P < 0.01, ***P < 0.001).

Table 2 Susceptibility of *L. braziliensis* wild-type and pTEXeGFP, pTEXCβSeGFP or pTEXCSeGFP transfected cell line to Sb^{III} and Sb^{V}

Cell line	IC_{50} (µg mL^{-1})	
	Sb^{III}	Sb^{V}
WT	4.8 ± 1.1	28.4 ± 2.49
pTEXeGFP	6.7 ± 0.6	26.9 ± 0.15
pTEXCβSeGFP	12.4 ± 0.11***	88 ± 17.7***
pTEXCSeGFP	23.2 ± 0.21***	134 ± 2.35***

Results are presented as mean IC_{50} ± SEM, significant differences detected by one-way ANOVA, followed by Bonferroni's Multiple Comparison Test (***P < 0.001). IC_{50} values for antimonial drugs are presented in terms of µg/mL (1 µg/mL of Sb^{III} = 1.63 µM).

Table S1 Primers and plasmids used in the present study

Gene name	Plasmids	Plasmid characteristics	Specific Primers / annealing temperature
CβS	pTEXCβSeGFP	Containing a partial sequence of gene (LbrM.17.0230), 0.36kb. Modified with EcoRI/EcoRV restriction sites. Used for overexpression analyses.	CBSLbECOI 5′-GAATTCATGACTTCCGCTAATC-3′ CBSLbECOV 5′-GATATCGGCTCCGAGGGAC-3′ 58 °C
CS	pET21a-LbrCS	Containing 1.0 kb ORF of LbrM.35.3820. Modified with NdeI/BamHI restriction sites and modified with a C-terminal 6×His-tag. Using for protein expression.	CSLbFN (5′-CATATGGCGGCACCGTTTGA-3′) CSLbRB (5′-GGATCCTGCAGCTCCGAGGCA -3′) 62 °C
	pTEXCSeGFP	Containing 1.0 kb ORF of LbrM.35.3820. Modified with EcoRI/EcoRV restriction sites. Using for overexpression analyses.	CSLbECOI (5′-- GAATTCATGGCGGCACCG-3′) CSLbECOV (5′- GATATCTCAGTCCTGCAGCTCC-3′) 62 °C

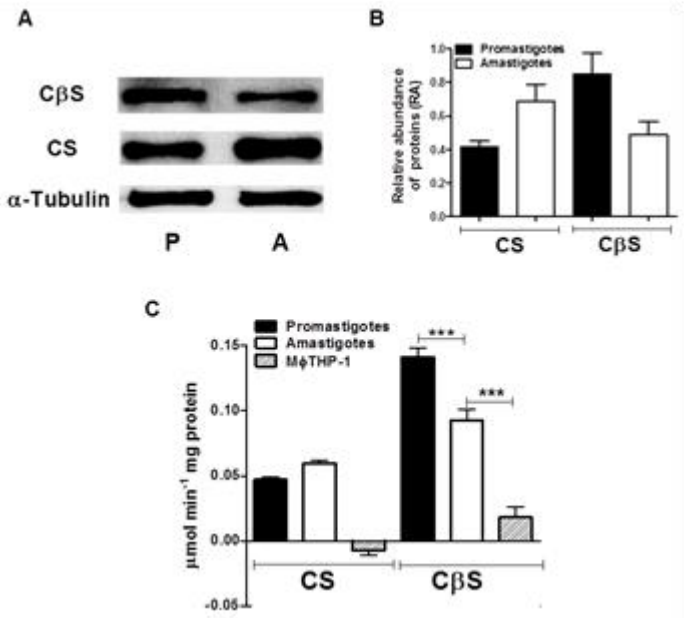

Figure 1. CS and CβS expression and activity in protein extracts of Leishmania braziliensis. (A). Western blot analysis of total extracts from promastigotes and amastigotes derived from human macrophages THP-1 (A). Equivalence in protein loading was controlled by immunodetection of α-tubulin. (B). Densitometric analysis of the CS and CβS expression using ImageJ. (C). CS and CβS activities were determined in soluble extracts of the parasite. Results represent the average of three independent experiments performed in triplicate ± SEM. Significant differences were determined by one-way ANOVA, followed by Bonferroni's Multiple Comparison Test (***P < 0.001). (P): promastigotes, (A): amastigotes

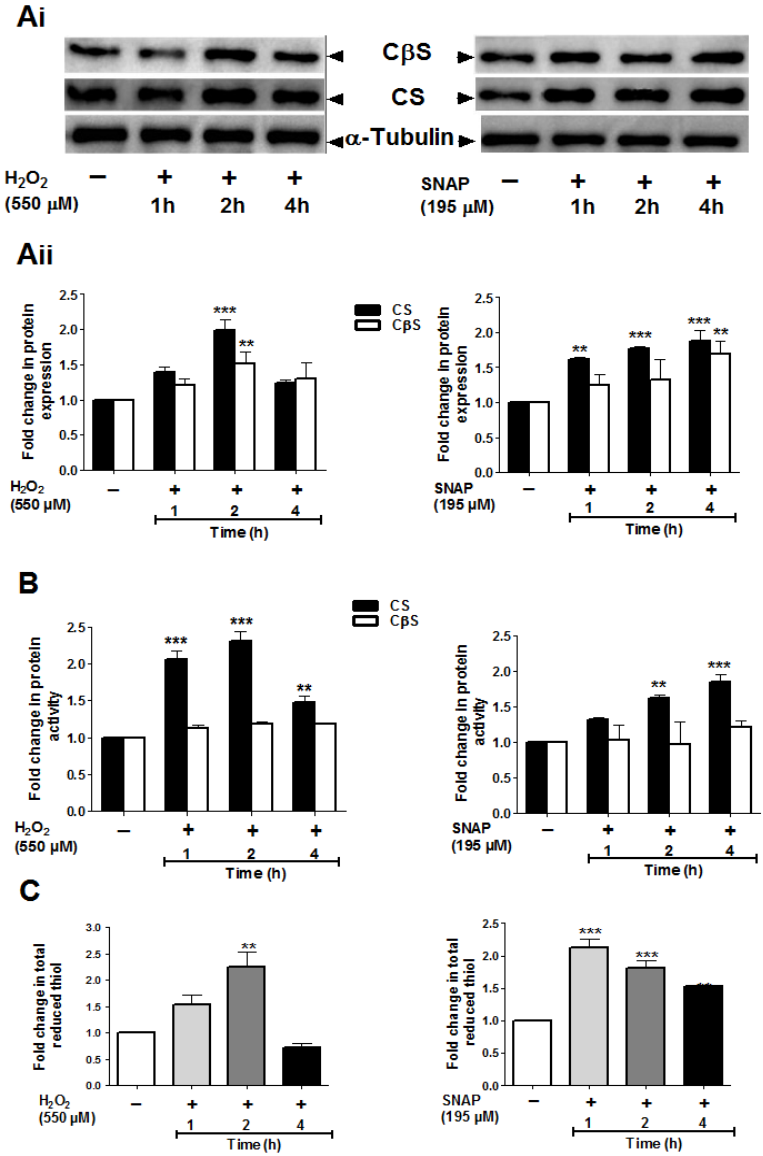
Ai
CβS
CS
α-Tubulin
H2O2 (550 μM)
SNAP (195 μM)
1h
2h
4h
Aii
Fold change in protein expression
CS
CβS
Time (h)
B
Fold change in protein activity
C
Fold change in total reduced thiol

Figure 2. Effect of oxidative and nitrosative stress on CS and CβS protein expression, enzymatic activity, and total reduced thiols levels in *L. braziliensis*. *(*Ai). Western blot analysis of soluble extracts obtained from promastigotes exposed to IC_{50} dose of H_2O_2 (550 µM) and IC_{50} dose of SNAP (195 µM). **(Aii).** Modulation of CS and CβS expression in *L. braziliensis* exposed to *in vitro* oxidative or nitrosative stress. Densitometric analysis of signals shown in panel A, was carried out with ImageJ **(B).** Changes of CS and CβS activity in promastigotes exposed to H_2O_2 and SNAP. The specific enzymatic activity was determined in soluble extracts from parasites. **(C)**. Total reduced thiol content (fold change) in promastigotes treated and untreated, the total thiol content was quantified using DTNB. Results represent the average of three independent experiments performed in triplicate ± SEM. Significant differences were determined by two-way ANOVA, followed by Bonferroni's Multiple Comparison Test (*P < 0.05, **P < 0.01, ***P < 0.001 compared to 0h). Fold change is the ratio of the untreated sample (Control with value 1) to treated samples.

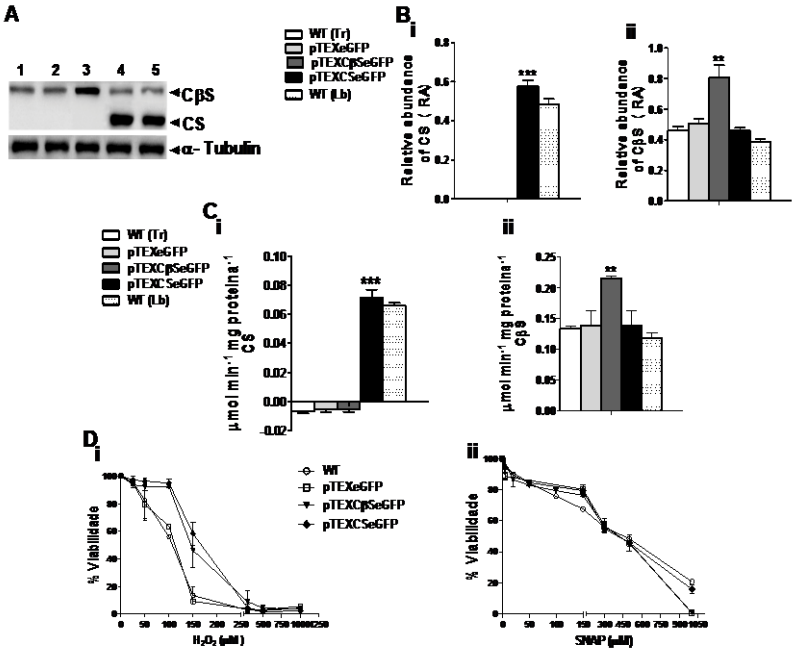

Figure 3. Functional rescue of CS activity and heterologous expression of *LbrCβS* in *T. rangeli*. (A). Western blot analysis of soluble extracts obtained from epimastigotes of *T. rangeli*. Line 1: WT; Line 2: pTEXeGFP; Line 3: pTEXCβSeGFP; Line 4: pTEXCSeGFP; Line 5: Positive control (promastigotes of *L. braziliensis*). **(B).** Densitometric analysis shows of fold change in CS **(Bi)** and CβS **(Bii)** expression determined by Western blot (Panel A). **(C).** Specific activity of CS **(Ci)** and CβS **(Cii)** was determined in total extract from transfected and WT parasites. **(D).** Susceptibility of transformed and WT *T. rangeli* epimastigotes after exposure to varying concentrations of H_2O_2 **(Di)** and SNAP **(Dii)** in triplicate wells, viability was assessed using Alamar blue (AB) assays. The results represent the average of three independent experiments performed in triplicate ± SEM. Significant differences were determined by one or two-way ANOVA, followed by Bonferroni's Multiple Comparison Test (*P < 0.05, **P < 0.01, ***P < 0.001 compared to WT).

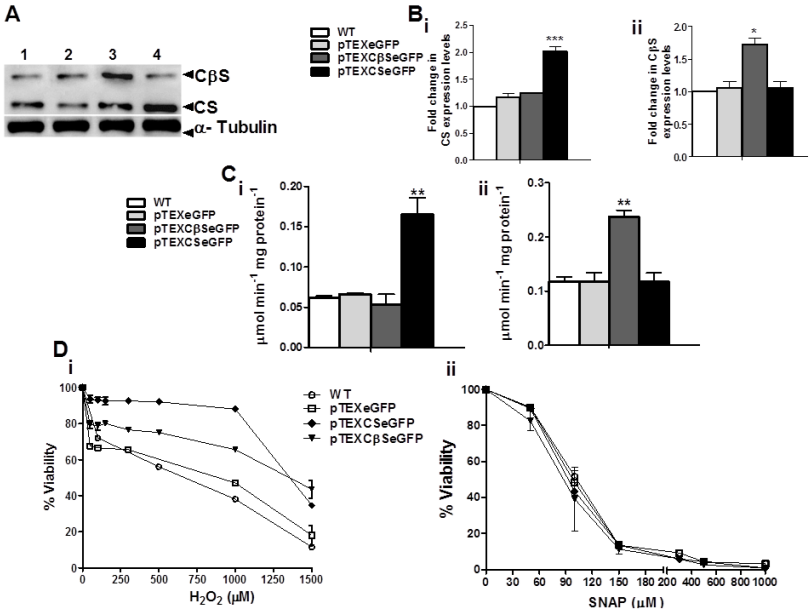

Figure 4. Functional characterization of CS and CβS in *L. braziliensis* transformed with pTEXeGFP, pTEXCSeGFP and pTEXCβSeGFP. *(*A). Western blot analysis of soluble extracts obtained from promastigotes of *L. braziliensis*. Line 1: WT; Line 2: pTEXeGFP; Line 3: pTEXCβSeGFP; Line 4: pTEXCSeGFP. **(B).** Densitometric analysis shows of fold change in CS **(Bi)** and CβS **(Bii)** expression determined by Western blot (Panel A). **(C).** Specific activity of CS **(Ci)** and CβS **(Cii)** was determined in total extract from transfected and WT parasites. **(D).** Susceptibility of transformed and WT promastigotes after exposure to varying concentrations of H_2O_2 **(Di)** and SNAP **(Dii)** in triplicate wells, viability was assessed using Alamar blue (AB) assays. The results represent the average of three independent experiments performed in triplicate ± SEM. Significant differences were determined by one or two-way ANOVA, followed by Bonferroni's Multiple Comparison Test (*P < 0.05, **P < 0.01, ***P < 0.001 compared to WT).

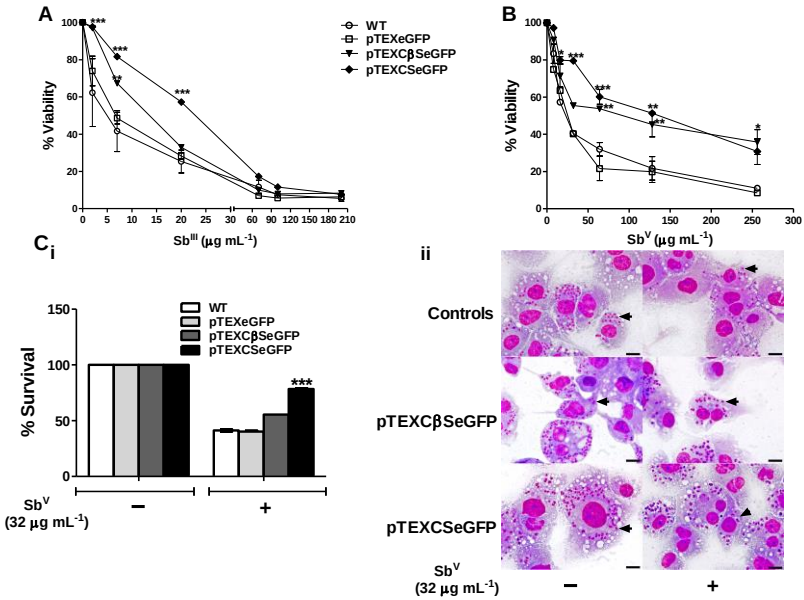

Figure 5. Effects of antimonial drugs on viability of *Leishmania braziliensis* overexpressing CS and CβS. Susceptibility to antimony was assessed in *L. braziliensis* WT and transfected cell line **A**. Dose-response curves in promastigotes treated with different doses of Sb^{III}. **B.** Dose-response curves in intracellular amastigotes treated with different doses of Sb^{V}. **Ci.** Intracellular survival of *L. braziliensis* amastigotes infecting THP-1 macrophages treated with Sb^{V} (32 µg/ mL)**. Cii.** Light micrograph of human macrophages THP-1 containing amastigotes (arrow) and treated with 32 µg/ mL of Sb^{V}. (Giemsa stain; scale bar = 20 µm). Results represent the average of three independent experiments performed in duplicate ± SEM. Significant differences were determined by one or two-way ANOVA, followed by Bonferroni's Multiple Comparison Test (***P < 0.001 compared to WT).

```
AtOASTL-Cyto   1   ------------------------------------------------------------
AtOASTL-plas   1   ------------------------------------------------MAATSSSAFLLNPLTSR---
AtOASTL-mito   1   ----------------------------------MVAMIMASRFNREAKLASQILSTLLGNRSCYTSMAATSSSALLLNPLTSSSSS

LbrCS          1   -----------MAAPFDTSK-------------------------------NVAESIDQLIGETPALVLNKLNH-TKAK---
LinfCS         1   -----------MAAPFDKSK-------------------------------NVAQSIDQLIGETPALVLNKLNN-TKAK---
LmjCS          1   -----------MAAPFDKSR-------------------------------NVAQSIDQLIGETPALVLNKLNN-TKAK---
TcCS           1   ----------MSVQEFDPRN-------------------------------NVAPSIDDLIGETPAVVLKKMND-TGAP---
EhCS           1   ----------MEQISINSPRK-------------------------------RIVHNILETIGETPLVSLHGVTEHPRIKKGTR
StCS A         1   ---------------MSK-----------------------------------IYEDNSLTIGETPLVLNRIGN---GR---
StCS B         1   ------------------------------------------------------MNTLEQIGETPLVKLQRLGPDNGSE----
TvCS           1   ------------------------------------------------------MHCNNILETIGETPLVRLNSLNPNPKVE----
AtOASTL-Cyto   1   ---------------MASR-----------------------------------IAKDVTEIIGETPLVYLNNVAEGCVGR----
AtOASTL-plas  17   --HRPFKYSPELSSLSLSSRKAAAFDVSSAAFTLKRQSRSDVVCKAVSIKPEAGVEGINIADNAAQLIGETPLVYLNNVVKGCVAS----
AtOASTL-mito  54   STLRRFRCSPEISSLSFSSASDFSLAMKRQSRSFADGSERDPSVVCEAVKRETGPDGINIADNVSQLIGETPLVYLNSIAKGCVAN----

LbrCS         37   IVLKMECENPMSSVKDRLALAIYDKAEKCGKLIRGKSIVVESTSGNTGVALAHIGAIRGYKVIITMPESMSIERRCLLRDFGAEVILTPA
LinfCS        37   VVLKMECENPMASVKDRLGFAIYDKAEKCGKLIPGKSVVVESSSGNTGVSLAHIGAIRGYKVIITMPESMSIERRCLLRDFGAEVILTPA
LmjCS         37   VVLKMECENPMASVKDRLGFAIYDKAEKCGKLIPGKSVVVESSSGNTGVSLAHIGAIRGYKVIITMPESMSIERRCLLRDFGAEVILTPA
TcCS          38   IVLKLECENPMASVKDRLAYAIYDKAEKCGKIIPGKSVIVESTSGNTGIALAHIGAIRGYKVIIIMPESMSIERRCLMRDFGAEVILTPA
EhCS          44   ILVKLEYPNPMSSVKDRLGFNIVYCAIKDGRLKPG-MEIIESTSGNTGIALCQAGAVGGYRVVIAMPSTMSVERMMIMKAFGAELILTEG
StCS A        28   ILKKMESRNPSFSVKGRLGANLIWDAEKRGWLKPG-VEIVESTSGNTGIALAIVAARGYKLTLTMPDNMSIERRALLKALGANLVLTEG
StCS B        27   IWVKLEGNNPAGSVKDRAALSMIVEAEKRGEIKPG-DALIESTSGNTGIALAMIAALKGYRMKLLMPDNMSIERRAAMRALGAELIVTK
TvCS          29   IWAKLEGFNPTGSVKDRIALKMIECAETEGKLKPGSSIIESTSGNTGIGLAMIGRTKGYSVIVVMSEAVSIERRQKMIKAFGEVILTDR
AtOASTL-Cyto  32   VAAKLEMMEPCSSVKDRIGFSMISDAEKKGLIKPGESVLIESTSGNTGVGLAFIAAKGYKVIITMPASMSIERRKILLRAFGAELVLTDP
AtOASTL-plas 102   VAAKLEIMEPCCSVKDRIGYSMITDAEEKGLITPGKSVLVESTSGNTGISLAFIAASKGYKVIITMPASMSIERRVLLRAFGAELVLTEP
AtOASTL-mito 140   IAAKLEIMEPCCSVKDRIGYSMVTDAEQKGFISPGKSVLVESTSGNTGISLAFIAASRGYRVIITMPASMSMERRVLLRAFGAELVLTDP

                                                                                                  ***β8***
LbrCS        127   ALGMKGAMAMAKKVVANPNAV-LAEQFATKYNALMHEETTGPEIWEQTHHSVDCFIAGVGTGGTLTGVGRFLKK--KVGSHAPIIAVEPV
LinfCS       127   ALGMKGAVAMAKKVVANPNAV-LADQFATKYNALIHEETTGPEIWEQTHNVDCFIAGVGTGGTLTGVGRFLKK--KMGSHAPIVAVEPT
LmjCS        127   ALGMKGAVTMAKKVVANPNAV-LADQFATKYNALIHEETTGPEIWEQTHNVDCFIAGVGTGGTLTGVGRFLKK--KMGSHAPIVAVEPM
TcCS         128   ALGMKGALEAAARVVSNPVAV-SANQFATKYNALIHEETTGPEIWEQTKGVDCFVAGVGTGGTITGVGRFLKK--SVGCGANIIAVEPA
EhCS         133   KKGMKGAIEEVNKMIKEPKKKEVANQFGNPDNTARHYT-AMEIWEDTDGEVDIVVSAVGTSGTVIGVAEKLK--EKKKGIKIIAVEPE
StCS A       117   AKGMKGAIQKAEEIVASDPQKYLLLQQFSNPANPEIHEKTTGPEIWEDTDGVDVFISGVGTGGTLTGVGRYIKSTKGKTDLITVAVEPT
StCS B       116   EQGMKGARDLAMSERGEGKLL--DQFNNPDNPYAHKTTTGPEIWEQTSGKIDFVSSMGTGTTIGVGRQLR--DQEKTVIIVGLQPE
TvCS         118   KLGMKGAIRKVAELVKENPAKYGNPMQFSNEYNKIAHKTTAEEIWQQTKGKVDFVAAVGTSGTLMGVGKNLR--EKNADIKIVEAQPT
AtOASTL-Cyto 122   AKGMKGAIAKAEEILAKTPNGY-MLQQFENPANPKIHEETTGPEIWKGTGKIDFVSGHGTGGTITGAGKYLK--EQNANVKLYGVEPV
AtOASTL-plas 192   AKGMKGAIQKAEEILKKTPNSY-MLQQFDNPANPKIHEETTGPEIWEDTRGKIDIIVAGHGTGGTITGVGRFIK--ERKPELKVIGVEPT
AtOASTL-mito 230   AKGMKGAVQKAEEILKNTPDAY-MLQQFDNPANPKIHEETTGPEIWDDTRGKVDIFVAGHGTGGTITGVGRFIK--EKNPKTQVIGVEPT

                          +   ++                  ***β9***
LbrCS        214   ESSVLSGG------KPGPHKIQGIGAGFVPAVLDRSLIDEVFCVSGDDAIETALKLTRSDGVCCGPSSGAVVAALRIAERPEMEGKIIV
LinfCS       214   ESSVLSGG------KPGPHKIQGIGFGFVPDVLDRSLIDEVLCVAGDDAIETALKLTRSDGVFLRSSCGAVVEALKQAERPEMEGKIIV
LmjCS        214   ESSVLSGG------KPGPHKIQGIGFGFVPDVLDRSLIDEVFCVAGDDAIETALKLTRSDGVFCGPSSGAVVAALKIAERPEMEGKIIV
TcCS         215   ESSVLSGG------KPGPHRIQGIGAGFVPEVFEAALVDEVIQVSGDDAIDTAQKLPRTDGIFCGPSSGAVVAALDIAKRPEMGGKIIV
EhCS         220   ESSVLEGK------AKGPHRIQGIGAGFIPDIYKKEVDEIIRIKTQDAVKMARAVVKYDGIMCGMSSGAAIIAALKQAKPEFEGKIIV
StCS A       207   DSSVIAQALAGEEIKPGPHKIQGIGAGFIPENIDLKLIDKVVGITNEEAISTARRMEEEGILAGISSGAAVAAALKQADEFTNKNIV
StCS B       202   ESSSIFG------------TRRWPTEYMPGIFNASLVDEVLDIHQNDAETVREIAVREGIPCGVSSGFAVGALRVARATPGA--IVV
TvCS         206   KGHYIQG------------LKSMEEAIVPAIYQADRIDEHILESEEAFAARERVIAKEGIFIGMSSGAAMIAALKVAEKLESG--VIV
AtOASTL-Cyto 209   ESAILSGG------KPGPHKIQGIGAGFIPSVINVDLIDEVVQVSSDDSIDMARQLALKEGLLVGHSSGAAAAAALKQARPEMGGKLIV
AtOASTL-plas 279   ESAILSGG------KPGPHKIQGIGAGFVPKNIDLAIVDEVIDISSEEAIETSKQLALQEGLLVGHSSGAAAAAALKVARPEMGGKLIA
AtOASTL-mito 317   ESDILSGG------KPGPHKIQGIGAGFIPKNIDGKIMDEVLDISSEEAIETAKQLALKEGLMVGISSGAAAAAALKVARPEMGGKLIA

LbrCS        298   TVIPSSGERYLSTVLYKNVRDEVSSLPVVDASELQD-
LinfCS       298   TSF-----RHSVSATSPPCCTGAFGMRCRPCP-----
LmjCS        298   TIIPSSGERYLSTVLYRSVRDEVSSLPVVDASELQD-
TcCS         299   TVIPSSGERYLSTALKSSIKDEVSALKVLSAADI---
EhCS         304   IIVPSCGERYLSTVLYKIKDEGTKIQILDSLLNE---
StCS A       297   VIIPSSGERYLSTALEADLPTEKELQQ----------
StCS B       277   AIICDRGERYLSTVLYGEEHFSQGAGI----------
TvCS         281   VLFADRGERYLSTKLEDTQ------------------
AtOASTL-Cyto 293   AIIPSSGERYLSTVLEDATRKEAEAMTFEA-------
AtOASTL-plas 363   VVIPSSGERYLSTVLEQSIREECEQMQPEL-------
AtOASTL-mito 401   VVIPSSGERYLSTALEQSIREEVEKMQPEV-------
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Figure S1. Multiple alignment of deduced amino acid sequences of CS from *L. braziliensis* and other representative organisms. The identity (black background) of the amino acid residues is shown. Red Box indicates the consensus amino acid residues of the putative pyridoxal phosphate-binding motif (PXXSVKDR); the lysine residues required for cysteine synthase activity are indicated with triangles. The β8–β9 loop at the entrance of the active site is indicated with an asterisk (*****), and the positively charged residues involved in binding with SAT are indicated with (+). LbrCS: *Leishmania braziliensis* (LbrM.35.3820); LmjCS: *Leishmania major* (LmjF.36.3590)*;* TcCS: *Trypanosoma cruzi* (Tc00.1047053507165.50); EhCS: *Entamoeba histolytica*; TvCS: *Trichomonas vaginalis* (XP001325874); StCS A: *Salmonella typhimurium* CysK (P0A1E4); StCS B: *Salmonella typhimurium* CysM (NP_456975).

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HsCBS     1   MPSETPQAEVGPTGCPHRSGPHSAKGSLEKGSPEDKEAKEPLWIRPDAPSRCTWQLGRPASESPHHPAPAKSPKIIPDIKKIGETPMV
ScCBS     1   ------------------------------------------------------------------------MKKSEQQADSRHNVIDLVGNTPLI
TcCBS     1   -----------MRRCGEWQPPSPPRNGCFHLLPFLFTLLLLLFMFPIGREDVQIMAQNPSAV-NNKKEVPDRRTCIIDTVDAIGETPCI
TraCBS    1   ------------------------------------------------------------MAQTHATD-CKGKEVPERQECIIDSVIDAIGETPCI
TbCBS     1   ------------------------------------------------------------------------------MKDQRTTIIDSVDTIGETPCI
LbrCBS*   1   ------------------------------------------------------------------------MTSANPHDHILSDAPEAVGNTPCI
LbrCBS    1   ------------------------------------------------------------------------MTSANPHDHILSDAPEAVGNTPCI
LmjCBS    1   ------------------------------------------------------------------------MASANPRNQILANAPEAVGNTPCI
LtaCBS    1   ------------------------------------------------------------------------MTSATPHDHILANAPEAIGNTPCI

HsCBS    91   RINKIGKKPGLKCELLAKCEFFNAGGSVKDRISLRNIEDAERDGTLKPG-DTIIEPTSGNTGIGLALAAAVRGYRCIIVMEEKMSSEKVD
ScCBS    25   ALKKIPKALGYKPQIYAKDEEYNPGGSIKDRIAKSMVEEAEASGRIEPSRSTLIEPTSGNTGIGLALIGAIKGYRTIITMPEKMSNEKVS
TcCBS    79   RINNVPKKHGVCCPVVAKCEFFNPGGSVKDRIAQMVLDAEASGRDRPN-QTIIEATSGNTGIGLSLVAAVKKYEMIITMPKKMSHEKEV
TraCBS   36   RINRPPQKHGVCCEVVAKCEFFNPGGSVKDRIAQMVLDAEASGRDRPN-SSLVEPTSGNTGIGLSMVAAVKGYEMVITMPKKMSHEKEV
TbCBS    23   RINRPPNMHGICCEVVAKCEFFNPGGSVKDRIALKMVLDAEASGRDPPN-SSLVEATSGNTGIGLSLVGSVRGPEVVITMPKKMSHEKEV
LbrCBS*  25   RINRVPQRHGICCEVVAKCEFFNPGGSVKDRIGKQMVADAEKGGTLKPG-SVIVEATSGNTGIGLSMAAAIRGYRIVITMPKKMSHEKET
LbrCBS   25   RINRVPQRHGICCEVVAKCEFFNPGGSVKDRIGKQMVADAEKGGTLKPG-SVIVEATSGNTGIGLSMAAAIRGYRIVITMPKKMSHEKET
LmjCBS   25   RINRVPQKHGIRCEVVAKCEFFNPGGSVKDRIGKQMVLDAEKGGTLKPG-SVIVEATSGNTGIGLSMAAAIRGYRMVITMPKKMSHEKET
LtaCBS   25   RINRVPQKHGIRCEVVAKCEFFNPGGSVKDRIGKQMVLDAEKGGTLKPG-SVIVEATSGNTGIGLSMAAAIRGYRMVITMPKKMSHEKET

HsCBS   180   VLRALGAEIVRTPTAARDDPESHVGVAMRLKNEIPNSHILDQYRNPSNPDAHYDTTADEILQCD-----GKLDMIVASVGTGGTITGI
ScCBS   115   VLRALGAEIIRTPTAAAWDPESHIGVAKKLEKEIPGVILDQYNMMNPDAHYPCTGPEIQRLEDLNLFDNLRAVVAGAGTGGTIISGI
TcCBS   168   TLRALGAEIRTETALPWDHPDSLIGVARRLEKEK-GYVLDQYRNPSNPKAHYEPTAQEIYDQCG-----GKVDMAVFTTGTGGTMAGV
TraCBS  125   VLRALGAEVIRTETALPWDHPESLIGVARRLEEEK-GYVLDQYKNPSNPKAHYEPTAQEIYDQCG-----GKVDMVVIGAGTGGTVAGV
TbCBS   112   VVRALGAEVIRTETSLAWDHPESLIGVARRLERER-GYVPLDQYRNPSNPGAHYESTGQEIYDQCG-----GKVDMVILGAGTGGTITGV
LbrCBS* 114   TVKSLGAEVIRTETALPWDHPDSLIGVARRLRDEK-GYVLLDQYSNPSNPGAHYEPTGQEIYQCG-----GKVDMVVISAGTGGTITGV
LbrCBS  114   TVKSLGA-------------------------------------------------------------------------------
LmjCBS  114   TLQSLGAEVIRTETSLPWDHPDSLIGVARRLRDEK-GYVLLDQYANPSNPGAHYEPTGQEIYDQCG-----GKVDMVVICAGTGGTITGV
LtaCBS  114   TLQALGAEVIRTETSLPWDHPESLIGVARRLRDEK-GYVLLDQYANPSNPGAHYEPTGQEIYDQCG-----GKVDMVVISAGTGGTITGV

HsCBS   265   ARKLKEKCPSCRIIGVDPEGSILAPPEELNQTEQTTYEVEGIGYDFPPVLDRIVVDKVMSKSNDEPRFTPARMLIAQEGLLCGGSAGSTV
ScCBS   205   SKVLKEQNDKIQIVGADPEGSILAPPENLNKTDITDYKVEGIGYDFVPQVLDRKLDDVMRKTDDKPSEKYARQLISNEGVLVGGSSGSAF
TcCBS   252   AKRLKELLPNIVIVGVDPEGSILADPS--VPLDPKPYLVEGIGYDFVPDVCERKYVDRWVKSADKESEALASEVHRPPALLVGGSSGAAM
TraCBS  209   SKRLKELLPNVIVVGVDPEGSILADPS--APASPTPYLVEGIGYDFVPSVCERPYVDRWVKSADKESEALASELHRPPALLVGGSSGAAM
TbCBS   196   AKKLKSLLPDVIVVGVDPEGSILADPAN-PPKDAKPYLVEGIGYDFVPDVCERPYVDKYVKSTDKESEALASQLHRPEGLLVGGSSGSAM
LbrCBS* 198   AKKLKELNPSITVVGVDPEGSILADPE--HVGEHVMYPVEGIGYDFVPDVCERKYVDRWVKTRDCESEDLAPELHRPEGLLVGGSSGSAM
LbrCBS  120   ------------------------------------------------------------------------------------------
LmjCBS  198   AKKLKELLPEVIVVGVDPEGSILADPE--HPGEPVMYPVEGIGYDFVPDVCERKYVDRWVKTRDCESEDLAPELHRPEGLLVGGSSGSAM
LtaCBS  198   AKKLKELLPEIIVVGVDPEGSVIADPE--HPCEPVMYQVEGIGYDFVPAVCERKYVDRWVKTRDQQSEDLARELHRPEGLLVGGSSGCAM

HsCBS   355   AVAVRAAQ---ELQEGQRCVVIIPDSVRNYMTKFLSDRYMLQKGFLKEPDLT-----------EKKPWWWPPRVQELGLSAPTVLPTI
ScCBS   295   TPVVKYCEDHPELTEPDVIVAIPDSIRSYLTKFVDDEALKKNNLWDDDVLARFDSSKLEASTTKYADVFGNATVKDLHLKPVVSVKPTA
TcCBS   340   WGVLCAAK---DLRPDQRCVVVPPDGIRNYMAKPPDKNWLVPAGLEEGE-----------VTRPTYGSIEACTEEAKKKLSEYPAK
TraCBS  297   WGVLCAAK---ELRPDQRCVVVPPDGIRNYMAKPPDKNWMIEHSLQEGE-----------VTRPTYDSILQACTEAAKKKLSKYPAK
TbCBS   285   WGVLCAAK---DLSPDQRCVVVPPDGIRNYMSKPPDVNWRIEKKLESGE-----------VTRPTYENLQAETECTRKKLAEYPAK
LbrCBS* 286   AGVLCAAK---DLRPDQRCVVLMADGIRNYMAKPADNNWMIEGFRECV-----------VTRPTYEALAKCTEEAKSKLAKYPSG
LbrCBS  120   ------------------------------------------------------------------------------------------
LmjCBS  286   AGVLCAAK---DLRPDQRCVVLMADGIRNYMCKPADVNWMIEGFRQGE-----------VTRPTYDLLKKETEEVKAKLAKYPSG
LtaCBS  286   AGVLCAAK---DLRPDQRCVVLMADGIRNYMCKPADVNWMIEGFRQGE-----------VTRPTYDLLKKETEEVKAKLAQYPSA

HsCBS   430   TCGHTIEILREKGFDQAPVVDEAGVILGMVTLGNMLSSLLAGKVQPSDQVGKVIYKQFKQIRLTDTLGRLSHILEMDHFALVVHEQIQYH
ScCBS   385   KVTDVIKILKDNGFDQLPVLTEDGKLSGLVTLSELLRKLSINN-SNNDNTIKGKYLDFKKLNNFNDVSSYNENKSGKKKFIKFDENSKLS
TcCBS   412   CGGSVEEGKRCCEKAK-----------------------------------------------------------------------
TraCBS  369   YGCNV---------------------------------------------------------------------------------
TbCBS   357   LGLIGK---------------------------------------------------------------------------------
LbrCBS* 358   AQ------------------------------------------------------------------------------------
LbrCBS  120   ------------------------------------------------------------------------------------------
LmjCBS  358   TQ------------------------------------------------------------------------------------
LtaCBS  358   AK------------------------------------------------------------------------------------

HsCBS   520   STGKSSQRQMVFGVVTAIDLLNFVAAQERDQK--
ScCBS   474   DLNRFFEKNSSAVITDGLKPIHIVTKMDLLSYLA
TcCBS   427   ----------------------------------
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Figure S2. Multiple alignment of deduced amino acid sequences of CβS from *L. braziliensis* and other representative organisms. The identity (black background) of the amino acid residues is shown. Red Box indicates the consensus amino acid residues of the putative pyridoxal phosphate-binding motif (PXXSVKDR), and other motifs vital for CβS activity are indicated with asterisks (**+**). The oxido-reductase motif of HsCβS is highlighted with (•). The lysine residues required for CS catalytic activity are marked with triangles. The positions of the heme-binding residues within the heme domain of the human CβS enzyme (Cys^{52} and His^{65}) are marked with (↓). HsCβS: Human (P35520); ScCβS: *Saccharomyces cerevisiae* (P32582); TcCβS: *Trypanosoma cruzi* (Tc00.1047053511691.20); TraCβS: *Trypanosoma rangeli*; TbCβS: *Trypanosoma brucei* (Tb11.02.5400); LbrCβS*: *Leishmania braziliensis* MHOM/BR/75/M2903 (LbrM.17.0230); LbrCβS: *Leishmania braziliensis* MHOM/BR/75/M2904 (LbrM.17.0230); LmjCβS: *Leishmania major* (LmjF.17.0250); LtaCβS: *Leishmania tarentolae* (LtaP17.0270).

5. DISCUSSÃO

O presente estudo constitui a primeira caracterização das vias de biossíntese de cisteína em *L. braziliensis e T. rangeli*, assim como o primeiro relato do importante papel protetor de CS e CβS de *L. braziliensis* sobre condições de estresse oxidativo, como demonstrado pelas análises genómicas, bioquímicas e funcionais. As análises bioinformáticas mostraram que *L. braziliensis* contém cópias únicas dos genes que codificam as duas enzimas CS e CβS, envolvidas na via *de novo* e RTS da biossínteses de L-cisteina, respectivamente, entanto que *T. rangeli* somente possui para CβS. Embora, estas enzimas (CS e CβS) estejam evolutivamente relacionadas com a CβS de humanos, nossas análises genômicas mostraram que LbrCS e LbrCβS não possuem o sitio cataliticamente essencial de ligação ao grupo heme ou o domínio carboxi-terminal regulador, normalmente encontrados na CβS de humanos (BANERJEE e ZOU, 2005; KOUTMOS et al., 2010; EREÑO-ORBEA et al., 2013). Além disso, nossos resultados demonstraram que LbrCS possui o domínio de ligação ao SAT descrito para outras CS em plantas, tipo CS A em bactérias e em *Leishmania spp* do Velho Mundo; no entanto, ausente na CβS de humanos (Figura S1 pag. 125) (WIRTZ et al., 2001; WILLIAMS, WESTROP e COOMBS, 2009; FELDMAN-SALIT et al., 2012). O domínio de ligação ao SAT é importante para formar o complexo enzimático CS-SAT, que tem sido referido como tendo um papel regulador antagonista funcional nestas enzimas, enquanto que SAT necessita estar unida a CS para a sua atividade formando o complexo CS-SAT, este complexo gera inibição da atividade de CS (CAMPANINI et al., 2005; WIRTZ e HELL, 2006; WILLIAMS, WESTROP e COOMBS, 2009). Estudos bioquímicos da cristalografia de CS de *L. major* e *L. donovani* confirmaram a interação entre SAT e CS, formando o complexo de regulação CS-SAT. Da mesma forma, estes estudos permitiram o desenho de péptidos com base no C-terminal de SAT como possíveis inibidores seletivos para a enzima CS (FYFE et al., 2012; RAJ, KUMAR e GOURINATH, 2012; SPYRAKIS et al., 2013).

As análises bioquímicas confirmaram que similar a outros tripanossomatídeos, *L. braziliensis* apresenta duas vias para a geração de cisteína, sendo ambas as enzimas (CS e CβS) ativas nas formas evolutivas do parasito promastigotas e amastigotas (NOZAKI et al., 2001; WILLIAMS, WESTROP e COOMBS, 2009; GIORDANA et al., 2014). Nossos resultados demonstraram uma associação estado-específica dos níveis de proteínas e atividade para LbrCS e LbrCβS,

onde na forma amastigota intracelular (forma evolutiva no hospedeiro mamífero), LbrCS mostrou um aumento nos níveis de expressão de proteína e na atividade específica da enzima. Este mesmo comportamento foi observado para LbrCβS mas na forma promastigota (forma evolutiva no inseto vetor). Estes resultados são consistentes com estudos de proteômica realizados em amastigotas axênicas de *L. panamensis*, nos quais foi encontrado que os níveis de expressão da proteína CS estavam aumentados exclusivamente na forma amastigotas axênica quando comparado com promastigotas (WALKER et al., 2006). Além disso, os resultados obtidos com *L. braziliensis* são coincidentes com aqueles obtidos em *T. cruzi*, outro parasito intracelular, onde os níveis mais altos de expressão e atividade da proteína CβS foram encontrados em epimastigotas (forma evolutiva no vetor) (ROMERO et al., 2014). Esse tipo de resposta tem sido demonstrado por outros autores para a CβS de *T. cruzi* (NOZAKI et al., 2001; MARCIANO, SANTANA e NOWICKI, 2012) embora, contrastam com nossos resultados em *T. rangeli*, nos quias nenhuma associação estágio-específica foi encontrada para esta proteína (ROMERO et al., 2014).

A regulação estágio-específica de ambas as enzimas (CS e CβS) em *Leishmania* e *T. cruzi* pode estar provavelmente associada com o complexo ciclo de vida destes parasitos, onde a via *de novo* através da regulação de CS, pode estar associada predominantemente na forma intracelular no hospedeiro mamífero, enquanto que a via de transsulfuração-reversa (RTS) através da regulação de CβS estaria mais associada às formas evolutivas dentro do inseto vetor. Esta observação é consistente com o fato que em *T.rangeli* e *T. brucei*, dois parasitos sem uma forma evolutiva intracelular dentro do hospedeiro mamífero, só possuem ativa a via RTS para a biossíntese de cisteína (OKALANG et al., 2013; ROMERO et al., 2014). Por sua vez, em parasitos com ciclos de vida exclusivo dentro do hospedeiro mamífero, tal como *Entamoeba* spp., tem sido demonstrado que possuem unicamente a via *de novo* ou assimilatoria para a biosinteses de cisteina (NOZAKI, ALI e TOKORO, 2005). O presente estudo constitue o primeiro relato da atividade da CS e CβS em *L. braziliensis*, embora para *L. major* (principal espécie do Velho Mundo) e *T. cruzi* já tenha sido confirmada que ambas as enzimas são ativas (NOZAKI et al., 2001; WILLIAMS, WESTROP e COOMBS, 2009; ROMERO et al., 2014). A redundância de ter duas vias para a biosínteses de cisteína observada em *Leishmania* spp. e *T. cruzi* continua a ser uma questão interessante a ser explorada. Neste sentido, WILLIAMS, WESTROP e COOMBS (2009) propuseram que à diferencia quanto a disponibilidade de nutrientes exógenos encontrada

nos diferentes hospedeiros, invertebrado e mamífero, pode explicar a necessidade de atividade das duas vias, *de novo* e RTS. No interior do trato digestivo do inseto vetor os parasitos residem em ambientes ligeiramente alcalinos ricos em glicose, mas escassos em aminoácidos. Enquanto que, dentro dos fagolisossomos de macrófagos humanos, os amastigotas sobrevivem em ambientes ácidos, onde a glicose é escassa e os aminoácidos são abundantes (ROSENZWEIG et al., 2008).

Em *Leishmania* spp. a presença de atividade das duas vias para a biossíntese de cisteína pode ser explicada pela necessidade constante deste aminoácido para a síntese da $T[SH]_2$, tiol antioxidante indispensável para resistir ao ambiente oxidativo no interior dos macrófagos do hospedeiro (FAIRLAMB e CERAMI, 1992; KRAUTH-SIEGEL e COMINI, 2008). Nossos resultados em *L. braziliensis*, corroboram com esta afirmativa, uma vez que os níveis de expressão da CS e CβS, assim como a atividade destas foram aumentados levando a consequente incremento da concentração total de tióis em resposta ao estresse oxidativo e nitrosativo *in vitro*, contrário ao obeservado nos tripanossomas onde a expressão das proteínas não apresentou modulação sob condições de estresse (Figura S2 pag. 126). De forma similar, em bactérias após exposição ao estresse oxidativo, um aumento dos níveis de cisteína e GSH tem sido demonstrado pela indução da expressão de genes do regulom cysB envolvidos na síntese de cisteína nestes microrganismos (POMPOSIELLO e DEMPLE, 2001; TURNBULL e SURETTE, 2010). Adicionalmente, amebas quando tratadas com óxido nítrico tiveram um aumento dos níveis de mRNA de CS acompanhado de incremento da atividade desta enzima (SANTI-ROCCA et al., 2012). O aumento da atividade da CS e da SAT da mesma forma, tem sido descrito positivamente correlacionado com a síntese de cisteína em plantas tolerantes a arsênico, e também com ao aumento de GSH em resposta ao estresse oxidativo por exposição a As^V (TRIPATHI et al., 2012; TALUKDAR, 2013).

A via de transsulfuração também tem sido associada na resposta ao estresse oxidativo em células humanas (NIU et al., 2014). Estudos de marcação metabólica revelaram um aumento na síntese de cistationina catalisada pela CβS, que por sua vez leva a um aumento nos níveis de glutationa em células hepáticas humanas (MOSHAROV, CRANFORD e BANERJEE, 2000; NIU et al., 2014). Além disso, em células epiteliais do cristalino e astrócitos humanos, tanto a expressão de proteína quanto a atividade de CβS foram gradualmente aumentadas após exposição a concentrações crescentes de H_2O_2 ou a um indutor de estresse oxidativo: como o terc-butil-hidroperóxido (PERSA et al.,

2004; McBEAN, 2012). Embora nossos resultados sobre a regulação de CS e CβS de *L. braziliensis* constituam o primeiro relato da ativação dessas enzimas sob estresse oxidativo e nitrosativo, o mecanismo molecular subjacente a esta regulamentação ainda não está claro e mais estudos são necessários para investigar essas associações.

A fim de compreender o papel de CS e CβS sob condições de estresse, a caracterização funcional dos genes *LbrCS* e *LbrCβS* foi realizado inicialmente um ensaio de complementação genética visando resgatar a atividade CS e expressão de proteínas heterólogas de CβS em *T. rangeli*. Este parasito foi escolhido pelos resultados obtidos na primeira parte de nosso trabalho onde demonstramos que o *T. rangeli* não possui a via *de novo* para a biossíntese de cisteína, possivelmente devido à presença de um códon de parada na sequência do gene *CS*, impedindo sua expressão e funcionalidade nas diferentes formas evolutivas do parasito (ROMERO et al., 2014). Outra característica importante deste parasito está associada com a sua maior sensibilidade ao estresse oxidativo e menor conteúdo de tióis totais observado na forma epimastigota quando comparado com as mesmas formas do *T. cruzi* (ROMERO et al., 2014). Nossos resultados de ensaios funcionais permitiram o resgate da atividade de CS em *T. rangeli* e mostraram que epimastigotas deste parasito expressando CS apresentaram um incremento na concentração de tiois totais (Figura S3 pag. 127), assim como um aumento da sobrevivência após a exposição a concentrações crescentes de peróxido de hidrogênio quando comparados com epimastigotas da cepa selvagem. Da mesma forma, os estudos de expressão heteróloga de LbrCβS mostraram que a superexpressão de CβS também confere resistência ao stresse oxidativo em epimastigotas. Em conjunto, estes resultados indicam não só que o *T. rangeli* é um modelo válido para estudos funcionais, mas também destaca o papel significativo da CS e CβS em resposta ao estresse oxidativo nestre parasito.

A relação entre os altos níveis de expressão das proteínas LbrCS e LbrCβS e a atividade das mesmas com a sobrevivência do parasito em condições de estresse foi ainda demonstrada pela capacidade de *L. braziliensis* superexpressando estas enzimas resistirem de maneira mais efetiva ao estresse oxidativo quando comparados com os parasitos controle. Estes dados confirmam as conclusões anteriores obtidas para *T. rangeli* expressando LbrCS e LbrCβS, onde observamos um aumento da sobrevivência destes parasitos sob condições de estresse oxidativo e nitrosativo e fornece um forte argumento de que estas proteínas podem desempenhar um papel importante na defesa da *L. braziliensis* contra o

estresse oxidativo. Estes resultados funcionais são consistentes com estudos anteriores em amebas, onde a superexpressão de CS e o aumento da atividade potencializou a resistência ao estresse oxidativo quando expostos a peróxido de hidrogênio (NOZAKI et al., 1999). Da mesma forma, a superexpressão de CS em plantas transgênicas induz uma maior tolerância a exposição a metais herbicidas como cádmio ou a metilviologênio (paraquat) (NOJI et al., 2001; YOUSSEFIAN et al., 2001; NING et al., 2010).

Os resultados de superexpressão da CβS em *L. braziliensis* sugerem que o aumento na expressão desta enzima pode conferir vantagens na sobrevivência dos parasitos sob condições de estresse oxidativo. Estes resultados constituem o primeiro relato de alteração fenotípica pela superexpressão de CβS e sua associação com a resposta em condições de estresse neste parasito. Recentemente, o envolvimento indireto da CβS e CSE (cistationina γ-liase) na defesa ao estresse oxidativo foi relatado através da superexpressão destas enzimas com aumento na produção de cisteína e consequentemente aumento nos níveis de H_2S nas células do mesângio glomerular de ratos (SEN et al., 2011). Estes autores demonstraram que ao restituir os níveis normais de H_2S gerados pela via de RTS, se induz uma inibição da expressão da NADPH-oxidase impedindo a geração de $O2\bullet^-$ e, consequentemente, o dano oxidativo nestas células (SEN et al., 2011). Outros estudos funcionais têm relatado que a inibição das enzimas CβS e CSE utilizando o aminooxiacetato (AOAA) e o D, L-propargilglicina (PAG), respectivamente, ou mediante silenciamento específico utilizando siRNA dos genes que codificam para estas proteínas aumentou a morte celular induzida por estresse oxidativo em células progenitoras mesenquimais (FOX et al., 2012).

A maior capacidade dos transfectantes de LbrCS e LbrCβS em suportar de maneira mais eficiente o estresse oxidativo induzido por H_2O_2 quando comparados aos parasitos com fenótipos selvagem ou transfectados com o vetor vazio, levantou a questão de como os parasitos tranfectados também poderiam sobreviver a diferentes estresses oxidativos, tal como aqueles induzidos por compostos antimoniais (MEHTA e SHAHA, 2006; MOOKERJEE BASU et al., 2006; MOREIRA, LEPROHON e OUELLETTE, 2011). Nossos resultados demonstraram uma resposta dose-dependente na redução da sensibilidade do parasito a exposição ao Sb^{III}, evidenciada pelo aumento nos valores da concentração que inibe 50% dos parasitos (CI_{50}) nos transfectantes com LbrCS ou LbrCβS. Estes resultados foram corroborados pela correlação positiva encontrada entre a expressão ou

atividade específica de CS e CβS quando comparada com a sobrevivência do parasito após a exposição ao Sb^{III}.

Os resultados do aumento na tolerância de promastigotas de *L. braziliensis* superexpressando LbrCS e LbrCβS ao Sb^{III}, foi da mesma forma explorada em amastigotas intracelulares em macrófagos humanos THP-1 expostos ao Glucantime® (Sb^{V}). Nossos resultados demonstraram claramente que, em amastigotas superexpressando LbrCS e LbrCβS, houve uma perda significativa da sensibilidade ao tratamento com Sb^{V}, ou seja, um aumento da resistência ao fármaco. Por sua vez, as análises de correlação corroboraram a relação entre expressão e atividade específica das proteínas com a sobrevivência dos parasitos após a exposição ao Sb^{V} (Tabela S1 pag. 124). Em conjunto, estes dados sugerem que o aumento da tolerância aos compostos antimoniais mediada pela CS e CβS, pode ser devido ao aumento observado na atividade destas proteínas, provavelmente induzindo à produção de cisteína para abastecer as necessidades de sintetizar os tiois glutationa e tripanotiona, necessários para a detoxificação de antimônio (WYLLIE, CUNNINGHAM e FAIRLAMB, 2004; WYLLIE e FAIRLAMB, 2006).

Como demonstrado em outros estudos, níveis aumentados de tióis tem sido considerado como um dos principais mecanismos relacionados à detoxificação de Sb^{III} em cepas de *Leishmania* spp. cuja resistência foi induzida em condições laboratoriais (MUKHOPADHYAY et al., 1996). Em promastigotas de *L. amazonensis* resistentes ao Sb^{III} foram relatados níveis significativamente aumentados de cisteína, glutationa e tripanotiona, enquanto que em linhagens de *L. infantum* resistentes ao Sb^{III}, apenas os níveis de cisteína se mostraram significativamente aumentados (EL FADILI et al., 2005; Do MONTE-NETO et al., 2011). Por sua vez, em isolados clínicos de *L. donovani*, os níveis dos tióis GSH e $T[SH]_2$ foram significativamente mais baixos em linhagens sensíveis quando comparados com os níveis encontrados em linhagens resistentes (MANDAL et al., 2007). Surpreendentemente, outro estudo demonstrou de forma contrária que não houve diferenças nos níveis de $T[SH]_2$ entre os isolados clínicos sensíveis e resistentes ao Sb^{V}, embora tenham observado um aumento significativo dos níveis de cisteína e glutationa nos isolados resistentes (MUKHERJEE et al., 2007). Estes autores explicaram que os níveis de $T[SH]_2$ não foram aumentados nos isolados resistentes, possivelmente devido ao aumento de efluxo deste tiol mediante a conjugação com Sb^{III}, um dos mecanismos de detoxificação de antimônio que também tem sido relatado em *L. donovani* (WYLLIE, CUNNINGHAM e FAIRLAMB, 2004). Similarmente, alguns estudos têm demonstrado um claro aumento na

expressão de outros genes como γ-glutamilcisteína sintetase, ornitina descarboxilase, tripanotiona sintetase, tripanotiona redutase e espermidina sintase, envolvidos nas vias de síntese de glutationa e tripanotiona, tanto em isolados clínicos resistentes a antimônio como em linhagens resistentes induzidas no laboratório (MUKHERJEE et al., 2007; Do MONTE-NETO et al., 2011). Em resumo, estes estudos, em conjunto com nossos resultados, demonstram a complexa regulamentação sobre as vias biossintéticas dos tióis, onde cisteína pode desempenhar um papel crucial nesta regulação.

Estudos *in vitro* utilizando linhagens resistentes de *L. tarentolae* e *L. infantum* induzidas no laboratório têm consistentemente demonstrado um aumento da expressão do gene da S-adenosil homocisteína hidrolase (SAHH), o qual está envolvido na conversão de S-adenosil homocisteína para homocisteína, sendo este um importante precursor da cisteína na via de RTS (GUIMOND et al., 2003; EL FADILI et al., 2005). Adicionalmente, em isolados clínicos naturalmente resistente ao Sb^V, foi observado um aumento nos níveis de expressão do gene que codifica para CβS, o que reforça a hipótese que cisteína pode estar direta ou indiretamente envolvida nos fenótipos de resistência (DECUYPERE et al., 2012). O aumento da resistência a compostos antimoniais (trivalente e pentavalente) observado em promastigotas e amastigotas de *L. braziliensis* superexpressando LbrCS e LbrCβS, demonstra claramente que a modulação nos níveis destas enzimas em *L. braziliensis* podem induzir alterações na sensibilidade ao antimônio, levantando a questão de, até que ponto, esta resposta também pode ocorrer em outras espécies do gênero *Leishmania*.

Devido ao fato de que cisteína forma o cerne básico para a síntese de tióis, nossos resultados mostraram que CS e CβS têm um papel importante na sobrevivência de *L braziliensis* em condições de estresse oxidativo *in vitro*. Ao contrario de patógenos intracelulares obrigatórios como *Leishmania* spp. e *T. cruzi* que possuem ativa as duas vias para a biossíntese de cisteína, demonstramos que o *T. rangeli* não possui a biosíntese *de novo* deste aminoácido em função do gene CS estar truncado em seu genoma. Com base em nossos resultados, apresentamos na Figura 10 um possível modelo da participação de CS e CβS na defesa antioxidante de tripanosomatideos. A associação estágio-específica da CS e CβS, permite hipotetizar que a via *de novo* estaria mais envolvida com a resposta ao estresse oxidativo nos patógenos intracelulares na sua forma amastigota, onde aumento na atividade da CS, leva ao incremento dos tióis totais, fornecendo as quantidades de $T[SH]_2$ necessarias para a detoxificação eficiente das ERO geradas durante o estresse oxidativo no

macrófago; ao contrário a via de RTS que estaria mais relacionada às formas evolutivas dentro o inseto vetor, sendo a CβS a enzima responsável pelo incremento da cisteína e dos tióis totais. A presença de uma só via ativa observada em *T. rangeli*, pode explicar a maior sensibilidade deste parásio quando expostos a condições de estresse oxidativo.

Interessantemente, nossos resultados demonstram que as mudanças nos níveis de expressão e atividade de proteínas CS e CβS podem estar relacionada com a eficácia do antimônio no tratamento das Leishmanioses, ou mesmo com a utilização de posologias distintas ou esquemas terapêuticos distintos. Em suma, podemos concluir que nossos resultados indicam ser a CS um alvo relevante e explorável para o desenho de novos fármacos, onde o domínio de ligação a SAT se constitui como um excelente candidato para o desenho racional de inibidores seletivos à resistência nas espécies de *Leishmania* do Novo Mundo, não representando conflito com rotas metabólicas de mamíferos uma vez que estes não possuem a via *de novo* para a biossíntese cisteína.

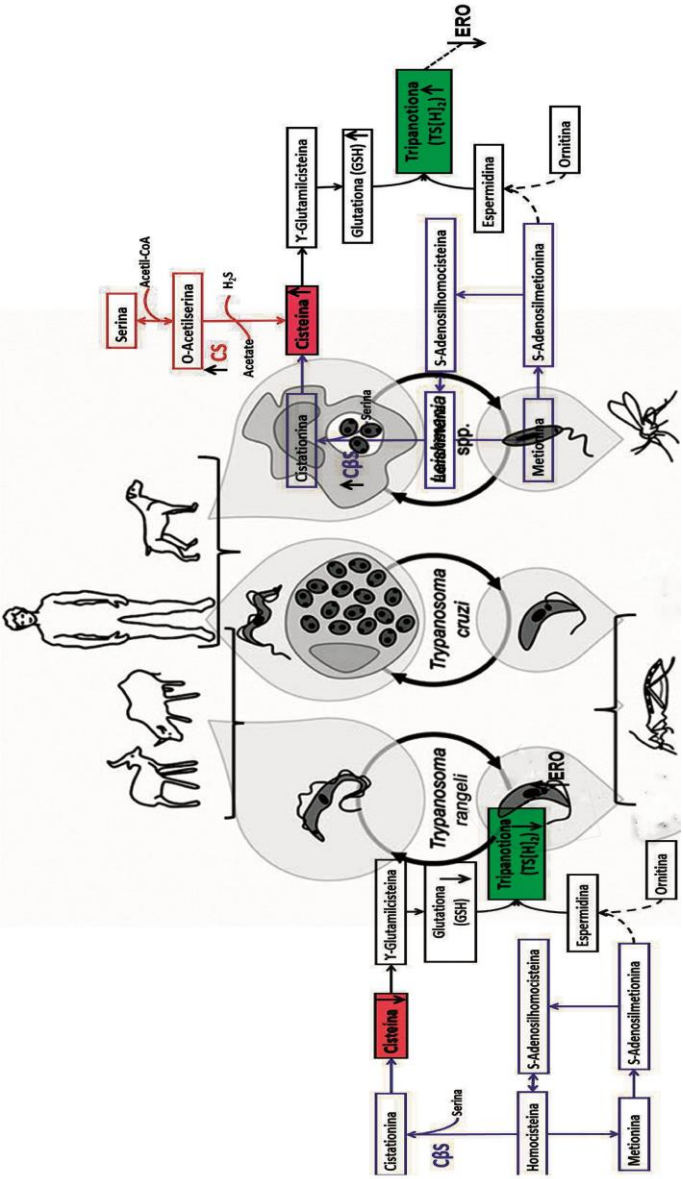

Figura 10. Modelo da participação de CS e CβS na defesa antioxidante nos tripanosomatideos. Fonte: Adaptado de TOMÁS E CASTRO 2012.

6. CONCLUSÕES

Em conjunto, nossos resultados nos permitem concluir que:

1. A CS e a CβS de *L. braziliensis* apresentam-se como cópias únicas no genoma, enquanto que em *T. cruzi* são genes multicópia;
2. Em *T. rangeli* a CβS está presente como cópia única no genoma, enquanto que a CS apresenta-se como um gene truncado em função da presença de um códon de parada, gerando uma proteína não funcional;
3. *L. braziliensis* e *T. cruzi* possuem as duas vias de biosíntese de cisteína ativas (*de novo* e RTS) e funcionais nas diferentes formas evolutivas, enquanto que em *T. rangeli* apresenta ativa só a via de RTS;
4. Houve uma regulação estágio-específica da expressão e da atividade de CS e CβS em *L. braziliensis* e *T. cruzi*, encontrando-se que a via *de novo* pode estar associada com estágios intracelulares e a via RTS com estágios de desenvolvimento no inseto vetor;
5. *T. rangeli* apresentou uma maior sensibilidade ao estresse oxidativo e menor concentração de tióis totais quando comparado com *L. braziliensis* e *T. cruzi* enquanto que *L. braziliensis* apresentou um incremento na tolerância ao estresse oxidativo quando comparada com os demais tripanosomatídeos analisados;
6. Houve um aumento na expressão e na atividade de CS e CβS de *L. braziliensis* em resposta ao estresse oxidativo, gerando um aumento na quantidade de tióis totais neste parasito;
7. O resgate funcional da atividade de CS em *T. rangeli* levou a um aumento da tolerância do parasito ao peróxido de hidrogênio e a um incremento dos níveis de tióis totais;
8. A superexpressão das enzimas CS e CβS por *L. braziliensis* levou a um incremento da tolerância do parasito ao estresse oxidativo induzido por peróxido de hidrogênio*;*
9. A superexpressão das enzimas CS e CβS reduziu a sensibilidade de *L. braziliensis* aos compostos antimoniais trivalente (Sb^{III}) e pentavalente (Sb^{V});
10. A regulação positiva das enzimas CS e CβS contribuem para a sobrevivência de *L. braziliensis* sob condições de estresse oxidativo e podem estar envolvidas na resistencia ao tratamento com compostos antimoniais.

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8. MATERIAL SUPLEMENTAR

Tabela S1 Análise de correlação da expressão e atividade das enzimas CS e CβS com a sobrevivência de *L. braziliensis* ao estresse oxidativo *in vitro*.

Indutores de estresse oxidativo	Expressão Vs Sobrevivência				Atividade Vs Sobrevivência			
	CS		CβS		CS		CβS	
	r	p	r	p	r	p	r	p
H_2O_2	0,9082	0,0018	0,9393	0,0005	0,8955	0,0026	0,7739	0,0242
Sb^{III}	0,8682	0,0052	0,7357	0,0375	0,8798	0,004	0,7611	0,0283
Sb^{V}	0,8894	0,0031	0,8867	0,0033	0,8714	0,0048	0,8305	0,0107

As análises de correlação foram feitas usando o teste de Pearson para determinação dos valores r e p.

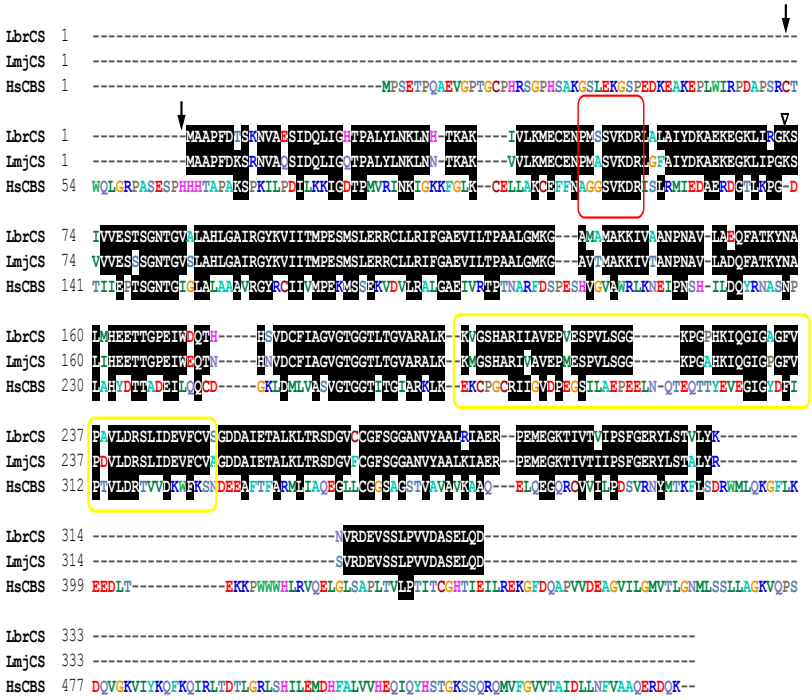

Figura S1. Alinhamento parcial das sequências aminoacídicas de CS de *L. braziliensis* e CβS de Homem. A caixa vermelha aberta indica o domínio de união ao cofator PLP (PXXSVKDR) conservado entre as proteínas da superfamília PLP. A caixa amarela indica o domínio de união a SAT presenta na CS; as setas o motivo de união ao grupo Hem presente na CβS. LbrCS: *Leishmania braziliensis* (LbrM.35.3820); LmjCS: *Leishmania major* (LmjF.36.3590); HsCβS: Humana (P35520).

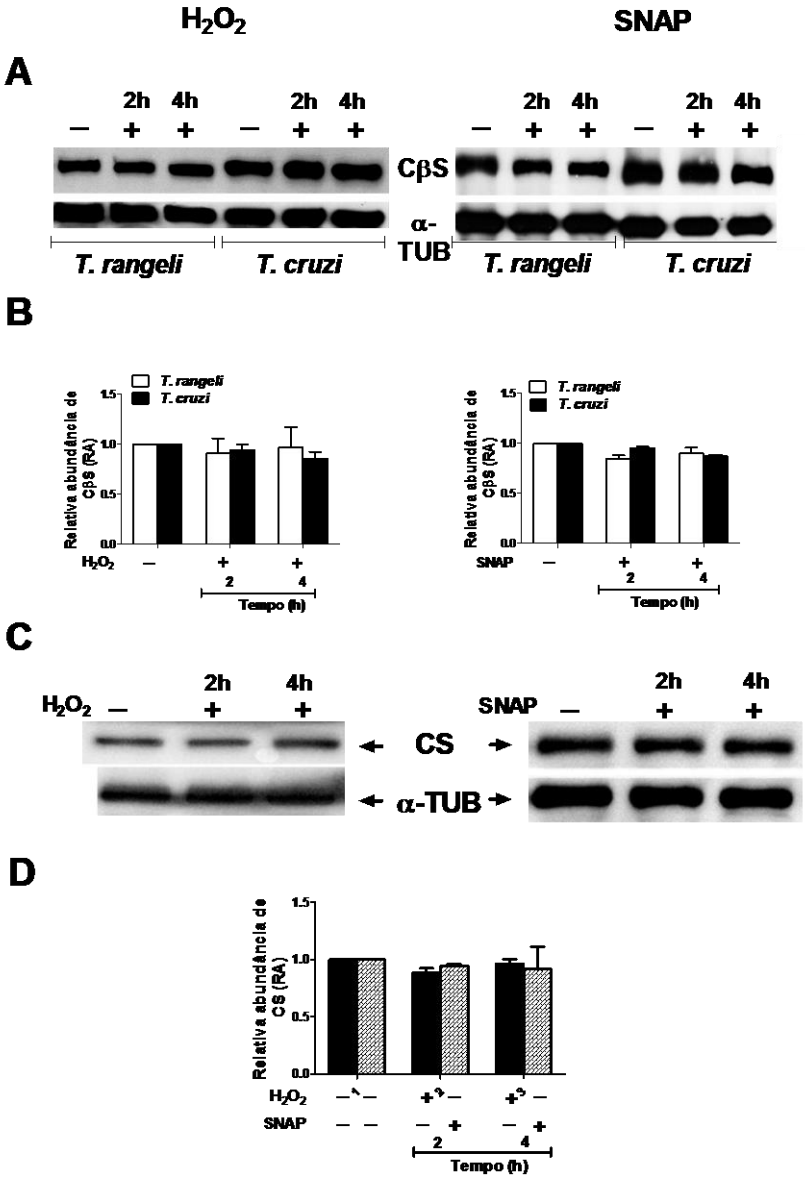
H2O2
SNAP
A
2h 4h 2h 4h
– + + – + +
CβS
α-TUB
T. rangeli
T. cruzi
B
T. rangeli
T. cruzi
Relativa abundância de CβS (RA)
1.5
1.0
0.5
0.0
H2O2
SNAP
2
4
Tempo (h)
C
2h 4h
H2O2 – + +
SNAP – + +
CS
α-TUB
D
Relativa abundância de CS (RA)
H2O2
SNAP
Tempo (h)

Figura S2. Efeito do estresse oxidativo e nitrosativo sobre a expressão das proteínas CS e CβS em *T. rangeli* e *T. cruzi*. *(*A). Análises de expressão por Western blot das proteínas CβS em *T. rangeli* e *T. cruzi* expostos a H_2O_2 e SNAP. (B). As análises densitométricas dos sinais apresentadas no painel forma realizadas usando o programa ImageJ. *(*C). Análises de expressão por Western blot da proteína CS a partir de epimastigotas de *T. cruzi* expostas a H_2O_2 e SNAP. (D). Análises densitométricas dos sinais apresentadas no painel. A equivalência de carga das proteínas foi controlada por imunodetecção da α-Tubulina. Os resultados apresentam a media de três experimentos ± DP.

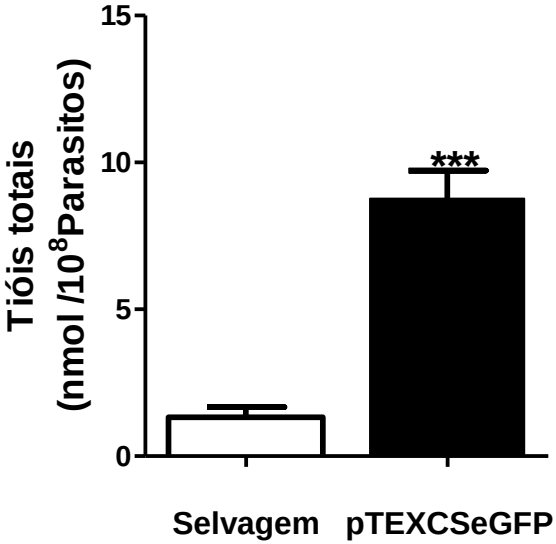

Figure S3. Níveis de tióis totais presentes em epimastigotas de *T. rangeli*. A concentração total de tióis em promastigotas de *T. rangeli* foi avaliada na cepa selvagem e a transfectada com pTEXCSeGFP. Os resultados apresentam a media de três experimentos ± DP. Diferenças significativas foram determinadas utilizando o teste t de *Student* (***P < 0.001).